This is to certify that the

dissertation entitled

A CD5$^+$ B CELL LINE PROVIDES HELP FOR HUMORAL RESPONSES: POTENTIAL ROLE FOR CD5$^+$ B CELLS IN IMMUNE REGULATION

presented by

Laurie Ann Iciek

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Microbiology

Kathryn Brooks
Major professor

Date July 14 1994

A $CD5^+$ B CELL LINE PROVIDES HELP FOR HUMORAL RESPONSES: POTENTIAL ROLE FOR $CD5^+$ B CELLS IN IMMUNE REGULATION

By

Laurie Ann Iciek

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1994

ABSTRACT

A CD5$^+$ B CELL LINE PROVIDES HELP FOR HUMORAL RESPONSES: POTENTIAL ROLE FOR CD5$^+$ B CELLS IN IMMUNE REGULATION

By

Laurie Ann Iciek

A unique population of B cells has been defined in both mice and humans which expresses CD5. In addition to exhibiting a unique phenotype (IgM^{bright}, IgD^{dull}, CD5$^+$), CD5$^+$ B cells appear early in ontogeny, at a site distinct from conventional B cells, in the fetal omentum. Furthermore, CD5$^+$ B cells are localized to specific anatomical sites in the adult mouse, including the peritoneal cavity and spleen. Currently, the role CD5$^+$ B cells play in the function of the immune network has not been clearly defined. However, the early appearance of CD5$^+$ B cells at distinct anatomical locations suggests these cells may be involved in regulating immune responses early in ontogeny. In addition, the frequency of CD5$^+$ B cells is increased in certain strains of autoimmune mice. Thus, CD5$^+$ B cells may also play a role in autoimmunity. One possible mechanism whereby CD5$^+$ B cells could alter immune responses in through direct ligand-receptor interactions between CD5 and its ligand CD72, which is expressed on B cells. In the present study, the ability of CD5$^+$ B cells to provide contact-dependent help to other B cells was investigated. The CD5$^+$ neoplastic B cell line, BCL_1-3B3, was irradiated and added to splenic B cells in the presence and absence of IL-2, IL-4 and IL-5. Whenever IL-2 was present, the addition of irradiated BCL_1-3B3 cells

markedly enhanced thymidine incorporation. This enhanced response to IL-2 was observed without the addition of a primary activator such as anti-Ig or dextran sulfate and was not dependent upon primary *in vivo* activation. Modest enhancement of IgM and IgG secretion was also observed, but only when IL-5 was present in addition to IL-2. The $CD5^+$ B cell-mediated help involves a contact-dependent signal since use of membrane-partitioned cultures completely inhibited the helper activity of the $CD5^+$ B cells. In addition, paraformaldehyde fixed $CD5^+$ B cells provided help equivalent to 70% of that seen with irradiated cells. Inhibition of B-cell-mediated help by antibodies reactive with various B cell surface molecules was investigated. Antibodies to ICAM-1 (CD54) and LFA-1 (CD11a, CD18) inhibited approximately 26 to 49 percent of the helper activity, respectively. Antibodies to CD5, CD40L, I-A and I-E had no inhibitory effect. These results suggest that neoplastic B cells can enhance responsiveness of resting and activated B cells to interleukin 2 in the absence of T-B interactions. These interactions involve the adhesion molecules LFA-1 and ICAM-1 but additional ligand/receptor pair interactions are likely. In addition to antibodies reactive with the adhesion molecules, anti-interleukin 6 and anti-interleukin 10 mAbs inhibited 56 and 42 percent of the helper activity, respectively. Thus, in addition to ligand-receptor interactions, B-cell-derived soluble factors may play a role in $CD5^+$ B-cell-mediated help. Developing a better understanding of the molecules directly involved in $CD5^+$ B-cell-mediated signalling may provide some exciting new insight about the functional role of $CD5^+$ B cells.

This work is dedicated to my parents Margaret and Stanley Iciek, in recognition of their continued love, support and guidance.

ACKNOWLEDGMENTS

I would sincerely like to thank my wonderful mentor **Dr. Kathryn Brooks** for her guidance, support, and unending patience, these past six year. I would also like to thank my committee members: Dr. Walter Esselman, Dr. Maria Patterson, Dr. Richard Schwartz, and Dr. Michael Zaroukian. In addition, I would like to thank my collaborators Drs. Marie Griffiths and Thomas Waldschmidt. Finally, Dr. Virginia Sanders has been very helpful and I would like to thank her for reviewing countless drafts of manuscripts.

I have been fortunate enough to encounter many valued colleagues and friends in the six years I have been at Michigan State University. This piece of work would not be complete without a sincere thank-you to the people who have helped me survive the ups and downs of graduate school, and life. Therefore, I am deeply indebted to: Dr. Sue Dagher, Dr. Trifon Ademedis, Houman Dehghani, Ron Nerio, Stacy Morradian, Dr. Denise Warren, Brenda Knotts, Larry Martin, Maggie Waldman and Craig Banotai. Finally, I would like to thank my dear sisters Celeste Iciek-Linder, and Christine Iciek, for their love, support and friendship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AO = acridine orange
B-1a = (IgM^{bright}, IgD^{dull}, $CD5^+$, $Fc_\epsilon R^-$) phenotype
B-1b = (IgM^{bright}, IgD^{dull}, $CD5^-$, $Fc_\epsilon R^-$) phenotype
B-2 = (IgM^{dull}, IgD^{bright}, $CD5^-$) phenotype
biotin = biotinylated
BrMRBC = bromelain-treated mouse red blood cells
cGvHD = chronic graft-versus-host disease
CIA = collagen-induced arthritis
CLL = chronic lymphocytic leukemia
CPM = counts per minute
DxS = dextran sulfate
ELISA = enzyme-linked immunosorbent assay
FACS = fluorescence-activated cell sorter
FITC = fluoresceinated
GAMIg = goat anti-mouse immunoglobulin
IL-2R = interleukin 2 receptor
mAb = monoclonal antibody
MAIDS = murine acquired immunodeficiency syndrome
MFI = mean fluorescence intensity
MuLV = murine leukemia virus
PE-AV = R-phycoerythrin avidin
PEC = peritoneal exudate cells
PHA = phytohemagglutinin
PI = post-infection
PKC = protein kinase C
PMA = phorbol 12-muristic 13-acetate
RF = rheumatoid factor
RIA = radioimmunoassay
SCID = severe combined immunodeficient
SEM = standard error of the mean
SI = stimulation index
sIg = surface immunoglobulin
SLE = systemic lupus erythematosus
T_H1 = type-1 T helper cell
T_H2 = type-2 T helper cell
TI-2 antigen = thymus-independent type 2 antigen

1.0 INTRODUCTION.

1.1 $CD5^+$ B cells defined.

1.1.1. History.

Historically, the CD5 antigen (murine Ly-1, human Leu-1), a 67-kDa glycoprotein, was thought to be exclusively expressed on helper T lymphocytes (Cantor and Boyse, 1975). With the availability of the anti-CD5 monoclonal antibody 53-7.3 and flow cytometric analysis, Ledbetter and colleagues showed that CD5 was also expressed, at lower levels, on cytotoxic T lymphocytes (Ledbetter et al., 1980). In addition, analysis of spleen tissue sections revealed the presence of CD5 positive, Thy-1 negative cells which were localized to the germinal centers of the spleen, an area believed to be comprised mainly of B cells (Ledbetter et al., 1980). In 1981, using the monoclonal antibody (mAb) 53-7.3, Lanier and colleagues showed that three B cell lymphomas (CH5, WEHI-55 and WEHI-259) were CD5 positive, providing support that transformed B cells, as well as T cells, could express CD5 (Lanier et al., 1981). Although each of these three B cell lymphomas expressed characteristic B cell surface antigens (surface Ig and Ia) and did not express Thy-1, it was unclear whether they represented a normal subset of $CD5^+$ B cells which had been transformed, or whether some transformation event had led to the expression of CD5.

The first evidence that CD5 was expressed on normal murine B cells was provided by Manohar and colleagues in 1982 (Manohar et al., 1982). Two-color flow cytometric analysis of splenic cells from a number of murine strains showed that a subset of IgM positive B cells expressed CD5, but did not express the T cell

antigen Thy-1. In a subsequent study Hayakawa and colleagues utilized two-color flow cytometry to confirm the presence of a murine splenic B cell subset which was surface Ig (sIg) positive, CD5 positive and lacked the expression of the T-cell associated antigens CD4, CD8, and Thy-1 (Hayakawa et al., 1983). In addition, Hayakawa's study showed that $CD5^+$ B cells were present in nude mice which lack a functional thymus, supporting the idea that CD5 is produced by the B cells as opposed to being acquired from the T cells. Furthermore, when CD5 was removed from the surface of the B cell lymphoma WEHI-259 by trypsin treatment, greater than 90% of the cells were CD5 positive within 18 hours (Lanier et al., 1981).

Concurrent with the discovery that certain transformed murine B cells lines expressed CD5, Wang and colleagues utilized two-color immunofluorescent staining to show CD5 was expressed on B cells from patients with chronic lymphocytic leukemia (CLL), (Wang et al., 1980). These findings were further supported by Royston and colleagues who showed not only did the anti-CD5 antibody T101 bind to sIg^+ cells from CLL patients, but it also immunoprecipitated a 65-kDa protein from the surface of CLL B cells (Royston et al., 1980). In 1982, Caligaris-Cappio et al. used immunofluorescent staining to show that not only was CD5 expressed on human B cells from patients with CLL, but approximately 2-5% of the normal B cells in human lymph nodes and tonsils coexpressed CD5, sIg, and HLA-DR (Caligaris-Cappio et al., 1982).

1.1.2. Phenotype.

In contrast to conventional murine B cells which are phenotypically sIgM^{dull}, sIgD^{bright}, Ly-1 (CD5) negative; murine CD5$^+$ B cells are sIgM^{bright}, sIgD^{dull} and Ly-1 positive (Manohar et al., 1982; Hayakawa et al., 1983, 1984; Hardy et al., 1983). Mac-1 (CDIIb), the C3bi receptor which is usually expressed on cells of the myelomonocytic lineage, is also expressed on CD5$^+$ B cells in the peritoneum; whereas, conventional B cells in the peritoneum are Mac-1 negative (Herzenberg et al., 1986). Waldschmidt and colleagues have shown that the Fc_ϵR (CD23) which is expressed on conventional B cells in both the spleen and peritoneum is not expressed on CD5$^+$ B cells (Waldschmidt et al., 1991). Although CD5 B cells express CD5 they do not express the T-cell associated antigens CD3, CD4, CD8 and Thy-1 (Manohar et al., 1982; Hayakawa et al., 1983). In addition to CD5, murine CD5$^+$ B cells express the CD19, CD20 and CD21 antigens, which are exclusive for lymphocytes of the B lineage (Hayakawa et al., 1983; Hardy et al., 1984, 1986). Two-color flow cytometric analysis studies have also shown that Ia and B220 [the B cell isomer of T200 (CD45)] are coexpressed on murine CD5$^+$ B cells (Hayakawa et al., 1983; Kipps, 1989).

In humans the phenotype of CD5$^+$ B cells parallels the murine phenotype with the expression of CD5, CD19, CD20, CD21 and HLA-DR (Gadol and Ault, 1986; Kipps and Vaughan, 1987). The T-cell associated antigens CD3, CD4, and CD8 are also not expressed on human CD5$^+$ B cells (Gadol and Ault, 1986; Kipps and Vaughan, 1987). In parallel to murine peritoneal CD5$^+$ B cells, Kipps and

Vaughan have previously shown human peripheral blood $CD5^+$ B cells coexpress CD11b (Kipps and Vaughan, 1987). A second myelomonocyte-associated surface antigen, CD14, has also been detected at low levels on human $CD5^+$ B cells (Kipps, 1989). In addition to the expression of unique surface antigens, when analyzed on a fluorescence-activated cell sorter (FACS), both murine and human $CD5^+$ B cells have a larger forward-and side-angle light scatter pattern than conventional B cells (Kipps, 1989). This finding is consistent with $CD5^+$ B cells being larger in size than conventional B cells.

1.1.3. Ontogeny.

In mice, Kearney and colleagues have shown that fetal omentum gives rise to $CD5^+$, but not conventional B cells, when grafted into adult severe combined immunodeficient (SCID) mice (Solvason et al., 1991). Thus, it appears that $CD5^+$ B cells in mice have a unique site of developmental origin which is associated with the mesodermally-derived peritoneal lining. In parallel to these findings, Hayakawa and colleagues have shown that $CD5^+$ B cells are one of the first B cell populations found in the peritoneum (Hayakawa et al., 1986). At seven days after birth, approximately 100% of the B cells in the mouse peritoneum express CD5. As the mouse ages the frequency of $CD5^+$ B cells in the peritoneum decreases, and $CD5^+$ B cells represent from 30- to- 50% of the peritoneal B cells by three months of age (Hayakawa et al., 1986). Although the frequency of peritoneal $CD5^+$ B cells declines, the actual number of $CD5^+$ B cells does not decrease, thus the apparent decline in frequency is due to increases in the number of

conventional B cells in the peritoneum. In the spleen, the appearance of $CD5^+$ B cells in the neonate coincides with the appearance of the first IgD-bearing B cells. Previously, Dexter and Corley have shown that $CD5^+$ B cells represent approximately 20% of the total splenic B cells five days after birth; a frequency which declines to approximately 5%, at three months of age (Dexter and Corley, 1987). Although there is a decrease in the percentage of splenic $CD5^+$ B cells from five days to three months of age, the actual number of splenic $CD5^+$ B cells increases during this period (Dexter and Corley, 1987). Thus, in parallel to the peritoneum, the apparent decline in splenic $CD5^+$ B cell frequency is due to an expansion of the conventional splenic B cell population, not a loss of $CD5^+$ B cells.

$CD5^+$ B cells have also been detected early in ontogeny in humans. Kearney and colleagues have shown that approximately 50% of the B cells in human fetal omentum express CD5, at fourteen weeks of gestation (Solvason et al., 1992). In addition to fetal omentum, $CD5^+$ B cells constitute a major B cell population in the fetal spleen, where approximately 50% of the B cells express CD5, at 22 weeks of gestation (Antin et al., 1986). In an extensive study of human B cell development, Bofill et al. showed that $CD5^+$ B cells are detectable in the fetal peritoneal cavity at fifteen weeks of gestation, in fetal lymph nodes around seventeen weeks of gestation, and in the fetal spleen at approximately twenty-two weeks of gestation (Bofill et al., 1985). Thus, the ontogeny of human $CD5^+$ B cells somewhat parallels that seen in mice, with an early detection of $CD5^+$ B cells in the fetal omentum and the fetal peritoneum in both species. Hayakawa et al. have

shown $CD5^+$ B cells also constitute a major B cell subpopulation in newborn cord blood, where they represent approximately 75% of the B lymphocyte population (Hardy and Hayakawa, 1986; Hardy et al., 1987). The frequency of $CD5^+$ B cells in the peripheral blood and spleens from normal adult humans is much lower than the frequencies reported for both human cord blood and fetal spleens. Thus, the previously noted shifts in the murine B cell populations which occur as the mouse matures may also occur in humans.

1.1.4. Anatomical localization.

In mice, the anatomical site with the greatest percentage of $CD5^+$ B cells is the peritoneum, where approximately 50% of all B cells express CD5 (Hayakawa et al., 1986). $CD5^+$ B cells also constitute approximately 2% of the total spleen cells in normal inbred murine strains such as BALB/c and approximately 5-10% of the spleen cells in autoimmune murine strains such as NZB/NZW F_1 (Hayakawa et al., 1983). Although a previous study indicated $CD5^+$ B cells were absent from the thymus (Hayakawa et al., 1983), a more sensitive study in which thymocytes were depleted found that over 70% of the thymic B cells coexpress CD5, Mac-1, sIgM, Ia and B220 (Miyama-Inaba et al., 1988). In contrast to conventional B cells, $CD5^+$ B cells have not been detected in the lymph nodes or bone marrow from normal mice (Hayakawa et al., 1983).

In normal adult humans, $CD5^+$ B cells comprise from 1% up to 30% of the B cells circulating in the peripheral blood (Plater-Zyberk et al., 1985; Gadol and Ault, 1986; Maini et al., 1987; Lydyard et al., 1987; Taniguchi et al., 1987; Hardy

and Hayakawa, 1986; Kipps and Vaughan, 1987). Less than 10% of human splenic B cells are $CD5^+$ (Freedman et al., 1987). In contrast to the murine lymph node B cells, up to 30% of the human lymph node B cells express CD5 (Freedman et al., 1987a). Although $CD5^+$ B cells have been detected in peritoneal washes from human fetuses as early as 15 weeks of gestation (Bofill et al., 1985), $CD5^+$ B cells have not been detected in adult peritoneal washings (Kipps, 1989). In parallel to mice, detectable levels of $CD5^+$ B cells are not present in human adult bone marrow (Kipps, 1989).

1.1.5. Strain distribution.

$CD5^+$ B cells comprise approximately 2% of the total spleen cells in a number of normal murine strains including BALB/c, DBA/2, CBA, SJA, BAB 14, C57BL/6, NFS, and B10.D2 (Manohar et al., 1982; Hayakawa et al., 1983). In addition to normal murine strains, immunodeficient nude mice such as CBA (*nu/nu*) and Balb/c (*nu/nu*), which are genetically T-deficient, have normal percentages of splenic $CD5^+$ B cells (Hayakawa et al., 1983). In autoimmune NZB strains, $CD5^+$ B cells are found at increased frequencies and comprise up to 10% of the normal spleen cells (Hayakawa et al., 1983). Murine strains which express the viable motheaten (*me*^{v}) or the motheaten (*me*) genes also have increased frequencies of $CD5^+$ B cells (Sidman et al., 1985). Although autoimmune NZB and motheaten viable mice have increased levels of $CD5^+$ B cells in both the spleen and the peritoneum, autoimmune murine strains which express the *lpr* gene, such as the MRL/*lpr* strain, have levels of $CD5^+$ B cells which are equivalent to the

levels found in normal mice (Hayakawa et al., 1983). In contrast to most normal murine strains, CBA/N mice which express the *xid* gene do not have detectable levels of $CD5^+$ B cells in the spleen or peritoneum (Hardy et al., 1983; Hayakawa et al., 1986a; Herzenberg et al., 1986).

1.1.6. Nomenclature.

In mice, there are distinct differences between conventional and $CD5^+$ B cells in terms of phenotype, function, anatomical localization, and Ig gene expression. In addition, a great deal of debate currently exists concerning the possibility that these two B cell populations represent separate B cell lineages. Thus, a new nomenclature scheme was devised in 1991 to separate these two B cell populations (Kantor, 1991). Under the new nomenclature what was previously considered a $CD5^+$ B cell is termed a B-1 cell, whereas, conventional B cells are termed B-2 cells.

The B-1 cell population has further been broken down into the B-1a and the B-1b cell populations, due to data obtained from a series of studies. In 1986, Herzenberg and colleagues noted the presence of a B cell population in the peritoneum which exhibited a $sIgM^{bright}$, $sIgD^{dull}$ phenotype, was Mac-1 positive and CD5 negative (Herzenberg et al., 1986). In addition to having a phenotype similar to that previously described for $CD5^+$ B cells, this subset of peritoneal B cells had a self-renewing capacity. Thus, this subset of B cells was considered the CD5 "sister" B cell lineage. Further support that CD5 and "sister" B cells are related came in 1991, when Waldschmidt and colleagues utilized two-color FACS analysis

to show that both the $CD5^+$, Mac-1^+ and the "sister" lineage ($CD5^-$, Mac-1^+) B cell populations in the peritoneum are phenotypically $Fc_\epsilon R$ negative (Waldschmidt et al., 1991). In addition to the $CD5^+$ B cells in the peritoneum, the splenic B cells which expressed CD5 were found to be $Fc_\epsilon R$ negative. Although both the $CD5^+$ and the "sister" B cell populations express similar phenotypes, Stall and colleagues have shown that each of these populations replenishes itself, but not the other when transferred into irradiated recipients with congenic bone marrow (Stall et al., 1992). Thus under the new nomenclature scheme those B cells which express CD5 are termed B-1a cells and the "sister" B cells are termed B-1b cells (Kantor, 1991). Although there are clearly functional differences between B-1a and B-2 cells in mice, there is currently no evidence of functional differences between B-1a and B-1b cells.

1.2 B cell lineage theories.

In mice, the B-1, B cell population exhibits a unique phenotype, ontogeny, and anatomical localization pattern. A number of researchers have theorized that B-1 and B-2 B cells represent separate B cell lineages which have arisen from unique, distinguishable, progenitors. Although the debate over separate B cell lineages is currently ongoing, three main theories have evolved : the single lineage theory, the separate lineage theory and the multiple lineage theory. A brief introduction of each of these theories and the studies which support them is presented.

1.2.1. Single lineage theory.

The model for the single lineage theory focusses on the existence of one progenitor B cell, the B-0 cell, which can differentiate into a B-1 or B-2 cell. In this model, differentiation of the progenitor B cell is based upon the antigenic signal delivered to the B-0 cell. If the B-0 cell receives a signal equivalent to a thymus-independent type 2 (TI-2) antigen it will differentiate into a B-1 cell. However, if the B-0 cell receives a signal from a T-cell-dependent antigen, concurrent with interactions with a type 2 T helper cell (T_H2), then it will differentiate into a conventional, B-2 cell. In support of the single lineage theory, Wortis and colleagues have previously shown that stimulation of splenic $CD5^-$ B cells with anti-Ig, a TI-2 antigen, induces CD5 expression (Ying-zi et al., 1991). In addition to acquiring a B-1 phenotype, these induced $CD5^+$ B cells were more resilient to *in vitro* culture than unstimulated, $CD5^-$ B cells. In contrast, splenic $CD5^-$ B cells that were stimulated with LPS remained $CD5^-$ and required added T-cell-derived lymphokines, such as IL-4, to retain viability when cultured *in vitro*.

Although the single lineage theory is based upon murine B cells, further support for the induction of CD5 expression on $CD5^-$ B cells has been provided by studies performed with human B cells. Miller and Gralow have previously shown that phorbol 12-myristic 13-acetate (PMA) induces CD5 expression on normal peripheral blood B cells and malignant B cells (Miller and Gralow, 1984). In addition, Freedman et al. have shown that 12-0-tetradecanoylphorbol 13-acetate (TPA) induces CD5 expression on human B cells (Freedman et al., 1987, 1989).

Finally, Lydyard and colleagues have shown that PMA enhances the expression of CD5 on peripheral blood B cells from patients with rheumatoid arthritis (Youinou et al., 1987). Thus, in order to ascertain a possible role for $CD5^+$ B cells in the immune network, it may be important to elicit the mechanisms involved in CD5 induction.

1.2.2. Separate lineage theory.

The separate lineage theory proposes the existence of two distinct B cell progenitors, one which gives rise to the B-1, B cell subset and one which gives rise to the conventional, B-2, B cell subset. One of the first pieces of evidence for two distinct B cell lineages has been provided by the cell-transfer experiments performed by Hayakawa and colleagues (Hayakawa et al., 1985). In these experiments, Hayakawa and colleagues demonstrated that the cotransfer of Igh^a allotype bone marrow and Igh^b allotype peritoneal B cells, into irradiated adult recipient mice, results in repopulation of B-1 cells which express the Igh^b allotype. In contrast to the B-1 cells, the conventional B cells expressed the Igh^a allotype. Thus, progenitors for B-1 and B-2 cells appear to have distinct anatomical localizations. Consistent with these findings, Hardy and colleagues have shown that although hematopoietic stem cells from fetal and neonatal liver repopulate the B-1 cell subset, hematopoietic stem cells from adult bone marrow do not repopulate the B-1 population when injected into irradiated SCID mice (Hardy and Hayakawa, 1992). Furthermore, in cell transfer studies utilizing 14-day fetal liver from mice which expressed the Igh^b allotype and adult bone marrow from mice

which expressed the Igh^a allotype, Kantor and colleagues have shown that conventional B-2 cells can be reconstituted from either fetal liver or adult bone marrow; however, B-1 cells can only be reconstituted from fetal liver (Kantor et al., 1992). Finally, Kearney and colleagues have shown that progenitors which give rise to B-1 cells, but not those that give rise to B-2 cells are present in 13-day fetal omentum (Solvason et al., 1991). Thus, B-1 cells, but not conventional B cells, develop at a distinct anatomical location, in the fetal omentum. Based on these findings, in order to elicit the role of $CD5^+$ B cells, further studies should be geared towards understanding how these cells interact with other cells in the developing immune system.

1.2.3. Multiple lineage theory.

The multiple lineage theory proposes that B-1a (IgM^{bright}, IgD^{dull}, $CD5^+$), B-1b (IgM^{bright}, IgD^{dull}, $CD5^-$) and B-2 (IgM^{dull}, IgD^{bright}, $CD5^-$) cells each represent a distinct B cell lineage. The first evidence supporting a separate lineage for B-1a and B-1b cells was provided by anti-IgM B cell suppression studies performed by Lalor and colleagues (Lalor et al., 1989). In a series of experiments, B cells were suppressed in newborn mice through treatment with anti-IgM until four weeks of age. Following the withdrawal of the anti-IgM treatment, FACS-analysis of the B-1 cell population revealed that the majority of the detectable B-1 cells which had arisen from the bone marrow expressed the B-1b phenotype. To further examine this phenomena, Kantor and colleagues performed a series of cell-transfer experiments utilizing fetal liver or adult bone marrow as the source of progenitor

cells (Kantor et al., 1992). Kantor's studies have shown that although progenitors for B-1a are abundant in fetal liver, they diminish as the mouse ages and are rare in adult bone marrow. In contrast, progenitors for B-1b cells which are present in the fetal liver exist into adulthood in the bone marrow. Thus, when bone marrow is utilized in cell-transfer studies peritoneal cells with the B-1b (IgM^{bright}, IgD^{dull}, Mac-1^{+}, $CD23^{-}$, $CD5^{-}$) phenotype are reconstituted. In further support of Kantor's findings, Hardy and colleagues have shown that pro-B cells which have undergone D_H-J_H but not V_H-$D_H J_H$ rearrangements isolated from both fetal liver and adult bone marrow will reconstitute the peritoneum with B cells which display the B-1b (IgM^{bright}, IgD^{dull}, $CD5^{-}$) phenotype (Hardy and Hayakawa, 1992). Although these studies provide support for three separate lineages, further functional studies will have to be performed to determine if the cells derived from fetal liver which express the B-1b phenotype perform the same functions as the B-1b cells derived from adult bone marrow.

1.3 Correlations between $CD5^{+}$ B cells and autoimmune pathogenesis.

1.3.1. Murine autoimmune disease.

Previous studies have shown that $CD5^{+}$ B cells are found at increased frequencies in autoimmune murine strains including NZB and NZB-related and Motheaten viable (*me/me*) strains (Hayakawa et al., 1983; Hayakawa et al., 1984; Sidman et al., 1986), suggesting a role for $CD5^{+}$ B cells in murine autoimmune pathogenesis. The autoimmune pathology in NZB mice includes increased levels of serum IgM and the production of autoantibodies reactive with ssDNA and

thymocytes (Shirai et al., 1971; Izui et al., 1978; DeHeer et al., 1978). In addition, splenic B cells from NZB mice spontaneously secrete IgM, when cultured *in vitro* in the absence of exogenous mitogens. To assess whether conventional ($CD5^-$), or $CD5^+$ B cells were responsible for this spontaneous IgM secretion and autoantibody production, Hayakawa and colleagues utilized two-color FACS analysis and sorting to separate conventional and $CD5^+$ splenic B cells, in NZB mice (Hayakawa et al., 1984). The results of Hayakawa's study indicate $CD5^+$ B cells secrete the majority of the IgM spontaneously secreted *in vitro* by splenic B cells. In addition, in NZB mice, virtually all of the IgM autoantibodies reactive with ssDNA and T cells is secreted by $CD5^+$ B cells. Furthermore, similar analysis of splenic B cells from BALB/c mice, a normal murine strain, revealed autoantibody reactive with BrMRBC is produced by the $CD5^+$ B cells in the spleen.

Analysis of serum IgM in a variety of murine strains has shown the levels of serum IgM corresponds to the frequency of $CD5^+$ B cells (Herzenberg et al., 1986). This correlation between serum IgM levels and $CD5^+$ B cell frequencies has been further demonstrated in NFS (*me/me*) *xid* mice (Scribner et al., 1987). In contrast to NFS (*me/me*) mice in which greater than 80% of the splenic B cells express CD5, NFS mice which carry both the *me* and the *xid* gene [NFS (*me/me*) *xid*], do not have detectable levels of splenic $CD5^+$ B cells (Scribner et al., 1987). Analysis of serum antibody production has shown NFS (*me/me*) *xid* mice exhibit reduced levels of serum IgM and autoantibodies to ssDNA, self-T lymphocyte surface antigens and BrMRBC when compared to homozygous (*me/me*) mice.

Furthermore, Ishida and colleagues have shown that the administration of neutralizing IL-10 antibodies to mice from birth to 8 weeks of age, results in the depletion of peritoneal $CD5^+$ B cells and a parallel reduction in serum IgM and anti-BrMRBC autoantibody production (Ishida et al., 1992, 1993).

A correlation between $CD5^+$ B cells and systemic autoimmunity has also been shown in one induced model of systemic autoimmune disease, the murine AIDS model. Hitoshi and colleagues have shown that B6 mice which bear the *xid* mutation and are devoid of $CD5^+$ B cells are resistant to disease induction when infected with the LP-BM5 murine leukemia virus (MuLV) which causes murine AIDS (Hitoshi et al., 1993). In addition, FACS-analysis of $CD5^+$ B cells from normal B6 mice which were infected with LP-BM5 MuLV has shown viral integration occurs in $CD5^+$ B cells. Furthermore, a number of B cell clones which have been established from the murine AIDS model express CD5 (Klinken et al., 1988).

1.3.2. Human autoimmune disease.

Since the identification of $CD5^+$ B cells in mice and the documentation of their high frequency in some spontaneous autoimmune murine strains, numerous clinical studies have been performed to determine the $CD5^+$ B cell frequencies in human autoimmune diseases. An increase in peripheral blood $CD5^+$ B cell frequency has been reported in rheumatoid arthritis (Hardy et al., 1987; Hara et al., 1988; Plater-Zyberk et al., 1988; Brennan et al., 1989), Sjögren's syndrome (Youinou et al., 1988; Dauphinée et al., 1988; Brennan et al., 1989), myasthenia gravis (Ragheb and Lisak, 1990), insulin-dependent diabetes mellitus (Nicoletti et

al., 1990), and Hashimoto's thyroiditis (Suranyi, et al., 1989). Evidence that disease pathology correlates with the frequency of $CD5^+$ B cells has been provided by Dauphinée et al. who have shown that clinical remission of primary Sjögren's syndrome (due to steroid therapy or combined chemotherapy and irradiation) is concurrent with decreases in $CD5^+$ B cell frequencies to normal levels (Dauphinée et al., 1988). Becker et al. have also shown that steroid therapy decreases the $CD5^+$ B cell frequencies in patients with rheumatoid arthritis (Becker et al., 1990). In addition, $CD5^+$ B cells have been found at increased frequencies in anatomical locations associated with disease pathology including the synovial fluid from rheumatoid arthritis patients and the cerebrospinal fluid from patients with multiple sclerosis (Hardy and Hayakawa, 1986; Mix et al., 1990). Furthermore, the monoclonal population of B cells which is expanded in CLL expresses CD5 (Wang et al., 1980; Royston et al., 1980; Calligaris-Cappio et al., 1982; Sthoeger et al., 1989).

One way in which $CD5^+$ B cells may be involved in the pathogenesis of human autoimmune diseases is through the production of autoantibodies. Two previous studies have shown that *in vitro* stimulation of peripheral blood and cord blood $CD5^+$ B cells, from normal individuals, results in the production of a polyreactive rheumatoid factor (RF) which binds IgG Fc with low affinity and exhibits cross-reactivity to ssDNA, insulin and thyroglobulin (Casali et al., 1987; Hardy et al., 1987; Bonagura et al., 1992). In addition, the $CD5^+$ B cells from patients with rheumatoid arthritis produce both a monoreactive RF, which binds

homologous IgG Fc with high affinity, and a polyreactive, low affinity RF (Burastero et al., 1988). Furthermore, although $CD5^+$ B cells from patients with CLL do not spontaneously secrete autoantibodies, *in vitro* stimulation of these $CD5^+$ B cells results in the production of both monospecific and polyspecific autoantibodies to IgG Fc, ssDNA and dsDNA (Sthoeger et al., 1989). Thus, the $CD5^+$ B cells in humans either spontaneously secrete, or can be stimulated to secrete, autoantibodies with specificities which parallel the specificities found in patients with rheumatoid arthritis and systemic lupus erythematosus.

1.4 Functional properties of $CD5^+$ B cells.

1.4.1. Capacity for self-renewal.

In previous studies Hardy and Hayakawa have shown that $CD5^+$ B cells are more resilient to *in vitro* culture than conventional, $CD5^-$ B cells (Hardy and Hayakawa, 1986; Herzenberg et al., 1986). When splenic B cells were cultured *in vitro* for extended periods without the addition of exogenous cytokines, the B cells that remained viable all expressed CD5. Furthermore, when murine splenic B cells were sorted into $CD5^+$ and $CD5^-$ populations before *in vitro* culture, the $CD5^+$ B cells survived longer than the $CD5^-$ B cells. In addition to *in vitro* studies, cell transfer studies performed in irradiated recipient mice have shown that the FACS-sorted $CD5^+$, IgM^+ B cell population in the peritoneum will reconstitute itself when injected into irradiated recipients (Hayakawa et al., 1985). This is in contrast to the conventional B cell population which must be replenished by self-renewing Ig^- progenitors in the adult spleen and bone marrow. The self-renewing capacity of

$CD5^+$ B cells may make these B cells more sensitive to transformation events resulting in increases in B cell lymphomas which express CD5. Previously, the BCL_1 lymphoma which occurs spontaneously in BALB/c mice, the CH lymphomas which appear in aged B10 mice, and a number of the B cell lymphomas which appear in aging NFS/N *v*-congenic mice have all been shown to express CD5 (Hardy et al., 1984; Lanier et al., 1982; Davidson et al., 1984).

1.4.2. Secretion of regulatory molecules.

Little is known about the functional role of $CD5^+$ B cells. One possible mechanism whereby $CD5^+$ B cells may regulate conventional B cells or other $CD5^+$ B cells is through the production of soluble cytokines. In a previous study Brooks et al. showed that supernatants from $CD5^+$, neoplastic, BCL_1 B cells contained a B cell growth factor which synergized with EL-4 supernatant to enhance the proliferation of IL-1-stimulated murine splenic B cells (Brooks et al., 1984). Further experimentation showed this factor, which had a m.w. of approximately 4500, did not have IL-1, IL-2 or IL-5 activity. Sidman et al. have also previously shown that splenic B cells from the C57BL/6J mice which express the viable motheaten gene produce a B-cell maturation factor which induces polyclonal IgM secretion from resting splenic B cell and WEHI-279 tumor B cells and has a m.w. of approximately 15,000 (Sidman et al., 1984). Furthermore, Sherr et al. have demonstrated that a hybridoma generated from $CD5^+$ idiotype specific B cells provides help for an idiotype dominant response to 4-hydroxy-3-nitrophenyl and

that this help is mediated by both a B cell-derived lymphokine and an anti-idiotypic antibody (Sherr et al., 1987).

In addition to lymphokines which effect proliferation and differentiation of B cells, O'Garra and colleagues have shown that $CD5^+$ B cells in the peritoneum produce IL-10, a lymphokine previously shown to suppress cytokine production by type-1 T helper (T_H1) cells (O'Garra et al., 1992). The production of IL-10 may provide some growth advantages to $CD5^+$ B cells, as two studies by Ishida and colleagues have shown that treating mice with neutralizing IL-10 antibodies from birth to 8 weeks of age results in the depletion of the $CD5^+$ B cell population in the peritoneum (Ishida et al., 1992, 1993). In contrast to the $CD5^+$ B cells, administration of anti-IL-10 antibodies did not alter the number, phenotype, or *in vitro* mitogenic responses of conventional B cells.

1.4.3. Autoantibody production.

Previous studies have shown that $CD5^+$ B cells from both mice and humans produce autoantibodies. In mice $CD5^+$ B cells produce IgM autoantibodies to bromelain-treated mouse red blood cells (BrMRBC), ssDNA, and thymocytes (Hayakawa et al., 1984; Ahmed et al., 1989). In humans, both peripheral blood and cord blood $CD5^+$ B cells can be stimulated to produce autoantibodies reactive with the Fc portion of IgG, ssDNA, insulin, and thyroglobulin (Casali et al., 1987; Hardy et al., 1987; Bonagura et al., 1992). In contrast to autoantibody production, previous studies indicate most of antibodies produced to exogenous antigens, such as sheep red blood cells (SRBC) and 2,4,6-trinitrophenyl conjugated keyhole

limpet hemocyanin (TNP-KLH), are produced by conventional B cells (Hayakawa et al., 1984).

A number of previous studies suggest that the repertoire of Ig genes expressed by $CD5^+$ B cells is unique. Examination of the Ig gene repertoire in mice has revealed that $CD5^+$ B cells preferentially express certain V heavy chain genes, including genes from the V_H11 family, the V_H12 family and the V_{11} gene from the S107 family (Pennel et al., 1988, 1989, 1990; Carmack et al., 1990). In addition, $CD5^+$ B cells from neonate mice contain fewer N sequence insertions in their $V_HD_HJ_H$ junctions than conventional B cells (Gu et al., 1990). Furthermore, a few reports have shown that the Ig secreted by $CD5^+$ B cells from NZB and Motheaten viable mice exhibits an increase in lambda light chain expression when compared to the Ig secreted by conventional B cells (Hardy et al., 1986; Hayakawa et al., 1986; Sidman et al., 1986; Slack et al., 1989).

1.5 The CD5-CD72 ligand/receptor pair.

1.5.1. CD5, a signal transducing molecule.

Similar to other T cell proteins that are involved in signal transduction, a number of previous studies utilizing mAb to CD5 have shown that CD5 can provide an activation signal to both murine and human T cells. In the mouse, anti-CD5 mAbs enhance IL-1-mediated T cell proliferation (Lögdberg and Shevach, 1985). In addition, anti-CD5 mAb synergistically augments murine thymocyte proliferation in response to the mitogen phytohemagglutinin, PHA (Lögdberg and Shevach, 1985). In the presence of PHA, mAb to CD5 increases both intracellular calcium

and the secretion of IL-2 from thymocytes (Lögdberg and Shevach, 1985; Stanton et al., 1986). Although anti-CD5 mAb can augment thymocyte responses to both IL-1 and the mitogen PHA, addition of anti-CD5 mAb alone does not induce thymocyte activation (Stanton et al., 1986). However, as shown by Stanton and colleagues, stimulation of thymocytes with anti-CD5 mAb in the absence of mitogens does induce IL-2R expression on a small proportion of thymocytes (Stanton et al., 1986).

In 1986, Ceuppens and Baroja demonstrated that anti-CD5 mAb could also provide a stimulatory signal to human peripheral blood T cells (Ceuppens and Baroja, 1986). Results from their study show that in the presence of CD3 receptor cross-linking, CD5 mAb initiates T cell proliferation and enhances IL-2R expression and IL-2 production. Two separate studies have shown that immobilized anti-CD5 mAb induces T cell proliferation in the presence of either the protein kinase C (PKC) activator phorbol 12-myristate 13 acetate (PMA) or IL-2 (Vandenberghe and Ceuppens, 1991; Verwilghen et al., 1990). In addition, stimulation of human peripheral blood T cells with CD5 mAb in the presence of monocytes induces a rise in intracellular calcium, activates PKC and tyrosine kinase activity, induces production of IL-2 and enhances IL-2R expression (Ledbetter et al., 1987; Ceuppens and Baroja, 1986; Verwilghen et al., 1990; Spertini et al., 1991; Alberola-Ila et al., 1992). Although anti-CD5 mAb can provide a co-stimulatory signal to human peripheral blood T cells in the presence of mitogens and/or monocytes, previous studies have shown that the mAb alone does not provide a strong

enough signal to activate T cells (Ceuppens and Baroja, 1986; Verwilghen et al., 1990; Spertini et al., 1991; Vandenberghe and Ceuppens, 1991).

In addition to the T cell activation studies, two previous studies have shown that stimulation of the T-cell antigen receptor (TCR) complex with anti-CD3 antibody leads to both the association of CD5 with the TCR complex and CD5 phosphorylation (Osman et al., 1992; Davies et al., 1992). This close association of CD5 with the TCR, and CD5 modification upon activation through the TCR complex, further supports a role for CD5 as a T cell signal transducing receptor. Currently, a similar role for CD5 on murine and human B cells has not been investigated. However, Fox and colleagues have previously shown that the CD5 molecule expressed on B cells is biochemically similar to the CD5 molecule expressed on T cells (Fox et al., 1982). Furthermore, both murine and human antibodies which were raised against the CD5 molecule expressed on T cells, will bind to and immunoprecipitate the CD5 protein which is expressed on B cells. Thus, CD5 may also provide some type of stimulatory signal to the B cells which express it.

1.5.2. CD72, the ligand for CD5.

In 1991, CD72 (Lyb-2) was identified as the ligand for human CD5 (Van de Velde et al., 1991). In a series of experiments, Van de Velde and colleagues utilized a protocol in which purified, biotin-labelled CD5 (biotin-CD5) was used to probe a variety of hematopoietic cell lineages. The results of their study showed that biotin-CD5 bound both pre-B and B cell lines; however it did not bind T cells,

monocytes, granulocytes or plasma cell lines. Further analysis revealed that co-incubation with an excess of the anti-CD5 mAb Leu-1 inhibited the binding of biotin-CD5 to human B cells. In addition, four antibodies specific for the B cell protein CD72 inhibited the binding of biotin-CD5. To further confirm their initial findings, Van de Velde and colleagues transfected both mouse L cells and Jurkat T cells with the cDNA for human CD72. Although biotin-CD5 did not bind to mouse L cells or Jurkat T cells which had not been transfected, biotin-CD5 did bind to the cells which were transfected with human CD72 cDNA. Using a similar protocol, Luo and colleagues have shown that Lyb-2 (CD72) is the ligand for murine CD5 (Luo et al., 1992). In addition, Luo's study showed that murine CD5 can bind human CD72, and human CD5 can bind murine Lyb-2.

1.5.3. Structural features of CD72.

The murine Lyb-2 (CD72) protein is a 45-kDa cell surface glycoprotein encoded by a single genetic locus on the mouse chromosome 4 (Sato and Boyse, 1976; Tung et al., 1977; Shen et al., 1977). Lyb-2 is exclusively expressed on cells of the B-lymphocyte lineage including pre-B cells, B cells and a variety of B cell lymphomas. Although present during the earlier stages of B cell development, neither Lyb-2 mRNA nor the Lyb-2 protein are expressed by antibody-secreting plasma cells (Yakura et al., 1980). Sequence analysis of cDNA from three allelic forms of Lyb-2 (Lyb-2^a, Lyb-2^b, and Lyb-2^c) has revealed that the cytoplasmic domain, transmembrane domain, and membrane proximal region of the extracellular domain are highly conserved, with approximately 95% sequence

identity between the three murine alleles (Robinson et al., 1992). In contrast, the membrane distal portion of the extracellular domain exhibits a high degree of polymorphism with approximately 75% sequence identity between the three alleles.

In 1990 cDNA for CD72, the human homolog of the murine Lyb-2 B cell antigen, was isolated and sequenced by Von Hoegen and colleagues (Von Hoegen et al., 1990). The expression of human CD72 parallels the expression of Lyb-2 on murine B cells in that it is not expressed on antibody secreting cells but is expressed on pre-B cells, B cells, and some EBV-transformed B cell lines (Von Hoegen et al., 1990). Sequence comparisons between the murine Lyb-2^a cDNA and the human CD72 cDNA demonstrate there is approximately 60% sequence identity between the mouse and human cytoplasmic domains, transmembrane domains and membrane proximal region of the extracellular domains (Von Hoegen et al., 1990). In contrast, in the membrane distal region of the extracellular domain there is only 31% sequence identity between the murine and human Lyb-2 cDNAs. Both human and murine CD72 (Lyb-2) represent receptors with inverted membrane orientation, thus their carboxyl terminus is exterior to the cell (Nakayama et al., 1989). This unique inverted membrane orientation has been shown for a variety of signal transducing receptors, including the B cell protein CD23 and the asialoglycoprotein receptor, suggesting CD72 may function as a signal transducing receptor.

1.5.4. CD72, a signal transducing molecule.

A number of previous studies in both mice and humans support a role for Lyb-2 (CD72) as a signal transducing receptor. Subbarao and Mosier have previously shown that anti-Lyb-2 mAb can transform resting splenic B cells into blast cells (Subbarao and Mosier, 1983). Antibody to murine Lyb-2 also induces B cell proliferation, increases in intracellular calcium and increases in surface Ia expression (Subbarao et al., 1988). Two previous studies have shown that stimulation with anti-Lyb-2 mAb and anti-IgM antibody results in synergistic increases in B cell proliferation (Yakura et al., 1986; Laurindo et al., 1987). Monoclonal antibody to Lyb-2 also synergizes with IL-4 and TNP-Ficoll to induce proliferation of TNP-specific B cells (Snow et al., 1986). In addition a number of studies have indicated that the f(ab) fragments of Lyb-2 mAbs provide stimulation equivalent to the entire Lyb-2 mAb molecule (Subbarao and Mosier, 1983; Laurindo et al., 1987; Subbarao et al., 1988). Thus, extensive cross-linking of the CD72 antigen does not appear to be required for the stimulatory signal.

Consistent with the studies performed in mice, Kamal et al., have shown that the stimulation of human tonsillar B cells with anti-CD72 mAb induces B cell proliferation, increased HLA-DR expression, and a G_0 to G_1 cell-cycle transition (Kamal et al., 1991). Proliferation studies have shown that anti-CD72 synergizes with IL-4, PMA, and immobilized anti-IgM signals to increase B cell proliferation (Kamal et al., 1991). In addition, Katira and colleagues have shown anti-CD72 mAb enhances IL-4 induced expression of both cell-associated and soluble CD23

(Katira et al., 1992). In parallel to the murine studies, antigen receptor cross-linking is not required to transduce a signal (Katira et al., 1992).

When combined with the previous studies which indicate CD5 may function as a signal transducing receptor on T cells, these studies suggest CD5 and CD72 may be involved in a bi-directional communication between T and B cells. Furthermore, the expression of CD5 on a unique subset of B cells in both mice and humans, and CD72 on pre- and mature B cells, provides a mechanism for B-B interactions. In order to more clearly understand the functions of CD5 and CD72 on murine and human B cells, further analysis are necessary.

1.6 Summary.

In the past decade a unique population of B cells has been defined in both mice and humans which expresses CD5. In addition to exhibiting a unique phenotype (IgM^{bright}, IgD^{dull}, $CD5^+$), $CD5^+$ B cells appear early in ontogeny at a site distinct from conventional B cells, in the fetal omentum, supporting the theory that $CD5^+$ and conventional B cells are derived from separate lineages. Currently, the role $CD5^+$ B cells play in the function of the immune network has not been clearly defined. However, the early appearance of $CD5^+$ B cells at distinct anatomical locations, in both mice and humans, suggests these cells may be involved in regulating immune responses early in ontogeny. In addition, correlations between increases in $CD5^+$ B cell frequencies and autoimmune pathology have been shown in both murine and human diseases which involve polyclonal B cell activation.

One way $CD5^+$ B cells may regulate conventional B cells and contribute to

autoimmune pathogenesis is through the production of soluble factors and/or the secretion of autoantibodies. Alternatively, B cells which express CD5 may directly interact with pre- and mature B cells which express CD72, the ligand for CD5, resulting in the stimulation of one or both B cell populations. In order to more clearly understand the role $CD5^+$ B cells play in the immune network, the studies presented in this dissertation were undertaken, based on the following *hypothesis:* **$CD5^+$ B cells regulate the functions of conventional B cells through direct interactions between CD5 and its ligand, CD72 and/or the secretion of soluble factors. Furthermore, if $CD5^+$ B cells contribute to the pathogenesis of autoimmune disease by providing a stimulatory signal to conventional B cells, then increases in $CD5^+$ B cell frequencies should correlate with disease progression.**

2.0 MATERIALS AND METHODS.

2.1 *In vitro.*

2.1.1. Preparation of B cells.

Splenic B cells from 13 to 22 week old BALB/c BYJ female mice (The Jackson Laboratory, Bar Harbor, ME) were depleted of T lymphocytes by anti-L3T4 and anti-Thy1.2 antibodies plus complement. To remove adherent cells, the T-depleted cell preparations were then passed over two G-10 Sephadex (Sigma Chemical Company, St. Louis, MO) columns and resuspended at $1X10^6$ cell/ml in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 2mM L-glutamine, 5 X 10^{-5}M 2-mercaptoethanol, and 10 μg/ml gentamicin. Resting and *in vivo*-activated splenic B cells were separated on a Percoll density gradient. Briefly, 10 ml aliquots of 75% (d=1.0975), 63% (d=1.082), 55% (d=1.0715) and 30% (d=1.039) Percoll in HBSS were layered in a 50 ml centrifuge tube. Approximately $2 X 10^8$ spleen cells (in 10 ml of medium) were layered on top of the 30% Percoll and centrifuged at 1600g, 30 min., 4°C. Separation into resting and activated populations was confirmed by staining $1 X 10^6$ cells with acridine orange (AO) as previously described (Traganos et al., 1977), followed by flow cytometric analysis on an Ortho-Diagnostics 50H cytofluorograf. B cells from the peritoneal cavity of BALB/c BYJ mice were also prepared by T cell depletion.

2.1.2. Neoplastic B cell clones.

BCL_1-3B3 cells and CH12.LX cells were maintained in 5% FCS RPMI 1640 media supplemented as above, at 37°C in an atmosphere of 6% CO_2 in air. Approximately three days prior to the experiment, both the BCL_1-3B3 cells and the CH12.LX cells were transferred to 3% FCS RPMI 1640 medium without 2-mercaptoethanol. 225-11 cells were maintained in 10% FCS RPMI 1640 medium supplemented as above, at 37°C in an atmosphere of 10% CO_2 in air. To eliminate background proliferation and ^{3}H-thymidine incorporation, all of the neoplastic B cells were irradiated with 4060R, prior to use. The three neoplastic B cell lines were negative for contaminating mycoplasma when tested with a mycoplasma rapid detection test kit (Gen-Probe Incorporated, San Diego, CA).

2.1.3. Pre-B cells.

High density bone marrow cells from BALB/c mice either uninfected, HDBM, or infected with a retroviral vector expressing *v-Ha-ras*, SV(X)-Ha-ras (Schwartz et al., 1986) were a kind gift of Dr. Richard Schwartz at Michigan State University. Both HDBM and SV(X)-Ha-ras were cultured by the procedure of Whitlock and Witte (1982). Following infection the pre-B cells were expanded on feeder layers of adherent bone marrow cells, as previously described (Whitlock et al., 1983). For use in experiments both HDBM and SV(X)-Ha-ras were resuspended in RPMI 1640, supplemented as above, and irradiated at 4060R.

2.1.4. Cytofluorometric analysis.

Surface expression of CD5 by each of the pre-B and neoplastic B cell clones was measured on an Ortho-Diagnostics 50H cytofluorograf. Briefly, $1X10^6$ cells were stained with fluoresceinated (FITC) anti-CD5 (clone 53-7.313, PharMingen, San Diego, CA) or a FITC-labelled rat IgG_{2a} isotype control (PharMingen, San Diego, CA). Following a 20 minute incubation with the FITC-labelled antibodies, cells were washed three times in PBS, resuspended in 1 ml of 1% formaldehyde fixative and stored at 4°C until analysis. To prevent non-specific Fc receptor binding, the cells were preincubated with Fc Block (PharMingen, San Diego, CA), for 5 min. at 4°C prior to staining.

2.1.5. B cell-induced B cell proliferation.

Triplicate cultures of BALB/c spleen cells were set up in a 96-well plate at $1X10^5$ cells/well in 100 μl of RPMI 1640, supplemented as above. BALB/c spleen cells were stimulated with 10 μg/ml dextran sulfate, DxS (Sigma Chemical Company, St. Louis, MO), or 5 μg/ml goat anti-mouse immunoglobulin, GAMIg (Jackson ImmunoResearch, West Grove, PA). Irradiated neoplastic B cells or pre-B cells were added to the splenic B cells at a concentration of $3X10^4$ cells/well, in the presence or absence of interleukins at the following concentrations: human rIL-2 (R&D Systems, Inc., Minneapolis, MN) 2.5 ng/ml; murine rIL-4 (R&D Systems, Inc., Minneapolis, MN) 2.0 ng/ml; murine rIL-5 (R&D Systems, Inc., Minneapolis, MN) 3.2 ng/ml; human rIL-6 (Genzyme, Boston, MA) 50, 100, 200, and 250 U/ml; and mouse rIL-10 (PharMingen, San Diego, CA) 2.5, 5, 10, and 20 U/ml. Cultures

were incubated at 37°C in an atmosphere of 10% CO_2 in air for 72 hrs. At 66 hrs. the cells were pulsed with 5μCi/ml ^{3}H-thymidine (NEN Dupont, Boston, MA). Following a 6 hr. pulse, cells were harvested and the counts per minute (CPM) for triplicate wells determined. Stimulation indices were calculated as follows: [(sample CPM - background CPM for irradiated cells in the presence of relevant lymphokines)] ÷ CPM for BALB/c only.

2.1.6. B cell-induced B cell differentiation.

For the differentiation assays, cells were cultured as described for the proliferation assay. Following 5 or 7 days of incubation, 100 μl of supernatant was collected from each well and IgM and IgG concentrations were determined with an enzyme-linked immunosorbent assay (ELISA).

2.1.7. ELISA.

Nunc-Immuno Plate MaxiSorp ELISA plates (VWR Scientific, Chicago, IL) were coated by overnight incubation (4°C) with 100 μl/well goat anti-mouse IgG + IgM (Jackson ImmunoResearch, West Grove, PA) at a concentration of 15 μg/ml in 0.1 M bicarbonate buffer (pH 9.6). Coated plates were washed three times with 0.01 M PBS containing 0.05% Tween 20 (PBS-Tween). To block nonspecific protein binding 1% (v/v) FCS in PBS (1% FCS-PBS) was added to each well and plates were incubated at room temperature for 90 min., followed by three washes in PBS-Tween. Prepared plates were stored at -20°C until use.

To determine Ig concentrations, Ig reference standards (Sigma Chemical Company, St. Louis, MO) or supernatant samples were diluted in 1% FCS-PBS and

100 μl was added to appropriate wells of the GAMIg coated ELISA plates. Plates were sealed and incubated for 90 min., at 37°C followed by three PBS-Tween washes. Alkaline phosphatase-conjugated goat anti-mouse IgM (Jackson ImmunoResearch) diluted 1:2500 in 1% FCS-PBS, alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:1500 in 1% FCS-PBS or alkaline phosphatase-conjugated goat anti-mouse IgA (Sigma Chemical Company) diluted 1:10,000 in 1% FCS-PBS were added to each well and the plates were incubated for 90 min., at 37°C followed by three PBS-Tween washes. Plates were developed by incubation with Sigma 104 phosphatase substrate (Sigma Chemical Company) at 1 mg/ml in substrate buffer, 60 min., 37°C. The enzyme reaction was stopped with 1 M NaOH and absorbance was measured at 405 nm on an ELISA plate reader (Molecular Devices, Palo Alto, CA).

2.1.8. Determining contact dependency of $BCL_1$3B3-mediated help.

Direct cell interactions between the B cell populations were studied using Transwells® (Costar, Cambridge MA). Briefly, 0.5 ml of $1X10^6$ BALB/c splenic B cells were cultured in a 24-well cluster plate with 0.1 ml of irradiated neoplastic B cells at $1X10^6$ cells/ml in the presence or absence of lymphokines. In some of the wells the two B cell populations were separated with a 0.4 μm polycarbonate membrane (Transwell®). The cells were incubated for 66 hrs. at 37°C, in an atmosphere of 10% CO_2 in air. At 66 hrs. the top chamber containing the irradiated neoplastic B cells was removed and discarded. Approximately 0.2 ml was transferred to each of three wells in a 96-well plate. The cells were then

pulsed and harvested as described above for the B cell proliferation assays.

2.1.9. Fixation of neoplastic B cells.

BCL_1-3B3 B cells were washed in PBS and resuspended at approximately 5 X 10^6 cells/ml. The BCL_1-3B3 cell suspension was incubated with an equal volume of 0.8% paraformaldehyde fixative (in double distilled H_2O) for 5 min. at room temperature. Following the incubation an equal volume of 0.2M lysine was added, the cells were washed three times in medium and resuspended. Fixed BCL_1-3B3 cells were incubated for 3 hr., at 37°C, washed, counted, and resuspended at 3 X 10^5 cells/ml for use in proliferation assays. As a control for protein synthesis 3 X 10^5 BCL_1-3B3 cells were cultured for 24 hrs with 2 μCi ^{3}H-leucine (ICN Radiochemicals, Irvine, CA) before or after fixation with paraformaldehyde. The mean count per minute for triplicate wells for BCL_1-3B3 was 1855 before fixation and 156 after fixation. Without the addition of ^{3}H-leucine the mean count per minute for triplicate wells was 35 and 31, respectively.

2.1.10. Antibody inhibition assays.

To determine if the B-cell-mediated help could be inhibited with mAb to various surface molecules or lymphokines, the irradiated or paraformaldehyde-fixed BCL_1-3B3 cells were preincubated for 45 min., 4°C, with the following: 12.5, 25, or 50 μg/ml anti-CD5 (clone 53-7.313, a rat IgG2a, PharMingen, San Diego, CA); 10 μg/ml anti-CD11a (clone 121/7, a rat IgG_{2a}, Endogen Inc., Boston, MA); 10 μg/ml anti-CD18 (clone 2E6, a hamster IgG, Endogen Inc.); 10 μg/ml anti-CD54 (clone 3E2B, hamster IgG, Endogen Inc.); 25 μg/ml anti-CD40L (clone MR1, a

hamster IgG, Mab was the kind gift of Dr. Randy Noelle, Department of Microbiology, Dartmouth Medical School, Lebanon NH); 25 μg/ml anti-I-A^d (clone AMS-32.1, a mouse IgG_{2b}, PharMingen); 25 μg/ml anti-I-E^k (clone 14-4-4s, a mouse IgG_{2a}, PharMingen); 75 μg/ml anti-murine IL-6 (clone MP5-32C11, a rat IgG_{2a}, Endogen); 75 μg/ml anti-murine IL-10 (clone JES5-2A5, a rat IgG_1, Endogen); or 25 μg/ml anti-human IL-2 (rabbit IgG, Endogen). Isotype matched controls included 25 μg/ml rat IgG_{2a} (PharMingen), 25 μg/ml hamster IgG (Jackson ImmunoResearch, West Grove, PA), 25 μg/ml mouse IgG_{2a} (Sigma Chemical Co., St. Louis, MO) and 75 μg/ml anti-murine IL-2 (clone JES6-1A12.9, a rat IgG_{2a}, Endogen). Following the preincubation with the mAb, the cells were cultured, pulsed and harvested as noted above for the proliferation assays.

2.1.11. Statistical analysis.

The Mann-Whitney statistical test for non-parametric data was used for statistical analysis.

2.2 *In vivo.*

2.2.1. Induction of murine acquired immunodeficiency syndrome (MAIDS).

Female C57Bl/6 mice were obtained at approximately 6 weeks of age from The Jackson Laboratory (Bar Harbor, ME). At 12 weeks of age, female C57Bl/6 mice were inoculated with 0.2 ml of LP-BM5 MuLV via the intraperitoneal route. LP-BM5 MuLV pool #3 was a kind gift of Dr. Donald Mosier, MBI, La Jolla, CA. Two mice were inoculated for each time point of study and lymphoid cells were pooled for analysis. Uninoculated female C57Bl/6 mice were used as age-

matched controls for each time point of study.

2.2.2. Induction of chronic graft-vs-host disease (cGvHD).

Female DBA/2 donor and C57Bl/6 x DBA/2 F_1 (BDF_1) recipient mice were obtained at approximately 8 weeks of age from the Jackson Laboratory (Bar Harbor, ME). The spleen and the axillary, cervical and mesenteric lymph nodes were removed from female DBA/2 donor mice. Single cell suspensions were prepared from spleen and lymph nodes in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 5mM HEPES, 5 X 10^{-5} M 2-mercaptoethanol, and 10 μg/ml gentamicin (M.A. Bioproducts, Walkersville, MD). The pooled donor cells were then washed three times in RPMI 1640 supplemented media and counted. To remove B cells approximately 1 X 10^8 pooled donor cells were incubated for 1 hour on tissue culture plates coated with 50 μg of goat anti-mouse IgG + IgM (GAMIg; Jackson ImmunoResearch, West Grove, PA). Following the incubation non-adherent cells were removed with a pasteur pipette, washed and counted. Fourteen to twenty week old BDF_1 mice were injected via the intraperitoneal route with 3.5 X 10^7 (Series I), 4.9 X 10^7 (Series II) or 3.3 X 10^7 (Series III) non-adherent DBA/2 donor cells. Two recipient mice were pooled for analysis. Non-injected female BDF_1 mice were used as age-matched controls for each time point of study.

2.2.3. Induction of collagen-induced arthritis (CIA).

Male B10.RIII (71NS)/SnJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). At approximately 8 weeks of age arthritis was induced via

intradermal injection at the base of the tail of 100 μg porcine type II collagen (PII), prepared as previously described (Griffiths et al., 1981) in 0.1 N acetic acid, emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI), 1:1. Seven days following the initial immunization the experimental mice were boosted with 100 μg PII collagen in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI). Two mice were immunized for each time point of study and lymphoid cells were pooled for analysis. Non-immunized male B10.RIII (71NS)/SnJ mice were used as age-matched controls.

2.2.4. Cell preparation.

Spleens were removed from treatment or age-matched control mice at the indicated time following infection or injection. The spleen were gently teased to make single cell suspensions. Lymphocyte viability was determined using trypan blue dye exclusion analysis. Splenocytes used for cytoflourometric analysis were layered over Lympholyte-M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and centrifuged at 943g for 20 min., 21°C, to deplete dead cells. Peritoneal exudate cells (PEC) were collected by rinsing the peritoneal cavity with Hanks Balance Salt Solution (M.A. Bioproducts, Walkersville, MD). The PEC were then washed and counted as described for the splenic cells.

2.2.5. Analysis of Ig secretion.

Splenic lymphocyte suspensions of 1 X 10^6 viable cell/ml in RPMI 1640 media (supplemented as described above) were cultured at 0.2 ml per well, in 96-well flat-bottom culture plates. Cells were incubated in an atmosphere of 10% CO_2

in air at 37°C. Supernatants were collected from the wells at 12 hour time intervals, from 12 to 84 hours after initiation of the *in vitro* culture. To determine the concentrations of spontaneously secreted Ig, supernatants were analyzed via a radioimmunoassay (RIA), or via an ELISA as described under *in vitro* materials and methods. Antibody specificities were also determined with an ELISA.

2.2.6. RIA.

Flexible U bottom plates (VWR Scientific, Chicago, IL) were coated by overnight incubation (4°C) with 100 μl/well goat anti-mouse IgG + IgM (GAMIg, Jackson ImmunoResearch, West Grove, PA) at a concentration of 10 μg/ml in PBS-azide. Coated plates were washed five times with PBS-azide. To block nonspecific protein binding, 200 μl of 1% (V/V) FCS in PBS-azide (1% FCS-PBS-azide) was added to each well and plates were incubated at room temperature for 2 hrs., followed by five washes with PBS-azide. Prepared plates were stored at 4°C, in PBS-azide until use.

To determine Ig concentrations, Ig reference standards (Sigma Chemical Company, St. Louis, MO) or supernatant samples were diluted in 1% FCS-PBS-azide and 100 μl was added to the GAMIg coated plates. Plates were incubated overnight at 4°C, followed by five washes with PBS-azide. ^{125}I-labelled GAMIg, μ chain specific antibody (Jackson ImmunoResearch) was added to the plates at 1 X 10^5 CPM/ml and the plates were incubated overnight, at 4°C. Following the overnight incubation, the I^{125}-labelled GAMIg was removed and the plates were washed five times with PBS-azide. The CPM for bound ^{125}I-labelled GAMIg were

determined on a Beckman 5500 gamma counter (Beckman Instruments, Inc., Fullerton, CA).

2.2.7. ELISA.

Antibody specificities were determined on plates coated with ssDNA, collagen, mouse IgG or TNP:BSA. Single stranded DNA plates were coated with 150 μl/well poly-l-lysine (U.S. Biochemical Corporation, Cleveland, OH) at 50 μg/ml in 0.1 M carbonate buffer (pH 9.6) for 90 min., at room temperature. Plates were washed three times with PBS-Tween and incubated overnight at room temperature with 150 μl/well of 3 mg/ml calf thymus ssDNA (Sigma Chemical Company) in 0.1 M carbonate buffer. To determine relative anti-ssDNA concentrations, samples were compared to an anti-DNA monoclonal antibody standard (Boehringer Mannheim Corporation, Indianapolis, IN). Anti-collagen concentrations were determined on plates coated overnight, 4°C, with 100 μl/well of 30 μg/ml porcine type II collagen in 0.15 M KPO_4 buffer, pH 7.6. A standard positive serum sample was used to verify collagen binding to the plates. IgG plates were coated overnight, 4°C, with 100 μl/well of 16 μg/ml mouse IgG (Jackson ImmunoResearch) in 0.1 M carbonate buffer. IgG binding to the plates was verified using an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Company). Anti-TNP:BSA levels were determined on plates coated overnight at 4°C with 100 μl/well of 16 μg/ml TNP:BSA (a gift of Linda Rasooly, Michigan State University, E. Lansing, MI) in 0.1 M carbonate buffer. To determine relative anti-TNP:BSA levels samples were compared to an anti-TNP

standard, MOPC 315 (Sigma Chemical Company). Conditions used to determine antibody concentrations in supernatant and serum samples were the same as described for the isotypes.

2.2.8. Antibodies.

The mAb used for three-color flow cytometric analysis were: B3B4, a rat IgG_{2a} anti-murine $Fc_{\epsilon}R$; b-7-6, a rat IgG_1 anti-murine IgM; 53.7.313, a rat IgG_{2a} murine Ly 1; GK1.5, a hybridoma IgG_{2b} anti-murine CD4; and 53.6.72, a rat IgG_{2a} anti-murine CD8. The antibodies were biotinylated (biotin) or fluoresceinated (FITC) following standard protocols. Cyanine labelled antibodies were conjugated with cyanine 5.18 dye (a kind gift of Dr. Alan Waggoner, Carnegie-Mellon University, Pittsburgh, PA) as previously described by Mujumdar et al., (1989). Biotinylated antibodies were revealed with R-phycoerythrin avidin (PE-avidin, Molecular Probes Inc., Eugene, OR). Rat IgG (Jackson ImmunoResearch) was used as a isotype matched control. To eliminate background staining due to Fc receptor binding Fc Block (PharMingen, San Diego, CA) was added to the tubes during staining.

2.2.9. Cytoflourometric analysis.

1 X 10^6 splenic or peritoneal exudate cells were resuspended in 20 μl of staining buffer (0.1% NaN_3, 5% calf serum, 1X HBSS) and incubated with mAbs for 20 min., at 4°C. Following the primary incubation the cells were washed in staining buffer and incubated with PE-avidin for 20 min., at 4°C. Stained cells were fixed in 1% formaldehyde fixative and stored at 4°C until the time of analysis. A

Becton Dickinson FACS 440 equipped with a primary argon laser and a secondary dye head (Rhodamine 6G) laser was utilized for two-color flow cytometric analysis. FITC/PE spectral overlap was electronically compensated. Data was analyzed using Electric Desk® software.

3.0 B CELL-MEDIATED, CONTACT-DEPENDENT HELP: $CD5^+$ NEOPLASTIC B CELLS ENHANCE B CELL RESPONSES TO INTERLEUKIN 2.

3.1 Rationale.

T helper cells are capable of stimulating B cell proliferation and differentiation by a two step process (Parker, 1993). The first step involves direct interaction between cell surface ligand/receptor pairs found on T and B cells, including but not limited to: TCR:CD3, CD4/MHC Class II, CD40L/CD40, LFA-1/ICAM-1, and CD2/LFA-3 (Parker, 1993). As a result of these direct T-B cell interactions, T cells are activated to secrete lymphokines and B cells express lymphokine receptors. Thus, the second step in T cell-mediated help is the stimulation of B cell growth and differentiation by T cell-derived lymphokines. In addition to T cell-mediated help, there have been a few reports of B cell-mediated help. Uher and Dickler (1988) have shown that irradiated, *in vivo*-activated, splenic B cells augment both proliferation and differentiation of anti-μ-stimulated splenic B cells. This B cell-mediated help is not genetically restricted and can be reconstituted with a combination of plasma membranes plus supernatant from the activated B cells, but not by either alone. Similarly, Armitage and Goff (Armitage and Goff, 1988) have shown that $CD23^+$ tonsillar B cells augment proliferative responses of $CD23^-$ tonsillar B cells to anti-IgM plus IL-4, anti-IgM plus anti-CDw40, 12-O-tetradecanoyl-phorbol 13-acetate and *Staphylococcus aureus* Cowan I. The B cell-mediated help observed by Armitage and Goff is also dependent upon both cell-cell contact and soluble factors of which soluble CD23 is a component. In addition, Saito et al. (Saito et al., 1991) have shown that mitomycin C-treated B cells from aged NZB mice are capable of stimulating young adult NZB B cells to secrete Ig. In contrast

to the previous studies, the stimulation provided by aged NZB B cells can be blocked by antibody to MHC class II antigens. An absolute requirement for cell-cell contact is brought into question by Sherr et al. (Sherr et al., 1987) who found that soluble factors played a dominant role. They demonstrated that a hybridoma generated from $CD5^+$ idiotype-specific B cells provides help for an idiotype dominant response to 4-hydroxy-3-nitrophenyl. This help is mediated by anti-idiotypic antibody and a B cell-derived lymphokine.

As is evident from the previous studies, there is controversy surrounding the mechanisms involved in B cell-mediated help. Specifically, it is unclear whether or not help occurs via direct cell-cell contact and/or soluble factors, and whether or not the B-cell-mediated help involves MHC class II molecules. Furthermore, the B cell population mediating this help has not been clearly defined. The B helper population reported by Sherr et al., was $CD5^+$. Murine $CD5^+$ B cells belong to a unique subset which is found at increased frequency in both the 60%/65% interface Percoll fraction used by Uher and Dickler, and in aged NZB mice (Kipps, 1989). However, in contrast to the B helper population described by Armitage and Goff, murine $CD5^+$ B cells are $CD23^-$ (Waldschmidt et al., 1991). Although $CD5^+$ B cells are prime candidates for the B helper population, such activity can not yet be definitively attributed to these cells. Thus, in the present study, a $CD5^+$ neoplastic B cell clone was utilized to evaluate the capacity of $CD5^+$ B cells to provide contact-dependent help for B cell responses.

3.2 Results.

3.2.1. $CD5^+$ neoplastic B cells enhance the responsiveness of unfractionated splenic B cells to IL-2.

Although a few previous studies imply B cells can provide help for the proliferation and differentiation of other B cells (Sherr et al., 1987; Uher and Dickler, 1988; Armitage and Goff, 1988; Saito et al., 1991), the B cell population capable of mediating this help has not been clearly defined. If the previous studies of B cell-mediated help were detecting the same cell population, the phenotype of this helper B cell would be a large, $CD23^+$, $CD5^+$ B cell. CD5 is the ligand for a B cell-restricted molecule CD72 (Luo et al., 1992), thus providing a ligand/receptor pair potentially suitable for mediating a B-B interaction.

Therefore, the helper activity of a $CD5^+$ B cell line, BCL_1-3B3, was evaluated in a manner parallel to the classic demonstrations of T cell-mediated help. Irradiated, $CD5^+$, BCL_1-3B3 B cells were combined with splenic B cells in the presence or absence of interleukins. To measure the helper capacity of the BCL_1-3B3 cells, proliferation of the splenic B cells was monitored by ^{3}H-thymidine incorporation. Whenever IL-2 was present, irradiated BCL_1-3B3 cells increased the proliferation of splenic B cells (Figure 1). There was a significant ($P < 0.05$) increase in the stimulation index when irradiated BCL_1-3B3 cells were added to IL-2 stimulated splenic B cells, with a mean fold increase of 11.8 ± 2.1 SEM (n=19). The IL-2/IL-4 combination allowed maximal proliferation of splenic B cells. The mean augmentation by CD5+ B cells of the IL-2/IL-4-mediated proliferation was

Figure 1. $CD5^+$ B cells stimulate increases in the proliferation of unfractionated splenic B cells, in the presence of IL-2. BALB/c splenic B cells at 5 X 10^5 cells/ml were cultured alone (diagonal hatched bars) or with 1.5 X 10^5 cells/ml irradiated (4060R) BCL_1-3B3 cells (solid bars), in the presence or absence of lymphokines, at 37°C in an atmosphere of 10% CO_2 in air. At 66 hrs. the cells were pulsed with 1 μCi/well of ^{3}H-thymidine. Following a 6 hr. pulse, cells were harvested and the counts per minute (CPM) for triplicate wells determined. Stimulation indices were calculated as follows: [(sample CPM - background CPM for irradiated BCL_1-3B3 cells plus interleukins)] ÷ CPM for BALB/c spleen cells. The mean CPM for BALB/c spleen cells was 400 ± 135. The mean background CPMs for irradiated BCL_1-3B3 cells in the presence of lymphokines were as follows: media = 514, IL-2 = 390, IL-4 = 264, IL-5 = 265, IL-2/IL-4 = 292, IL-2/IL-5 = 804, IL-4/IL-5 = 307, and IL-2/IL-4/IL-5 = 298. Lymphokine concentrations were: IL-2, 2.5 ng/ml; IL-4, 2.0 ng/ml; and IL-5, 3.2 ng/ml. The experiment shown is representative of nineteen experiments.

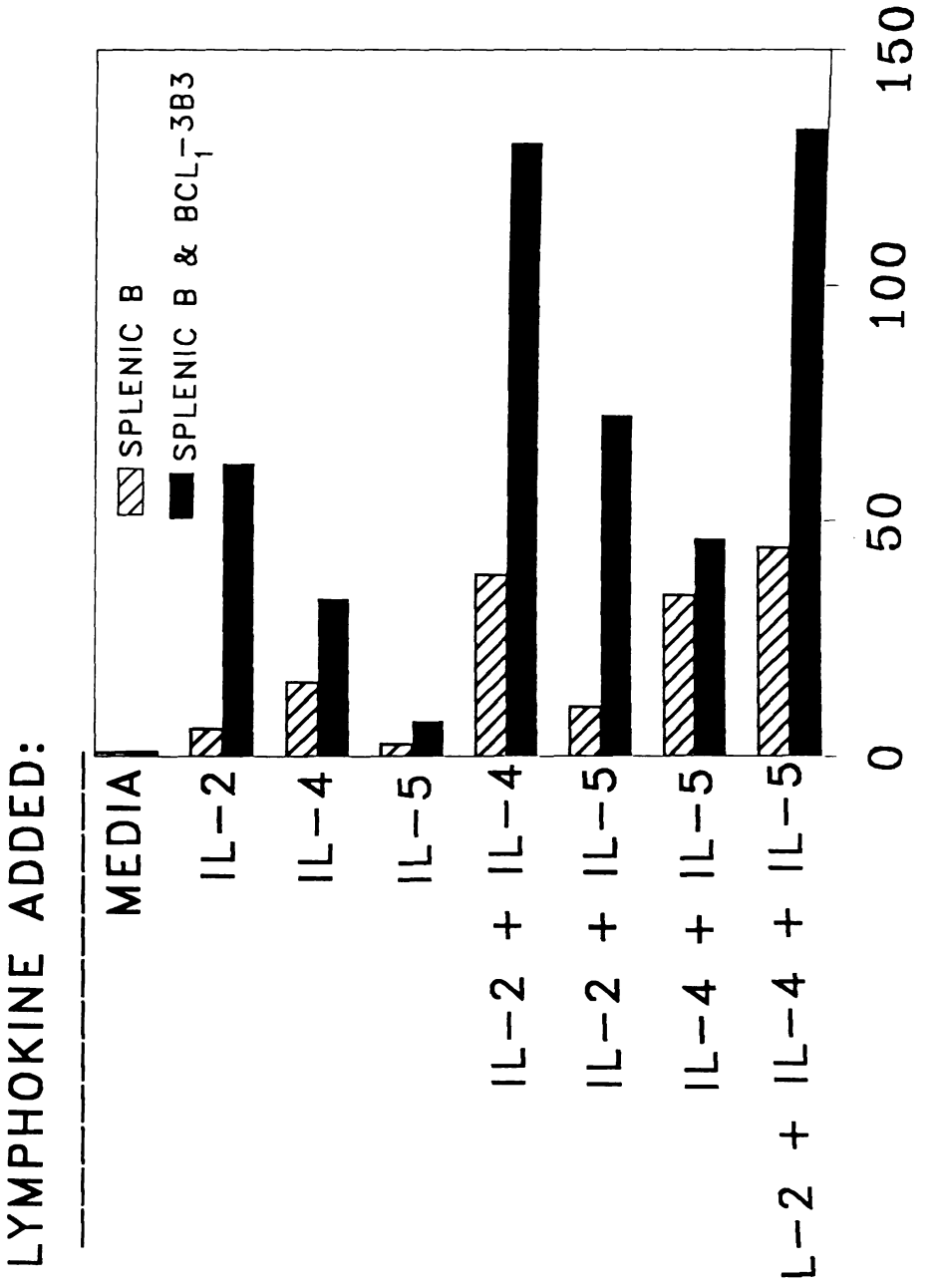
LYMPHOKINE ADDED:
MEDIA
IL-2
IL-4
IL-5
IL-2 + IL-4
IL-2 + IL-5
IL-4 + IL-5
IL-2 + IL-4 + IL-5
SPLENIC B
SPLENIC B & BCL1-3B3
0
50
100
150
STIMULATION INDEX

Figure 1

3.4 ± 0.4 SEM (n=19).

In contrast to lymphokine combinations which included IL-2, BCL_1-3B3 cells did not significantly alter B cell proliferation in the presence of IL-4 or IL-5 alone or in combination (Figure 1). BCL_1-3B3 cells did not significantly augment the IL-5 plus dextran sulfate or IL-4 plus anti-Ig-induced proliferation of splenic B cells (Figure 2). Thus, BCL_1-3B3-mediated augmentation of splenic B cell proliferation is IL-2-specific and does not require a preceding activation signal *in vitro*.

3.2.2. Both resting and *in vivo*-activated B cells proliferate in response to B cell-mediated help.

To determine if the IL-2 specific, BCL_1-3B3-mediated augmentation of splenic B cell proliferation requires a preceding *in vivo* activation signal, a Percoll density gradient was utilized to separate enriched populations of resting or *in vivo*-activated splenic B cells. Acridine orange staining and flow cytometric analysis were performed to verify that the Percoll separation resulted in enriched populations of resting and *in vivo*-activated cells. A representative histogram from the acridine orange analysis is shown in Figure 3. Both the 75% Percoll fraction (94% in G_0, 5% in G_1) and the 55% Percoll fraction (61% in G_0, 39% in G_1) exhibited increased proliferation in the presence of irradiated BCL_1-3B3 cells and IL-2 (Figure 4). There was no significant difference in the proliferative response of these two splenic B cell fractions. Thus, the IL-2 specific, BCL_1-3B3-mediated enhancement of B cell proliferation does not require prior *in vivo* activation.

Figure 2. $CD5^+$ B-cell-mediated help does not synergize with Anti-Ig or DxS. BALB/c splenic B cells at 5 X 10^5 cells/ml stimulated *in vitro* with 10 μg/ml dextran sulfate or 5 μg/ml goat anti-mouse immunoglobulin were cultured alone (diagonal hatched bars) or with 1.5 X 10^5 cells/ml irradiated (4060R) BCL_1-3B3 cells (solid bars) in the presence or absence of lymphokines. At 66 hrs. of culture the cells were pulsed with 1 μCi/well of ^{3}H-thymidine. Following a 6 hr. pulse, cells were harvested and the mean CPM for triplicate wells determined. Stimulation indices were calculated as indicated in Figure 1. The mean CPM for BALB/c spleen cells was 400 ± 135. The mean background CPMs for irradiated BCL_1-3B3 cells in the presence of lymphokines were as follows: media = 514, IL-2 = 390, IL-4 = 264, IL-5 = 265, IL-2/IL-4 = 292, IL-2/IL-5 = 804, IL-4/IL-5 = 307. and IL-2/IL-4/IL-5 = 298. Interleukin concentrations are as indicated for Figure 1. The experiment shown is representative of three experiments.

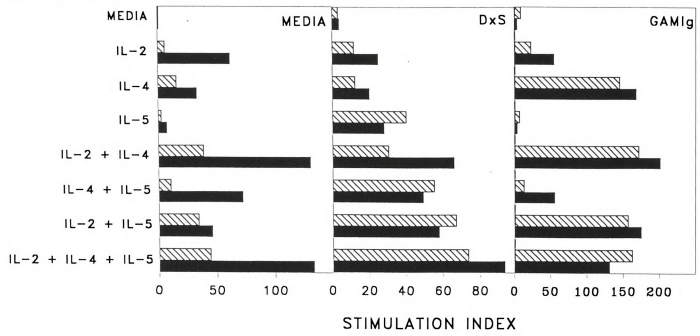
LYMPHOKINE ADDED:
MEDIA
IL-2
IL-4
IL-5
IL-2 + IL-4
IL-4 + IL-5
IL-2 + IL-5
IL-2 + IL-4 + IL-5
MEDIA
DxS
GAMIg
0
50
100
0
20
40
60
80
0
50
100
150
200
STIMULATION INDEX

Figure 2

Figure 3. Acridine orange analysis of peritoneal, splenic and Percoll fractionated splenic B cells. 1 X 10^6 peritoneal, splenic or Percoll-fractionated splenic B cells were stained with acridine orange, followed by analysis on an Ortho-Diagnostics 50H cytofluorograf. RNA versus DNA content is indicated.

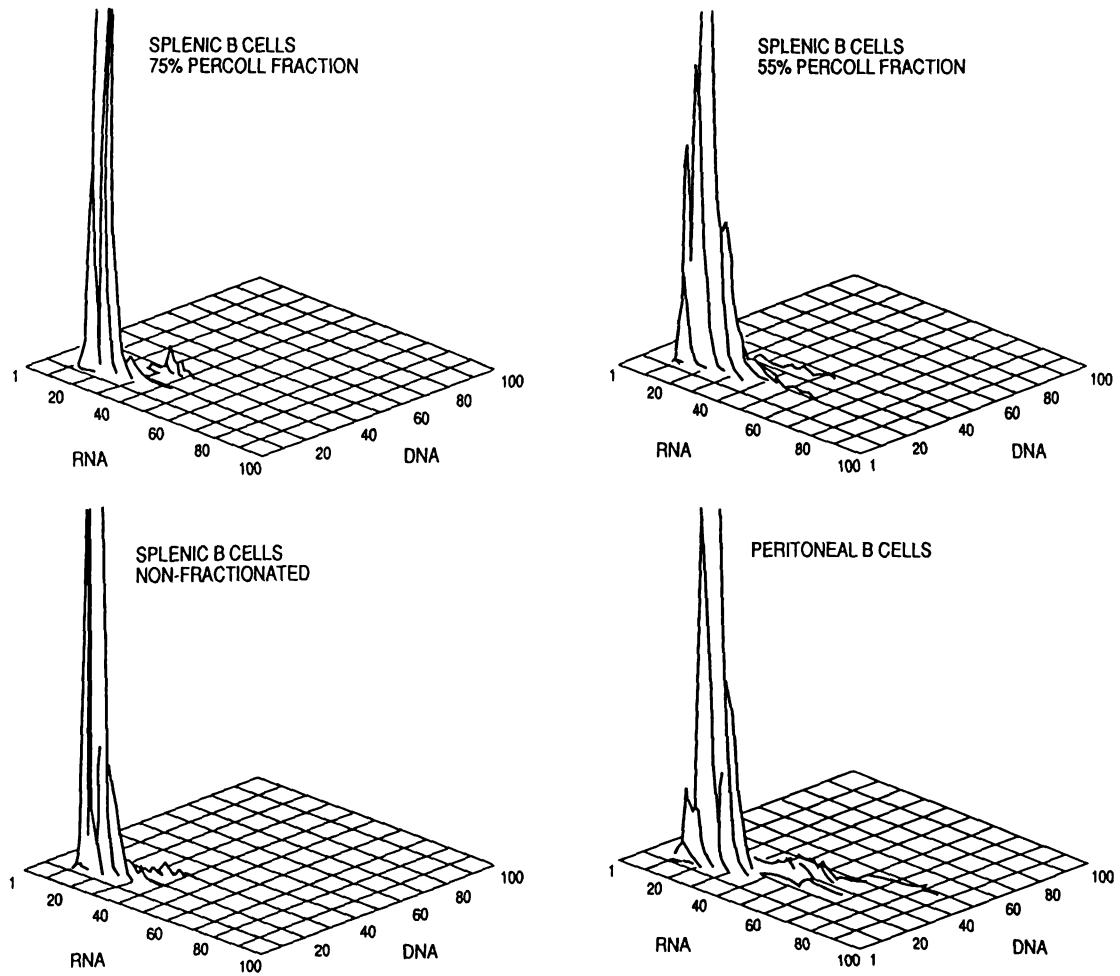
SPLENIC B CELLS
75% PERCOLL FRACTION
SPLENIC B CELLS
55% PERCOLL FRACTION
SPLENIC B CELLS
NON-FRACTIONATED
PERITONEAL B CELLS
RNA
DNA
1
20
40
60
80
100

Figure 3

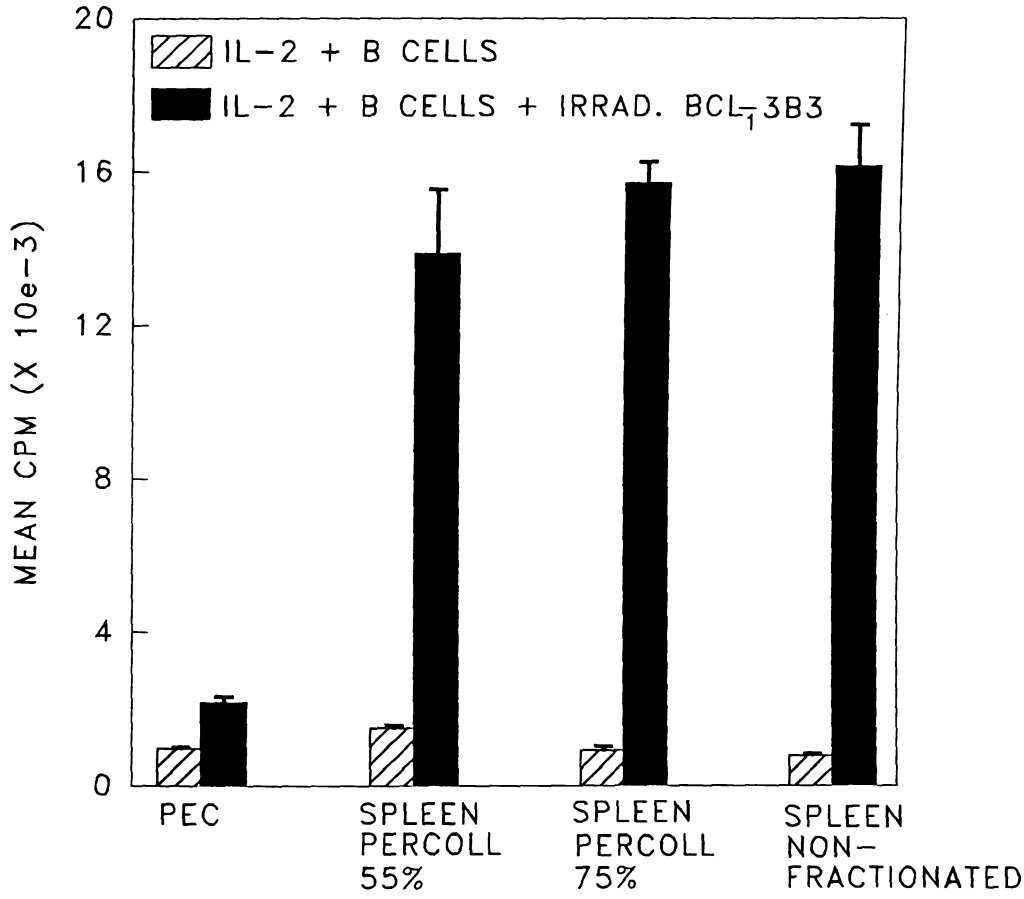

Figure 4. Both resting and *in vivo*-activated splenic B cells proliferate in response to help from BCL_1-3B3, but peritoneal exudate B cells do not. Splenic B cells were separated into resting (75% Percoll fraction) and *in vivo*-activated (55% Percoll fraction) fractions using a discontinuous Percoll density gradient. 5 X 10^5 splenic B cells from the 75% Percoll band or the 55% Percoll band or 5 X 10^5 peritoneal exudate (PEC) B cells were combined with 2.5 ng/ml IL-2 (diagonal hatched bars) or 2.5 ng/ml IL-2 plus 1.5 X 10^5 irradiated BCL_1-3B3 cells/ml (solid bars). Cells were incubated for 66 hrs. at 37°C in an atmosphere of 10% CO_2 in air. ^{3}H-thymidine was added at 1 μCi/well and cells were harvested following a 6 hr. pulse. The mean CPM for triplicate wells was determined. The mean CPMs for unstimulated cells were: PEC = 662 ± 240, 55% Percoll = 413 ± 107, 75% Percoll = 521 ± 49, and Non-Fractionated = 392 ± 135. The mean background CPMs for 3B3 and 3B3 plus IL-2 were 207 ± 108 and 781 ± 95, respectively. The experiment shown is representative of four experiments. Error bars indicate standard error of mean (SEM).

In contrast to the splenic B cell fractions, peritoneal exudate B cells did not proliferate in response to the help provided by irradiated BCL_1-3B3 cells. Although both the 55% Percoll fraction and the peritoneal exudate B cells represent *in vivo*-activated populations of B cells, cell cycle analysis profiles for the two populations suggest that the peritoneal exudate B cells are at a different stage of activation with 10% of the cells in G_{1A}, and 57% in G_{1B} (compared to 12% in G_{1A} and 27% in G_{1B} for the 55% Percoll fraction). Thus, the BCL_1-3B3 mediated help may play a more important role in the differentiation, as opposed to the proliferation, of peritoneal exudate B cells.

3.2.3. BCL_1-3B3 B cells enhance the differentiation of both peritoneal exudate and splenic B cells.

To determine if BCL_1-3B3 cells could provide help for the differentiation of B cells, supernatants from *in vitro* cultures of splenic or peritoneal exudate B cells plus irradiated BCL_1-3B3 cells and lymphokines were analyzed by ELISA to detect changes in antibody concentration. For both the peritoneal exudate B cells (Figure 5A,C) and splenic B cells (Figure 5B,D) differentiation was enhanced in the presence of irradiated BCL_1-3B3 cells and the IL-2/IL-5 lymphokine combination. The most marked changes in differentiation were in IgG, with mean-fold increases in secretion of 3.1 ($\pm$ 0.95 SEM, n=4) and 5.0 ($\pm$ 2.02 SEM, n=4) for the PEC and splenic B cells, respectively. Although the splenic and peritoneal exudate B cells demonstrated differences in their proliferative responses, both splenic and peritoneal exudate cells showed enhanced differentiation in the presence of

Figure 5. $CD5^+$ B cells provide help for the differentiation of peritoneal exudate and splenic B cells. Peritoneal exudate, PEC, B cells at 5 X 10^5 cells/ml (panels A, C) or splenic B cells at 5 X 10^5 cells/ml (panels B, D) were cultured with 1.5 X 10^5 irradiated (4060R) BCL_1-3B3 cells in the presence of 2.5 ng/ml IL-2 (open bars) or 3.2 ng/ml IL-5 (diagonal hatched bars) or 2.5 ng/ml IL-2 + 3.2 ng/ml IL-5 (solid bars). Supernatant was collected at 5-7 days of culture and analyzed with ELISA to determine IgM (panels A, B) and IgG (panels C, D) concentrations. Results represent the mean for triplicate wells ± SD. The experiment is representative of four experiments.

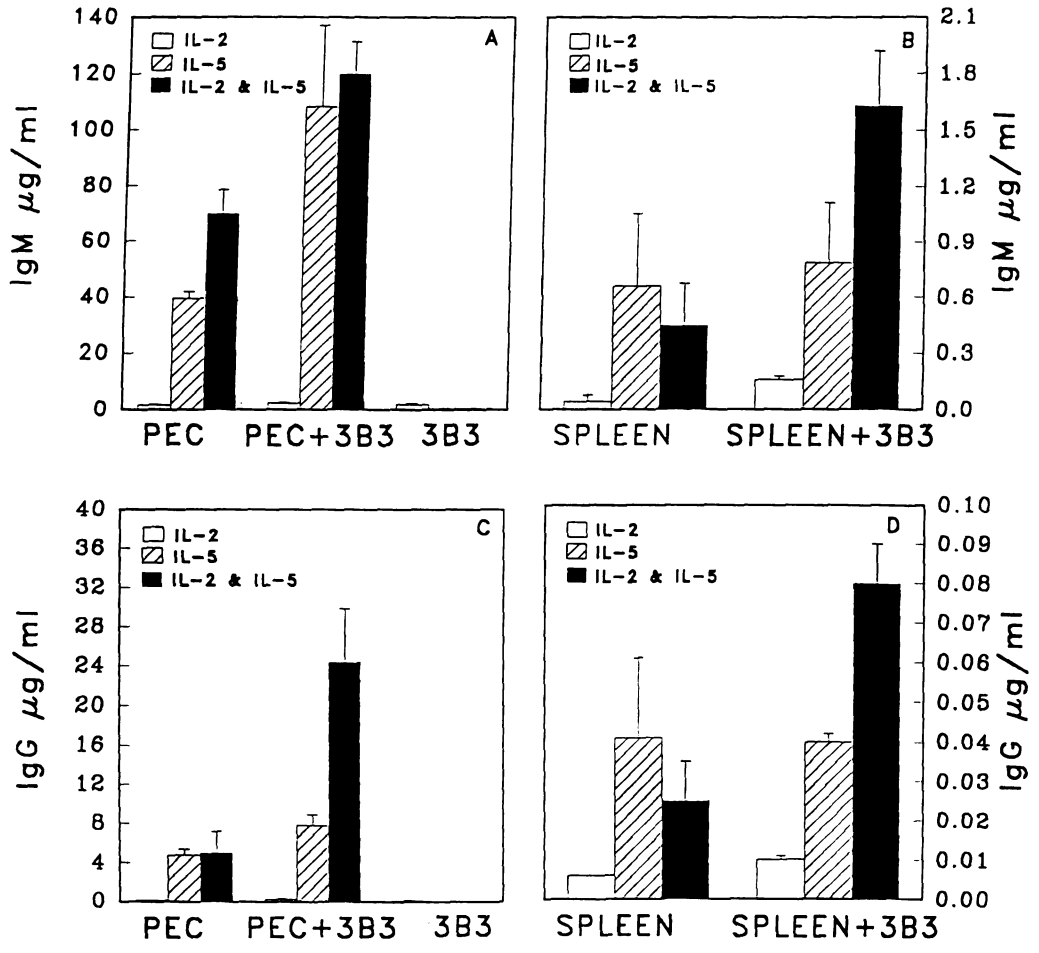
IL-2
IL-5
IL-2 & IL-5
A
B
C
D
IgM µg/ml
IgG µg/ml
PEC
PEC+3B3
3B3
SPLEEN
SPLEEN+3B3

Figure 5

irradiated BCL_1-3B3 cells.

3.2.4. The help provided by $CD5^+$ BCL_1-3B3 is contact-dependent.

To determine if direct cell-cell contact was necessary to stimulate the proliferation of splenic B cells, the irradiated BCL_1-3B3 cells were cultured together with the splenic B cells in the same well or separated from the splenic B cells by a polycarbonate membrane Transwell®, in the presence or absence of IL-2. The proliferation of splenic B cells in the presence of irradiated BCL_1-3B3 cells and IL-2 was reduced to background levels when the two cell populations were physically separated by the polycarbonate membrane (Figure 6). Thus, the Transwells® were capable of completely inhibiting the helper activity provided by BCL_1-3B3.

3.2.5. Paraformaldehyde-fixed BCL_1-3B3 cells retain helper capacity.

Although cell-cell contact is necessary for BCL_1-3B3-mediated help, it is unclear whether that contact is sufficient to activate the B cell. To determine if BCL_1-3B3 cells that were no longer capable of secreting soluble factors retained their helper function, paraformaldehyde-fixed BCL_1-3B3 cells were combined with splenic B cells in the presence or absence of IL-2. Following fixation, BCL_1-3B3 cells retained approximately 70% of their helper capacity (Figure 7). Thus, soluble factors may provide some enhancement of BCL_1-3B3-mediated help, but, direct cell-cell contact is the dominant mechanism by which help is mediated.

3.2.6. B helper activity correlates with CD5 expression.

To determine whether B helper activity is unique to the BCL_1-3B3 cell line, the helper capacity of five B cell lines was compared. These lines differed in both

Figure 6. BCL_1-3B3-mediated help requires direct cell-cell contact. Irradiated (4060R) BCL_1-3B3 cells at 1 x 10^5 cells/ml were cultured in the presence of 5 X 10^5 cells/ml splenic B cells (solid bars) or separated from the splenic B cells with a 0.4 μm polycarbonate membrane (cross-hatched bars), with the addition of 2.5 ng/ml IL-2 as indicated. At 66 hrs. of culture transwells were discarded, 0.2 ml of cell suspension was transferred from the 24-well cluster plate to a 96-well plate and pulsed with 1μCi/well ^{3}H-thymidine. Cells were harvested following a 6 hr. pulse and the mean CPM for triplicate wells was determined. Stimulation indices were calculated as shown for Figure 1. The mean CPM for splenic B cells was 1195 ± 164. The mean background CPMs for the irradiated BCL_1-3B3 cells were: media = 124, IL-2 = 285, Transwell = 100, Transwell plus IL-2 = 189. The experiment shown is representative of eight experiments.

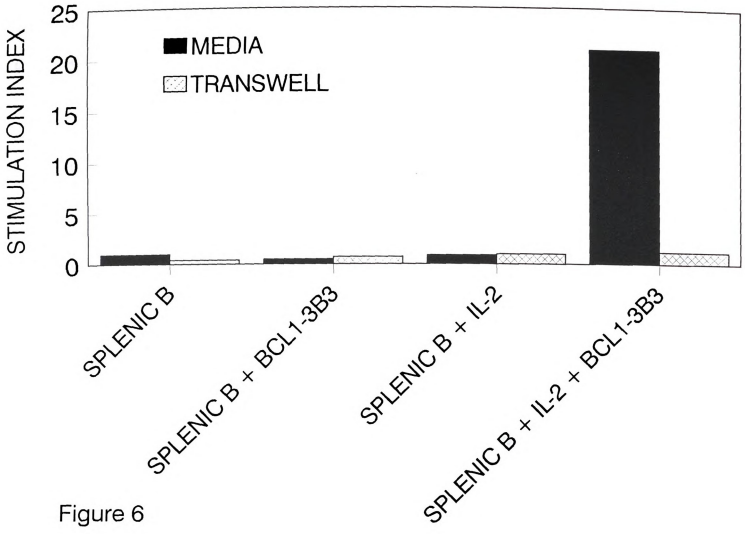
STIMULATION INDEX
25
20
15
10
5
0
MEDIA
TRANSWELL
SPLENIC B
SPLENIC B + BCL1-3B3
SPLENIC B + IL-2
SPLENIC B + IL-2 + BCL1-3B3

Figure 6

STIMULATION INDEX

Figu
Sple
irrad
BCL
66 h
6 hr.
Stim
cells
71, i
plus

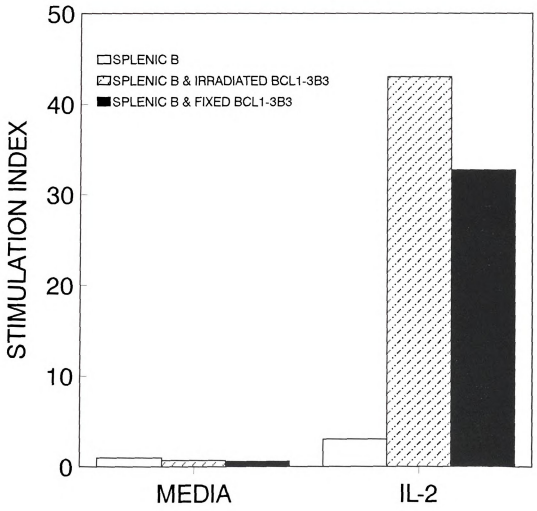

Figure 7. Fixed BCL_1-3B3 cells retain helper capacity for proliferation. Splenic B cells at 5 X 10^5 cells/ml were cultured alone (open bars), with 1.5 X 10^5 irradiated BCL_1-3B3 cells/ml (dotted bars) or with 1.5 X 10^5 paraformaldehyde-fixed BCL_1-3B3 cells/ml (solid bars) in the presence or absence of 2.5 ng/ml IL-2. At 66 hrs. of incubation, cells were pulsed with 1 μCi/well ^{3}H-thymidine. Following a 6 hr. pulse, cells were harvested and the mean CPM for triplicate wells determined. Stimulation indices were calculated as for Figure 1. The mean CPM for spleen cells was 606 ± 152. The mean background CPMs were: irradiated BCL_1-3B3 = 71, irradiated BCL_1-3B3 plus IL-2 = 456, fixed BCL_1-3B3 = 130 and fixed BCL_1-3B3 plus IL-2 = 191. The experiment shown is representative of four experiments.

the

Thel

HDE

BCL

com

(MF

of th

B ce

8B).

B ce

1.02

lines

prov

tran

of a

Tra

(Fig

lines

Ha-

rem

surf

the level of CD5 surface expression (Figure 8A) and their maturational state. TheBCL_1-3B3, CH12.LX, and 225-11 cell lines represent mature B cells, whereas HDBM and SV(X)-Ha-ras are pre-B cell lines. As shown in Figure 8A, 83.4% of the BCL_1-3B3 cells were surface $CD5^+$ (mean fluorescence intensity, MFI = 120), compared to 41.4% for the CH12.LX cells (MFI = 26), 11.1% for the 225-11 cells (MFI = 26), and only 3.5% for the SV(X)-Ha-ras pre-B cells (MFI = 31). All three of the mature B cells lines were able to provide help for the proliferation of splenic B cells in the presence of IL-2 and the IL-2/IL-4 lymphokine combination (Figure 8B). The mean-fold increases in the stimulation indices of IL-2 stimulated splenic B cells for three experiments were: BCL_1-3B3, 6.3 ± 1.65 SEM; CH12.LX, 4.1 ± 1.02 SEM; and 225-11, 2.0 ± 0.30 SEM. All three of these neoplastic, mature B cell lines were derived from different strains of mice. Thus, the B-cell-mediated help provided does not appear to be either MHC-restricted or the result of a unique transformation event. This lack of MHC restriction is consistent with the absence of a TCR-MHC interaction. As seen with BCL_1-3B3, polycarbonate membrane Transwells® blocked the help provided by CH12.LX and 225-11 neoplastic B cells (Figure 9). Thus, the B-cell-mediated help provided by each of the mature B cell lines was contact-dependent.

In contrast to the three mature B cell lines, neither the HDBM nor the SV(X)-Ha-ras pre-B cells were capable of mediating B cell help (Figure 8B). Although it remains unclear as to whether this lack of helper function is due to either low surface CD5 expression or the maturational stage of the pre-B cell lines, the results

Figure 8. B-cell-mediated help corresponds to the level of expression of surface CD5. Two pre-B cell lines (Panel A, HDBM and SV(X)-Ha-ras) and three mature, neoplastic B cell lines (Panel A, 225-11, CH12.LX and BCL_1-3B3) were stained with FITC-anti-CD5 (solid lines) or FITC-rat-IgG_{2a} isotype control (dashed lines). Both the percentage of the cells that were CD5 positive and the mean fluorescence intensity (MFI) are indicated in Panel A. The five B cell lines were also irradiated (4060R) and cultured at 1.5 X 10^5 cells/ml with 5 X 10^5 splenic B cells/ml (Panel B), in the presence of media only (open bars), 2.5 ng/ml IL-2 (cross hatched bars), 2.0 ng/ml IL-4 (diagonal hatched bars) or 2.5 ng/ml IL-2 + 2.0 ng/ml IL-4 (solid bars). At 66 hrs. of incubation, cells were pulsed with 1μCi/well ^{3}H-thymidine. Following a 6 hr. pulse, cells were harvested and the mean CPM for triplicate wells determined. Stimulation indices were calculated as for Figure 1. The mean CPM for splenic B cells was 637 ± 64. The mean background CPMs for the irradiated cells were: HDBM (media) = 654, (IL-2) = 397, (IL-4) = 276, (IL-2/IL-4) = 205; SV(X)-Ha-ras (media) = 205, (IL-2) = 264, (IL-4) = 376, (IL-2/IL-4) = 269; 225-ll (media) = 774, (IL-2) = 503, (IL-4) = 725, (IL-2/IL-4) = 424; CH12.LX (media) = 272, (IL-2) = 175, (IL-4) = 263, (IL-2/IL-4) = 228; and BCL_1-3B3 (media) = 335, (IL-2) = 645, (IL-4) = 339, (IL-2/IL-4) = 274. The experiment shown is representative of three experiments.

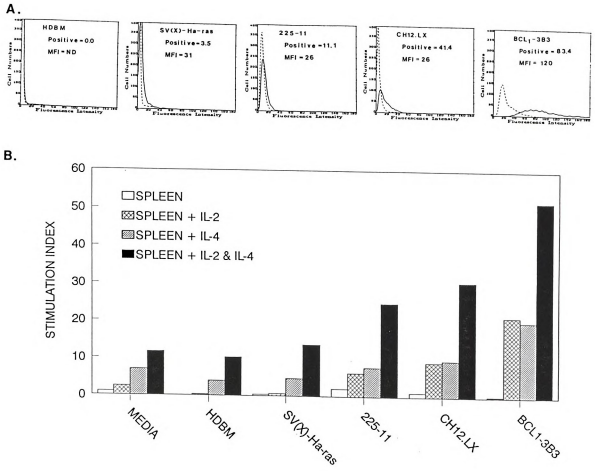
A.
HDBM
Positive = 0.0
MFI = ND
SV(X)-Ha-ras
Positive = 3.5
MFI = 31
225-11
Positive = 11.1
MFI = 26
CH12.LX
Positive = 41.4
MFI = 26
BCL1-383
Positive = 83.4
MFI = 120
Cell Numbers
Fluorescence Intensity
B.
SPLEEN
SPLEEN + IL-2
SPLEEN + IL-4
SPLEEN + IL-2 & IL-4
STIMULATION INDEX
0
10
20
30
40
50
60
MEDIA
HDBM
SV(X)-Ha-ras
225-11
CH12.LX
BCL1-3B3

Figure 8

Figure 9. Helper activity of mature B cell lines is contact-dependent. Three neoplastic B cell lines: BCL_1-3B3, CH12.LX and 225-11 were irradiated (4060R) and cultured at 1 X 10^5 cells/ml with 5 X 10^5 splenic B cells/ml in the presence of 2.5 ng/ml IL-2 (dotted bars). In parallel wells the irradiated neoplastic B cells were separated from the splenic B cells with a 0.4 μm polycarbonate membrane Transwell® in the presence of 2.5 ng/ml IL-2 (solid bars). At 66 hrs. of culture the Transwells® were discarded, 0.2 ml of cell suspension was transferred from the 24-well cluster plate to a 96-well plate and pulsed with 1 μCi/well ^{3}H-thymidine. Cells were harvested following a 6 hr., pulse and the mean CPM for triplicate wells determined. Stimulation indices were calculated as indicated in Figure 1. The mean CPM for spleen cells was 902 ± 237. The mean background CPMs for each of the irradiated neoplastic B cell lines were as follows: BCL_1-3B3 plus IL-2 = 351, CH12.LX plus IL-2 = 739, and 225-11 plus IL-2 = 1130. The results shown are representative of three experiments.

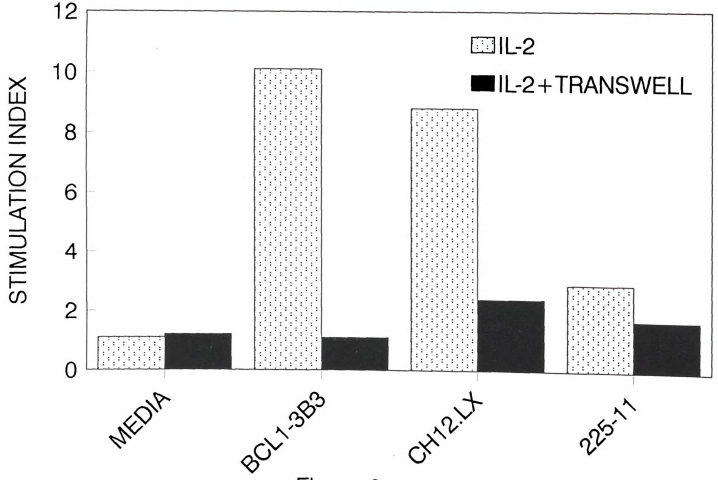
STIMULATION INDEX
12
10
8
6
4
2
0
IL-2
IL-2+TRANSWELL
MEDIA
BCL1-3B3
CH12.LX
225-11

Figure 9

indicate that the IL-2 mediated B cell helper function is not a universal property of proliferating B cells.

3.2.7. Multiple surface molecules are involved in B-cell-mediated help.

As indicated by Figure 8, the levels of help provided by the different neoplastic B cell lines corresponds to the levels of surface CD5 expression for each cell line; therefore, monoclonal antibody against surface CD5 was used to determine if the helper activity of BCL_1-3B3 could be inhibited. As shown in Figure 10, anti-CD5 mAb at concentrations from 12.5-50 μg/ml did not inhibit the helper activity of either irradiated or fixed BCL_1-3B3 cells.

A variety of ligand/receptor pairs including CD40L/CD40, Ia/TCR and ICAM-1/LFA-1 have previously been shown to play a role in T-cell-mediated help for humoral responses (Parker, 1993). Therefore, to examine a potential role for these surface molecules during B-cell-mediated help monoclonal antibodies reactive with CD11a, CD18, CD54, CD40L, I-A, and I-E were added to the B cell cultures (Figure 11a,b). The LFA-1 antibodies CD11a and CD18, and the ICAM-1 antibody CD54 inhibited the BCL_1-3B3-mediated-help by 48.7%, 33.0% and 26.3%, respectively (Figure 11c). As shown in Figures 11a and c, a mAb to CD40L did not significantly inhibit the B-cell-mediated help. In the presence of mAb to I-A and I-E there was a 10- and 20-fold increase (from a SI of 1.6 to 15.5 and 32.6, respectively), in the proliferative responses of IL-2 stimulated B cells. Thus, the adhesion molecules appear to be important for the interaction but additional ligand/receptor pairs appear to play a role in cell signalling.

Figure 10. BCL_1-3B3-mediated B cell help is not inhibited by antibody to CD5. 1.5 X 10^5 BCL_1-3B3 cells which had been irradiated with 4060R (diagonal hatched bars) or fixed in 0.4% paraformaldehyde (solid bars) were preincubated for 45 minutes, at 4°C with anti-CD5 antibody (clone 53-7.3, rat IgG_{2a}) or a rat IgG_{2a} isotype control antibody at the indicated concentrations. Following the preincubation with antibody 5 X 10^5 splenic B cells/ml and 2.5 ng/ml IL-2 were added and the cells were incubated at 37°C, in an atmosphere of 10% CO_2 in air. At 66 hrs. of culture the wells were pulsed with 1 μCi/well ^{3}H-thymidine. Following a 6 hr. pulse the cells were harvested and the mean CPM for triplicate wells determined. Stimulation indices were determined as described for Figure 1. The mean CPM for splenic B cells was 527. The mean background CPM's for irradiated BCL_1-3B3 cells in the presence of IL-2 and various antibody concentrations were as follows: media = 4129; anti-CD5 at 12.5 μg/ml = 4800; anti-CD5 at 25.0 μg/ml = 4768; anti-CD5 at 50.0 μg/ml = 5341; and rat IgG_{2a} at 50 μg/ml = 3629. The mean background CPM's for fixed BCL_1-3B3 cells were: media = 113; 50 μg/ml anti-CD5 = 308; and 50 μg/ml rat IgG_{2a} = 104.

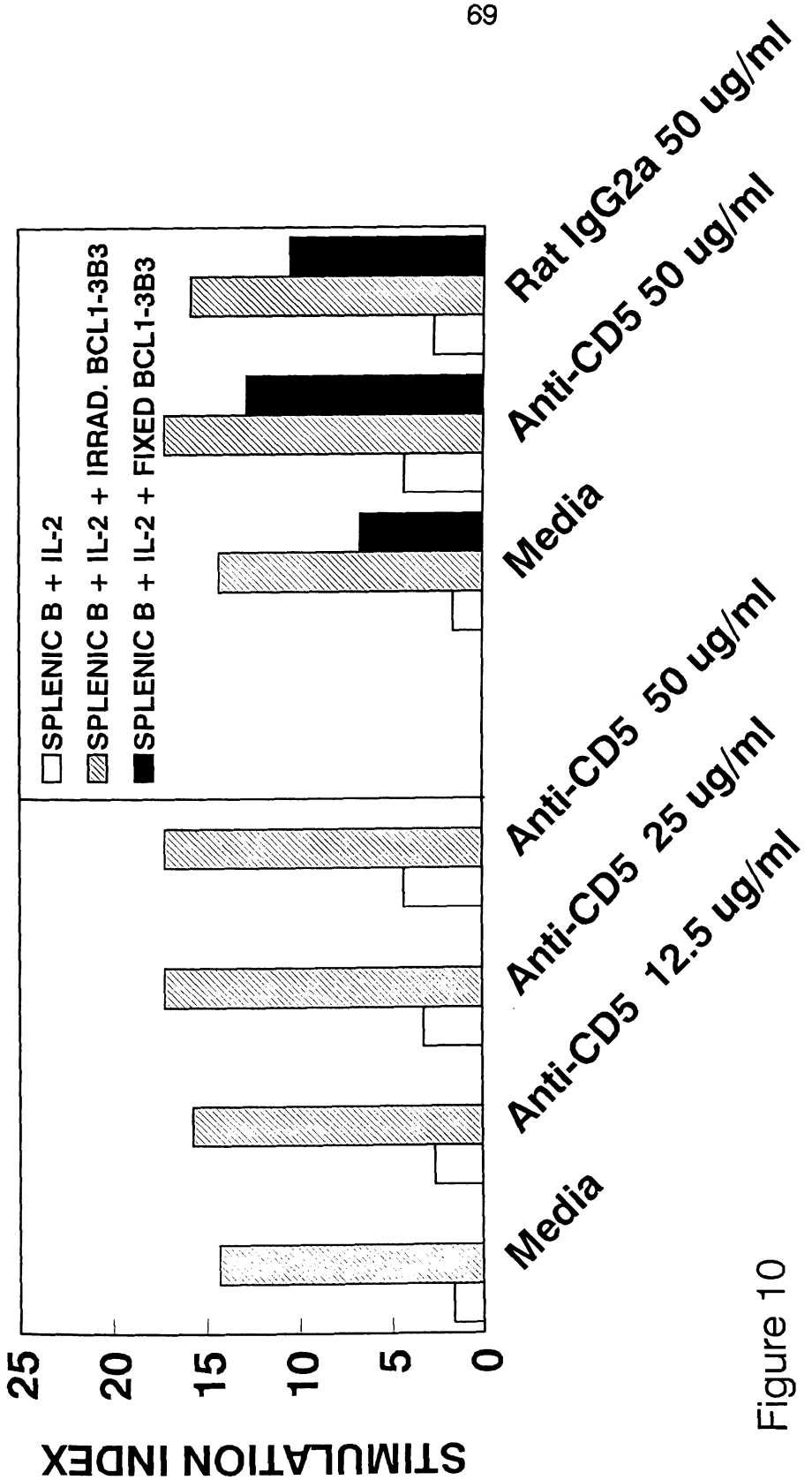
STIMULATION INDEX
25
20
15
10
5
0
SPLENIC B + IL-2
SPLENIC B + IL-2 + IRRAD. BCL1-3B3
SPLENIC B + IL-2 + FIXED BCL1-3B3
Media
Anti-CD5 12.5 ug/ml
Anti-CD5 25 ug/ml
Anti-CD5 50 ug/ml
Media
Anti-CD5 50 ug/ml
Rat IgG2a 50 ug/ml

Figure 10

Figure 11. Antibodies to the adhesion molecules LFA-1 and ICAM-1 inhibit BCL_1-3B3-mediated B cell help. BCL_1-3B3 B cells were fixed in 0.4% paraformaldehyde, cultured at 1.5 X 10^5 cells/ml, and preincubated with 10 or 25 μg/ml of the indicated antibodies for 1 hr., at 4°C. The antibodies used were: CD11a/LFA-1α (clone 121/7, rat IgG_{2a}), CD18/LFA-1β (clone 2E6, hamster IgG), CD54/ICAM-1 (clone 3E2B, hamster IgG), CD40L (clone MR1, hamster Ig), I-A^d (clone AMS-32.1, a mouse IgG_{2b}) and I-E^k (clone 14-4-4s, a mouse IgG_{2a}). Following the preincubation with antibody 5 X 10^5 splenic B cells/ml and 2.5 ng/ml IL-2 were added and the cells were incubated at 37°C, in an atmosphere of 10% CO_2 in air. At 66 hrs. of culture the wells were pulsed with 1 μCi/well ^{3}H-thymidine. Following a 6 hr. pulse the cells were harvested and the mean CPM for triplicate wells determined. Stimulation indices were determined as described for Figure 1. The stimulation indices for isotype matched control hamster IgG, rat IgG_{2a}, and mouse IgG_{2a} were 9.8, 9.5 and 11.4, respectively. The mean CPM for splenic B cells was 1251 ± 325 (panel a) or 527 ± 120 (panel b). The mean CPM's for fixed BCL_1-3B3 B cells in the presence of IL-2 and the various antibodies were as follows: media = 373 (panel a) or 113 (panel b), CD11a = 63, CD18 = 146, CD54 = 183, CD40L = 149, I-A^d = 852, I-E^k = 258. The experiment shown in panel (a) is representative of three experiments. The experiment shown in panel (b) is representative of two experiments. Panel (c) represents the mean of three experiments with the percent inhibition for each experiment calculated as follows: 100% - [(SI in presence of IL-2, antibody and fixed BCL_1-3B3 ÷ SI in presence of IL-2 and fixed BCL_1-3B3) (100)]. Error bars indicate standard error of the mean (SEM).

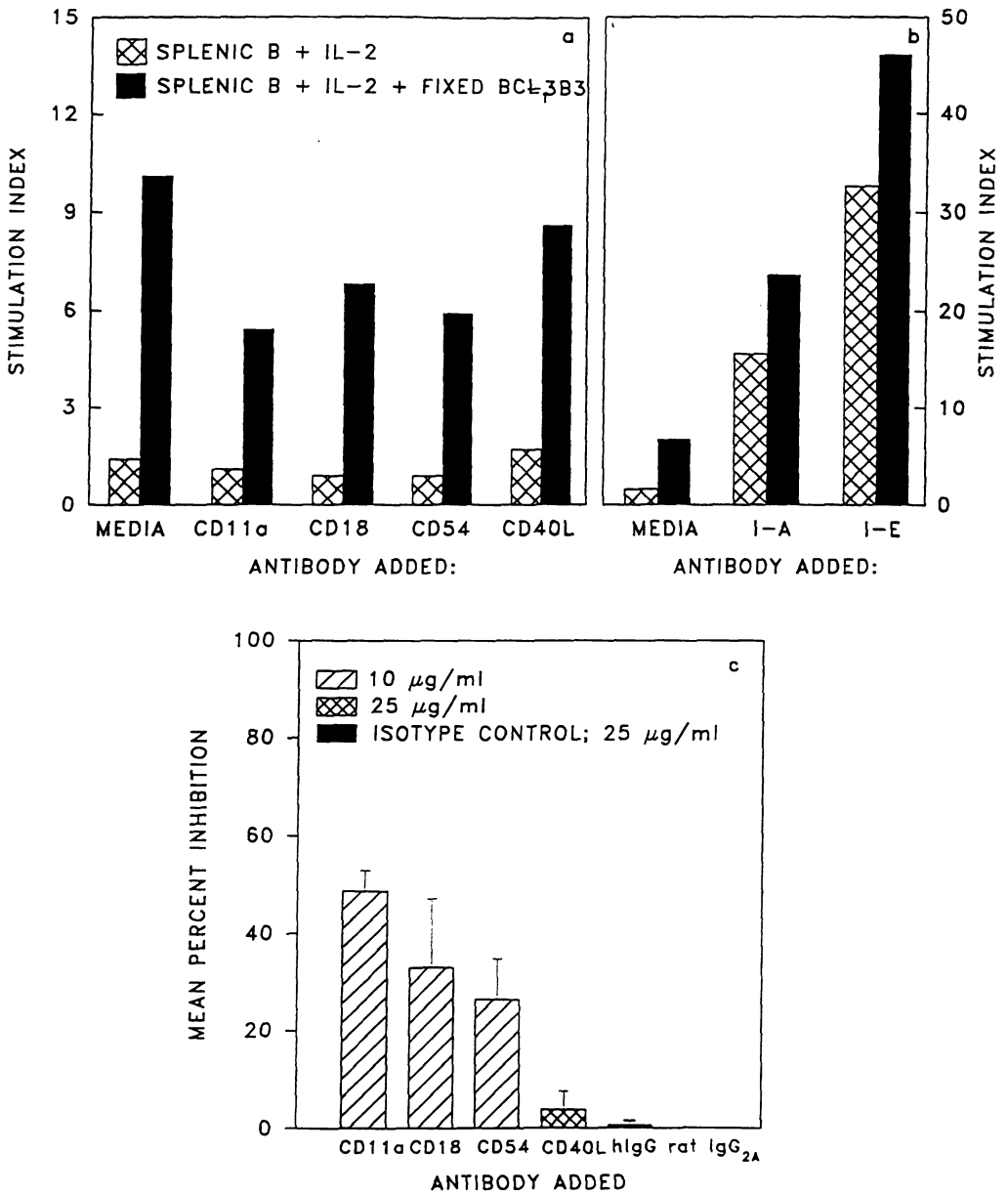
a
SPLENIC B + IL-2
SPLENIC B + IL-2 + FIXED BCL1 3B3
STIMULATION INDEX
15
12
9
6
3
0
MEDIA
CD11a
CD18
CD54
CD40L
ANTIBODY ADDED:
b
50
40
30
20
10
0
STIMULATION INDEX
MEDIA
I-A
I-E
ANTIBODY ADDED:
c
10 μg/ml
25 μg/ml
ISOTYPE CONTROL; 25 μg/ml
MEAN PERCENT INHIBITION
100
80
60
40
20
0
CD11a
CD18
CD54
CD40L
hIgG
rat IgG2A
ANTIBODY ADDED

Figure 11

3.3

man
depe
expr
that
cells
CDE
in th
the
incc
of a
upo
was
B ce
part
add
that
app
to i
inhi
mol

3.3 Summary.

Activation of B lymphocytes can occur in either a thymus-independent manner in which antigen extensively cross-links membrane Ig or in a thymus-dependent manner involving bidirectional signalling through ligand/receptor pairs expressed on the surface of T helper cells and B cells, respectively. The possibility that B cells expressing CD5 may also provide contact-dependent help to other B cells via interactions between CD5 and its ligand CD72, was investigated . The $CD5^+$ neoplastic B cell line, BCL_1-3B3, was irradiated and added to splenic B cells in the presence and absence of IL-2, IL-4 and IL-5. Whenever IL-2 was present, the addition of irradiated BCL_1-3B3 cells markedly enhanced thymidine incorporation. This enhanced response to IL-2 was observed without the addition of a primary activator such as anti-Ig or dextran sulfate and was not dependent upon primary *in vivo* activation. Modest enhancement of IgM and IgG secretion was also observed, but only when IL-5 was present in addition to IL-2. The $CD5^+$ B cell-mediated help involves a contact-dependent signal since use of membrane-partitioned cultures completely inhibited the helper activity of the $CD5^+$ B cells. In addition, paraformaldehyde fixed $CD5^+$ B cells provided help equivalent to 70% of that seen with irradiated cells. The help provided by a panel of B cell lines appeared to correspond to the level of surface CD5 expression; however, attempts to block the helper activity with anti-CD5 antibody were unsuccessful. Thus, inhibition of B-cell-mediated help by antibodies reactive with various B cell surface molecules was investigated. Antibodies to ICAM-1 (CD54) and LFA-1 (CD11a,

CD1

In a

effe

of re

The

add

CD18) inhibited approximately 26 to 49 percent of the helper activity, respectively. In addition to anti-CD5 mAb, antibodies to CD40L, I-A and I-E had no inhibitory effect. These results suggest that neoplastic B cells can enhance responsiveness of resting and activated B cells to interleukin 2 in the absence of T-B interactions. These interactions involve the adhesion molecules LFA-1 and ICAM-1 but additional ligand/receptor pair interactions are likely.

4.0 BCL_1-3B3-MEDIATED HELP: POTENTIAL ROLE OF CYTOKINES.

4.1

the

exo

enal

Alte

allo

IL-2

BCL

BCL

vitro

with

dete

obs

nur

Fur

stin

car

BC

IL-

pa

4.1 Rationale.

Initial studies of BCL_1-3B3-mediated help showed help was dependent upon the addition of exogenous IL-2. One possible explanation for the requirement of exogenous IL-2 is that IL-2 provides an initial signal to splenic B cells which then enables them to respond to stimulatory signals provided by BCL_1-3B3 cells. Alternatively, BCL_1-3B3 cells could provide an initial signal to splenic B cells which allows them to proliferate in response to IL-2. In addition to exogenously added IL-2, comparison studies performed with irradiated and paraformaldehyde-fixed BCL_1-3B3 cells indicate a BCL_1-3B3-derived soluble factor(s) may be involved in BCL_1-3B3-mediated help. Previously O'Garra and colleagues have shown that *in vitro* cultured BCL_1 lymphoma cells secrete IL-10 (O'Garra et al., 1992). Consistent with O'Garra's findings, concentrations of IL-10 as high as 10U/ml have been detected in supernatants from BCL_1-3B3 cells (H. Dehghani, unpublished observations). In addition to IL-10, O'Garra and colleagues have shown that a number of $CD5^+$ B cell lymphomas secrete IL-6 (O'Garra et al., 1990). Furthermore, peritoneal $CD5^+$ B cells secrete both IL-10 and IL-6, following LPS stimulation (O'Garra et al., 1990). Thus, both IL-6 and IL-10 appear to be likely candidates for the BCL_1-3B3-derived soluble factor(s).

To more clearly understand the mechanisms involved in IL-2-dependent, BCL_1-3B3-mediated B cell help, a series of experiments were performed in which IL-2 was either washed away from splenic B cells prior to culture with paraformaldehyde-fixed BCL_1-3B3 cells, or added into cultures which contained

both

hou

mec

para

stim

4.2

4.2.

mec

18,

con

hou

incu

3B3

BCI

resu

me

cell

res

3B3

both splenic B cells and paraformaldehyde-fixed BCL_1-3B3 cells, at 3, 6, 18 and 24 hours. In addition, the potential role for IL-6 and IL-10 enhancement of BCL_1-3B3-mediated help was further investigated by stimulating splenic B cells with paraformaldehyde-fixed BCL_1-3B3 cells in the presence of IL-6 and IL-10 and stimulating splenic B cells in the presence of mAbs reactive with IL-6 and IL-10.

4.2 Results.

4.2.1. BCL_1-3B3 cells provide the initial signal in IL-2-dependent, BCL_1-3B3-mediated B cell help.

To clarify the signalling events involved in IL-2-dependent, BCL_1-3B3-mediated B cell help, splenic B cells were cultured in the presence of IL-2 for 3, 6, 18, and 24 hours. The IL-2 was then washed away and the splenic B cells were combined with paraformaldehyde-fixed BCL_1-3B3 cells, for the remainder of the 72 hour culture period. As shown in Figure 12a, when splenic B cells were pre-incubated with IL-2, followed by the removal of IL-2, paraformaldehyde-fixed BCL_1-3B3 cells did not stimulate splenic B cell proliferation. These results suggest that BCL_1-3B3 cells deliver a signal which renders the splenic B cells responsive to IL-2, resulting in proliferation. Alternatively, it is possible that the IL-2 and BCL_1-3B3-mediated signals must be delivered simultaneously, in order to achieve splenic B cell proliferation.

To further clarify that BCL_1-3B3 cells mediate a signal which confers IL-2 responsiveness, splenic B cells were combined with paraformaldehyde-fixed BCL_1-3B3 cells and IL-2 was added in at 3, 6, 18, and 24 hours of culture. In contrast

Figure 12. BCL_1-3B3 cells provide the initial signal in IL-2-dependent, BCL_1-3B3-mediated enhancement of splenic B cell proliferation. In two separate experiments, splenic B cells were cultured with IL-2 at 2.5 ng/ml. At the indicated time intervals, the IL-2 was removed and the splenic B cells were cultured in media only (○, □) or in the presence of paraformaldehyde-fixed BCL_1-3B3 (●, ■) at 1.5 X 10^5 cells/ml (panel a). In parallel cultures, 5 X 10^5 splenic B cells/ml were cultured with 1.5 X 10^5 paraformaldehyde-fixed BCL_1-3B3 (●, ■), and 2.5 ng/ml IL-2 was added at the indicated time intervals (panel b). The cells were incubated at 37°C and at 66 hrs. of culture the cells were pulsed with 1 μCi/well ^{3}H-thymidine. Following a 6 hr. pulse, the cells were harvested and the mean CPM for triplicate wells was determined. Stimulation indices were calculated as shown for Figure 1. The mean CPM for splenic B cells was 232 ± 79 (dashed lines) and 187 ± 55 (solid lines).

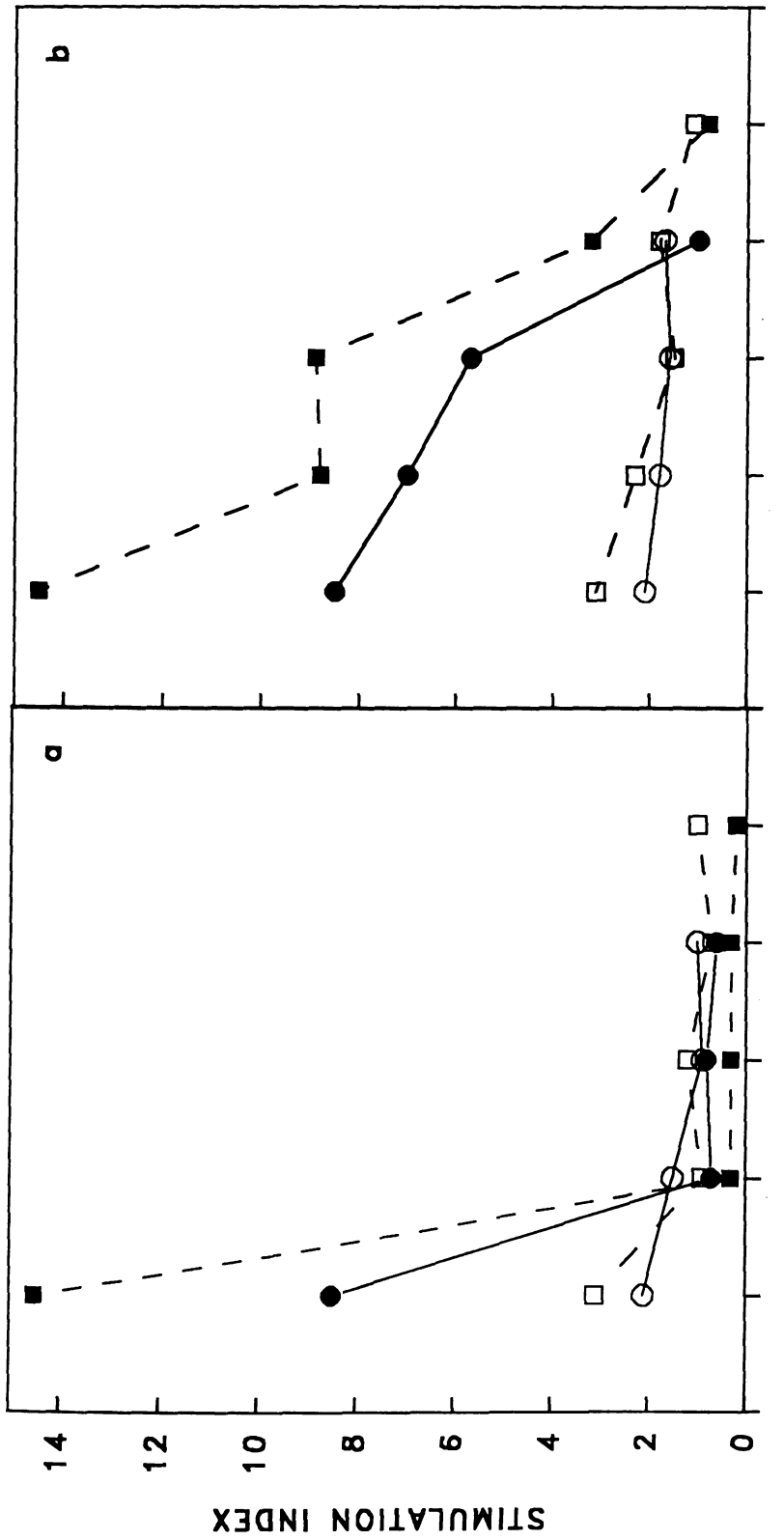
a
b
STIMULATION INDEX
0
2
4
6
8
10
12
14
Control
3hr
6hr
18hr
24hr
Time of IL-2 Removal
Time of IL-2 Addition

Figure 12

to the results obtained when IL-2 was removed prior to culture with BCL_1-3B3 cells, paraformaldehyde-fixed BCL_1-3B3 cells stimulated the proliferation of splenic B cells when the addition of IL-2 was delayed by 3 and 6 hours (Figure 12b). However, when the addition of IL-2 was delayed by 18 and 24 hours, there was a marked reduction in splenic B cell proliferation in response to paraformaldehyde-fixed BCL_1-3B3 cells. When the addition of paraformaldehyde-fixed BCL_1-3B3 cells was delayed by 18 and 24 hours the proliferative responses were retained (Figure 13). Thus, it is unlikely that the decrease in proliferation noted at 18 and 24 hours in Figure 12b represents a temporal shift in cell proliferation. These results suggest BCL_1-3B3 cells provide the initial signal to splenic B cells which enables them to respond to IL-2 and proliferate. Furthermore, there appears to be a window of IL-2 responsiveness within the first 18 hours of BCL_1-3B3-mediated help.

4.2.2. IL-2 is required in the first six hours of BCL_1-3B3-mediated B cell help.

To more clearly understand the kinetics of the IL-2 responsiveness, a series of experiments were performed in which the addition of IL-2 was delayed by 1, 2, 3, 4, 5, 6, 15, 18 and 24 hours. In two separate experiments, maximal stimulation of proliferation (SI = 5.9 and 5.7) was obtained when IL-2 was added in the first hour of culture (Figure 14). When the addition of IL-2 was delayed by six hours, this stimulation declined (SI = 2.6 and 3.3, respectively). Furthermore, when the addition of IL-2 was delayed by fifteen hours, the stimulation was below background levels (SI = 1.1 and 1.0, respectively). Thus, the IL-2 signal must be delivered within the first six hours following the BCL_1-3B3-mediated signal.

Figure 13. Delayed addition of paraformaldehyde-fixed BCL_1-3B3 cells does not alter B-cell-mediated help. In two separate experiments, splenic B cells were cultured at 5 X 10^5 cells/ml in the presence of 2.5 ng/ml IL-2 (○, □). 1.5 X 10^5 paraformaldehyde-fixed BCL_1-3B3 cells/ml were added (●, ■) at the indicated time intervals and the cells were incubated at 37°C, in an atmosphere of 10% CO_2 in air. At 66 hrs. of culture the wells were pulsed with 1 μCi/well ^{3}H-thymidine. Following a 6 hr. pulse the cells were harvested and the mean CPM for triplicate wells determined. Stimulation indices were determined as described for Figure 1. The mean CPM for splenic B cells was 232 ± 79 (solid line) and 187 ± 55 (dashed line).

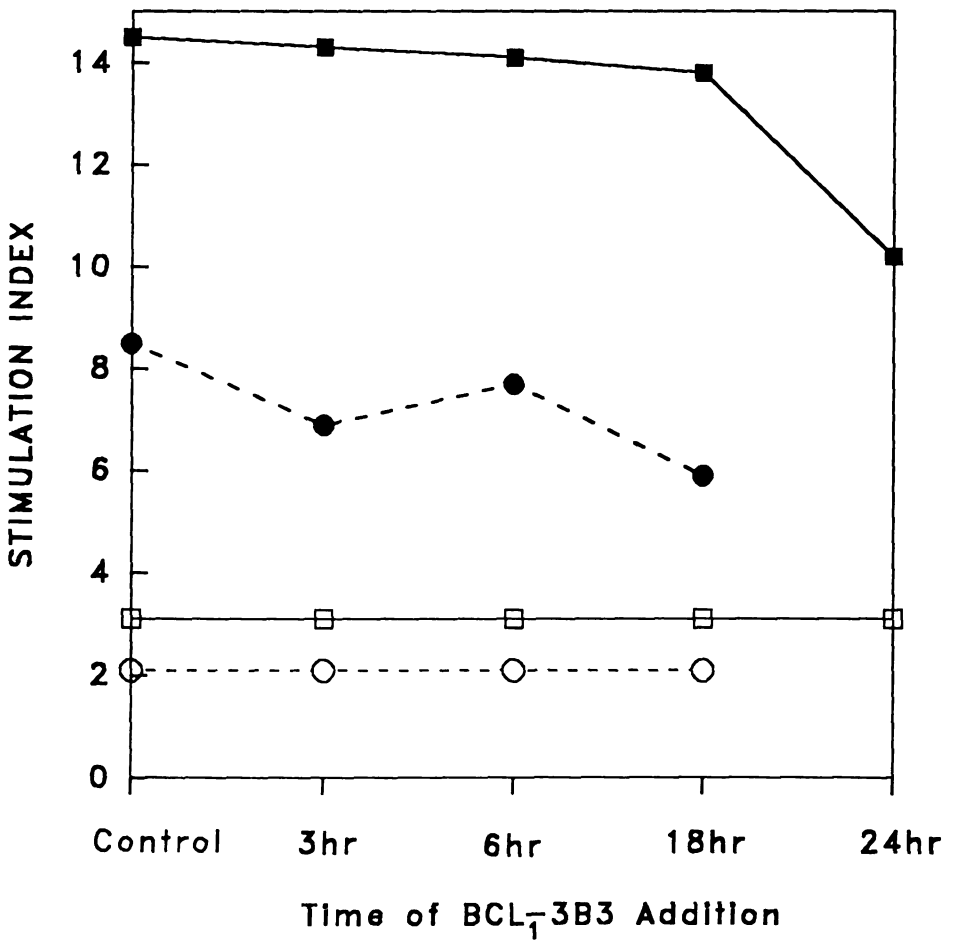
STIMULATION INDEX
14
12
10
8
6
4
2
0
Control
3hr
6hr
18hr
24hr
Time of BCL1-3B3 Addition

Figure 13

Figure 14. IL-2 is required in the first six hours of BCL_1-3B3-mediated B cell help. In two separate experiments, splenic B cells at 5 X 10^5 cells/ml were cultured alone (○, □) or with 1.5 X 10^5 cells/ml paraformaldehyde-fixed BCL_1-3B3 B cells (●, ■) at 37°C, in an atmosphere of 10% CO_2 in air. At the indicated time intervals, 2.5 ng/ml IL-2 was added to the cultures. At 66 hrs. of culture the cells were pulsed with 1 μCi/well of ^{3}H-thymidine. Following a 6 hr. pulse, cells were harvested and the mean CPM for triplicate wells determined. Stimulation indices were calculated as shown for Figure 1. The mean CPM for splenic B cells was 1264 ± 685 (solid lines) and 1568 ± 475 (dashed lines). The mean background CPMs for fixed BCL_1-3B3 cells plus IL-2 at various time points were as follows: 0 hr. = 178, 1 hr. = 250, 2 hr. = 294, 3 hr. = 305, 4 hr. = 406, 5 hr. = 213, 6 hr. = 490, 15 hr. = 299, 18 hr. = 305, and 24 hr. = 274 (solid line); and 0 hr. = 435, 1 hr. = 454, 2 hr. = 611, 3 hr. = 559, 4 hr. = 340, 5 hr. = 265, 6 hr. = 262, 15 hr. = 210, 18 hr. = 99, 24 hr. = 328 (dashed line).

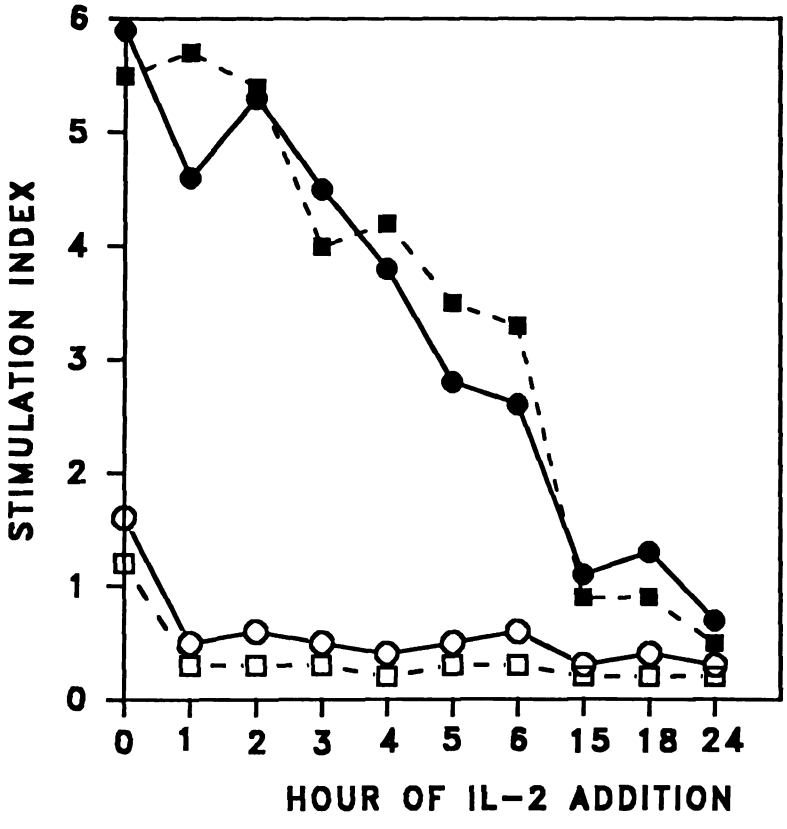
STIMULATION INDEX
0
1
2
3
4
5
6
0 1 2 3 4 5 6 15 18 24
HOUR OF IL-2 ADDITION

Figure 14

4.2.

cell
we
plu
by
ac
(S
sti
10
pa
by
IL
of

in
o
3
th
h
a

4.2.3. Interleukin 10 (IL-10) enhances the helper capacity of paraformaldehyde-fixed BCL_1-3B3 B cells.

To determine if the helper capacity of paraformaldehyde-fixed BCL_1-3B3 cells could be enhanced by soluble factors, paraformaldheyde-fixed BCL_1-3B3 cells were combined with splenic B cells in the presence of IL-2, IL-2 plus IL-10, and IL-2 plus IL-6. The addition of exogenous IL-10 markedly enhanced the help provided by paraformaldehyde-fixed BCL_1-3B3 cells (Figure 15). Maximal proliferation was achieved in the presence of 10 U/ml of IL-10, with a mean CPM of 26,228 ± 1407 (SEM). The addition of exogenous IL-10 resulted in a two-fold increase in the stimulation provided by paraformaldehyde-fixed BCL_1-3B3 cells [mean CPM of 10,915 ± 1164 (SEM)]. The stimulation provided by the combination of IL-10 and paraformaldheyde-fixed BCL_1-3B3 cells was roughly equivalent to the help provided by irradiated BCL_1-3B3 cells [mean CPM of 22,069 ± 2478 (SEM)]. In contrast to IL-10, the addition of exogenous IL-6 did not markedly enhance the helper capacity of paraformaldehyde-fixed BCL_1-3B3 cells [mean CPM of 13,856 ± 902 (SEM)].

The role of IL-10 in $CD5^+$ B-cell-mediated help was confirmed by culturing irradiated BCL_1-3B3 cells with splenic B cells and IL-2, in the presence or absence of antibody to IL-10. Antibody to IL-10 inhibited 42% of the help provided by BCL_1-3B3 cells (Figure 16). Surprisingly, although soluble IL-6 did not markedly enhance the help provided by fixed BCL_1-3B3 cells, antibody to IL-6 inhibited 56% of the help provided by irradiated BCL_1-3B3 cells. In addition, when antibodies to IL-10 and IL-6 were combined in a **preliminary** experiment, 93% of BCL_1-3B3-mediated

Figure 15. IL-10 enhances the helper capacity of paraformaldehdye-fixed BCL_1-3B3 cells. Splenic B cells at 5 X 10^5 cells/ml were cultured with 1.5 X 10^5 cells/ml irradiated BCL_1-3B3 cells (diagonal hatched bars) or 1.5 X 10^5 cells/ml paraformaldehyde-fixed BCL_1-3B3 cells (solid bars), in the presence of 2.5 ng/ml IL-2. In addition to IL-2, IL-6 and IL-10 were added at the indicated concentrations. At 66 hrs. of culture the wells were pulsed with 1 μCi/well ^{3}H-thymidine. Following a 6 hr. pulse the cells were harvested. The mean CPM for triplicate wells is shown. Error bars represent the standard error of the mean. The results shown are representative of three experiments.

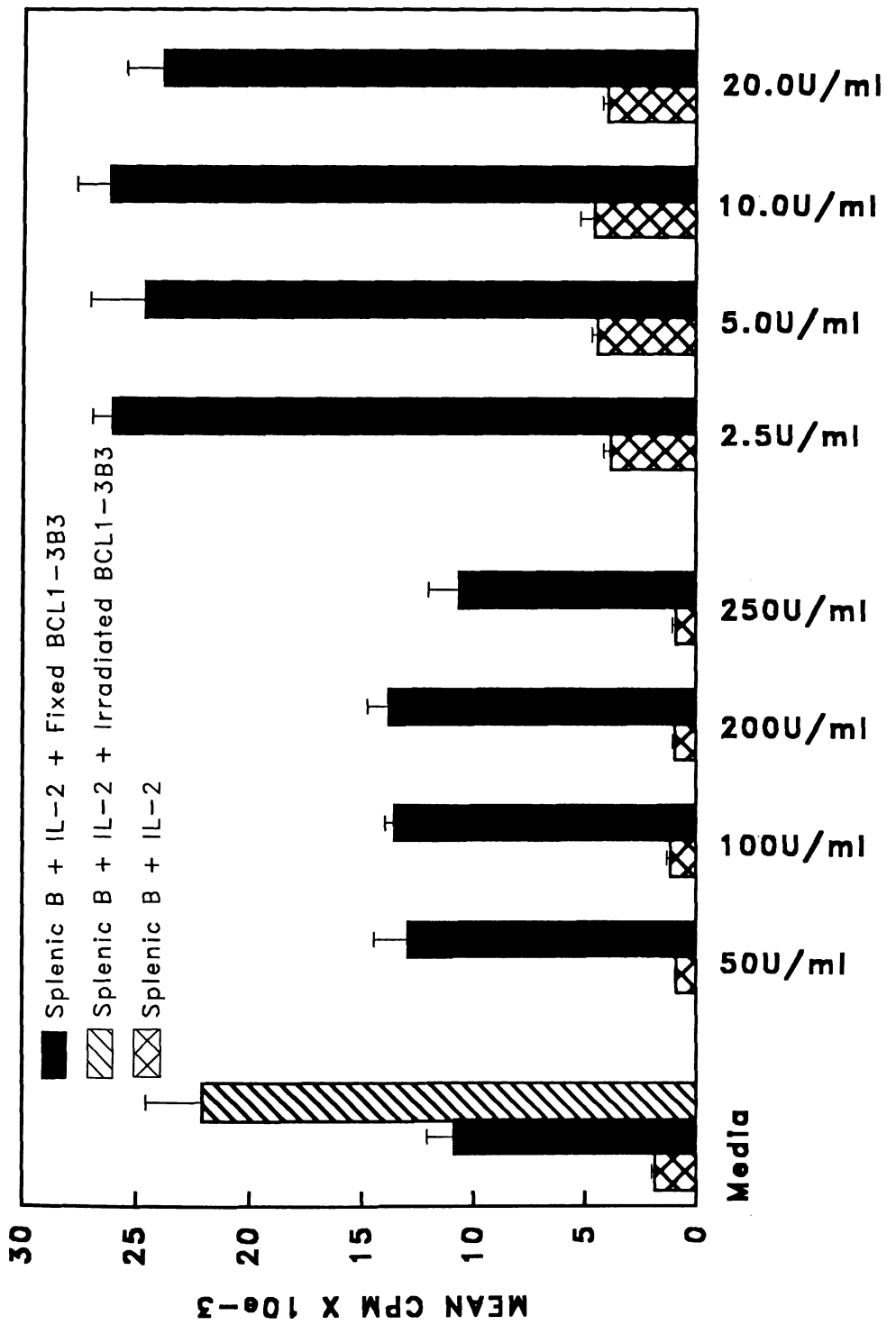

Figure 15 Interleukin-6 Added: Interleukin 10 Added:

Figure 16. Antibodies to IL-6 and IL-10 inhibit BCL_1-3B3-mediated help. Irradiated (4060R) BCL_1-3B3 cells were cultured at 1.5 X 10^5 cells/ml and preincubated with 75 μg/ml anti-IL-10 mAb or anti-IL-6 mAb for 1 hr., at 4°C. Following the preincubation with antibody, 5 X 10^5 cells/ml splenic B cells and 2.5 ng/ml IL-2 were added and the cells were incubated at 37°C, in an atmosphere of 10% CO_2 in air. At 66 hrs. of culture the wells were pulsed with 1 μCi/well ^{3}H-thymidine. Following a 6 hr. pulse the cells were harvested and the mean CPM for triplicate wells was determined. Stimulation indices were determined as described for Figure 1. The data shown represents the mean from five experiments with the % inhibition for each experiment calculated as follows : 100% -[(SI in presence of IL-2, anti-IL-6 or anti-IL-10 and irradiated BCL_1-3B3 cells / SI in the presence of IL-2 and irradiated BCL_1-3B3 cells) (100%)]. Error bars indicate the standard error of the mean.

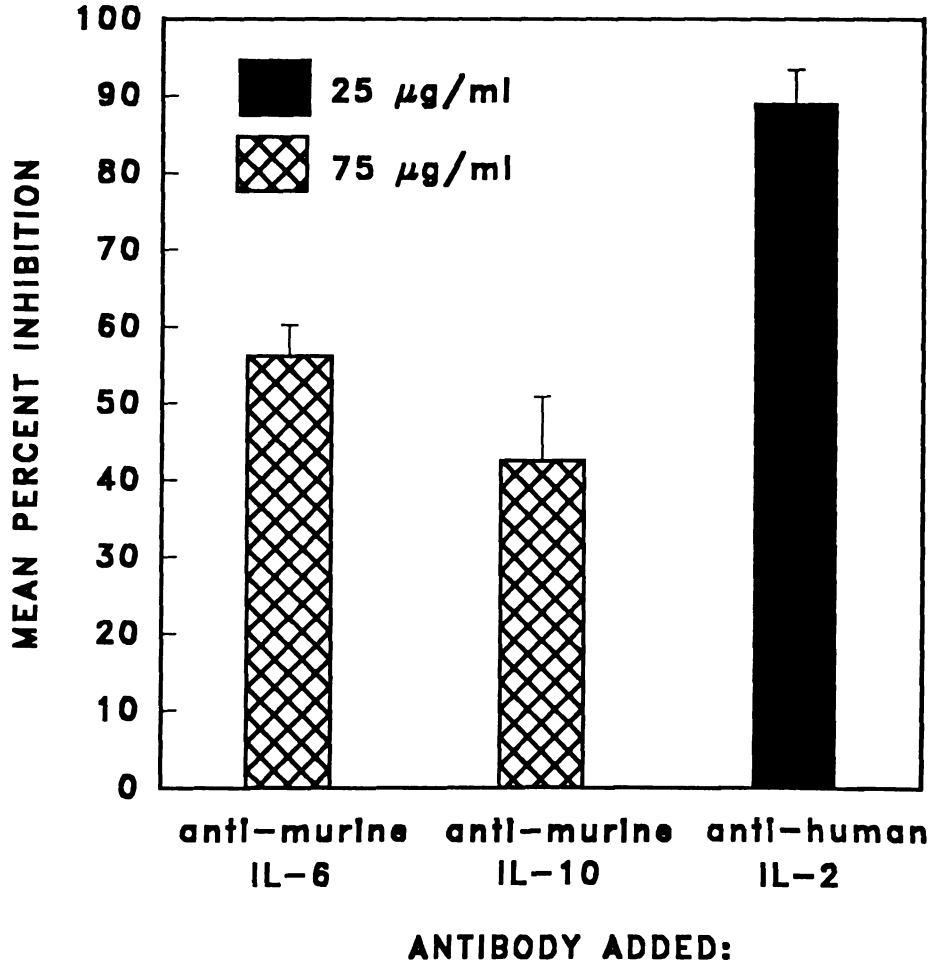
100
90
80
70
60
50
40
30
20
10
0
MEAN PERCENT INHIBITION
25 µg/ml
75 µg/ml
anti-murine IL-6
anti-murine IL-10
anti-human IL-2
ANTIBODY ADDED:

Figure 16

help was inhibited. Consistent with our previous experiments which have shown BCL_1-3B3-mediated help is dependent upon the presence of IL-2, neutralizing antibodies to the exogenously added recombinant-human IL-2 inhibited 89% of the BCL_1-3B3-mediated help.

4.3 Summary.

Analysis of the sequence of signalling events involved in IL-2-dependent, BCL_1-3B3-mediated help has revealed that BCL_1-3B3 cells provide an initial signal to splenic B cells which enhances their proliferative responses to IL-2. This enhanced IL-2 responsiveness appears to be transient and the addition of IL-2 is most critical in the first six hours of BCL_1-3B3-mediated help. In the presence of IL-2, IL-10 markedly enhanced the helper capacity of paraformaldehyde-fixed BCL_1-3B3 cells. In addition, anti-IL-10 mAb inhibited 42% of the stimulation provided by irradiated BCL_1-3B3 cells. These findings suggest that in addition to the contact-dependent signal, BCL_1-3B3-derived IL-10 may play a role in B-cell-mediated help. Although exogenous IL-6 did not markedly enhance the stimulatory capacity of paraformaldehyde-fixed BCL_1-3B3 cells, anti-IL-6 mAb inhibited 56% of the stimulation provided by irradiated BCL_1-3B3 cells. Consistent with these findings, the splenic B cells may be secreting IL-6 which functions as an autocrine factor and enhances splenic B cell proliferation. Alternatively, it is possible that BCL_1-3B3 cells express a membrane form of IL-6 which is directly involved in mediating the contact-dependent signal. In order to more clearly understand BCL_1-3B3-mediated help, further evaluations of both of these possibilities are necessary.

5.0 ANALYSIS OF B-1 FREQUENCIES FOLLOWING INDUCTION OF THREE MURINE SYSTEMIC AUTOIMMUNE DISEASES: MURINE ACQUIRED IMMUNODEFICIENCY SYNDROME (MAIDS), CHRONIC GRAFT-VERSUS-HOST DISEASE (cGvHD) AND COLLAGEN-INDUCED ARTHRITIS (CIA).

5.1 Rationale.

Since the identification of $CD5^+$ ($Ly\text{-}1^+$) B cells in mice and the documentation of their high frequency in some spontaneous autoimmune mouse strains, numerous clinical studies have been performed to determine the $CD5^+$ B cell frequency in human autoimmune diseases. An increase in peripheral blood $CD5^+$ B cell frequency has been reported in rheumatoid arthritis (Hardy et al., 1987; Hara et al., 1988; Plater-Zyberk and Maini, 1988; Brennan et al., 1989), Sjögren's syndrome (Youinou et al., 1988; Dauphinée et al., 1988; Brennan et al., 1989), myasthenia gravis (Ragheb and Lisak, 1990), insulin-dependent diabetes mellitus (Nicoletti et al., 1990), and Hashimoto's thyroiditis (Suranyi et al., 1989). Although patients with rheumatoid arthritis have consistently been shown to have increased $CD5^+$ B cell levels (Hardy et al., 1987; Hara et al., 1988; Plater-Zyberk and Maini, 1988; Youinou et al., 1988; Dauphinée et al., 1988; Brennan et al., 1989), there is still some controversy concerning whether this increase correlates with disease activity (Brennan et al., 1989; Smith and Olson, 1990; Kazbay and Osterland, 1990; Becker et al., 1990). Furthermore, variable changes in the $CD5^+$ B cell frequency in systemic lupus erythematosus (SLE) have been reported (Dauphinée et al., 1988; Suzuki and Sakane, 1989; Smith and Olson, 1990), including a decline in $CD5^+$ B cells (Suzuki and Sakane, 1989).

To help clarify the significance of changes in $CD5^+$ B cell frequency which have previously been observed in human systemic autoimmune diseases, three-color flow cytometric analysis of the B-1 and B-2 cell populations was performed

in three murine models of systemic autoimmunity. In order to more clearly address how a normal system may advance to an autoimmune state, induced murine models were chosen, as opposed to genetically inherited autoimmune murine models. Each of the three models chosen differ significantly in terms of the inducing signal and shares certain pathological features with a human autoimmune disease. The first model, murine acquired immunodeficiency syndrome (MAIDS), has been purported to result from superantigen stimulation of T cells by the Murine leukemia virus (MuLV) gag protein (Hügin et al., 1991) and its pathology has been compared to the ARC phase of AIDS (Mosier, 1986). The second model, chronic graft-versus-host disease (cGvHD), which exhibits pathology similar to SLE in humans (Gleichmann, et al., 1982), involves the reaction of parental DBA2 T cells to MHC Class II molecules on C57BL/6 x DBA2 (BDF1) B cells and monocytes (Portanova and Kotzin, 1988). The third model, type II collagen-induced arthritis (CIA) involves a cross-reactive response to foreign collagen (Courtenay, et al., 1980) and exhibits pathology that resembles human rheumatoid arthritis (Holmdahl et al., 1989). In addition to B-1 frequency analysis, spontaneous *in vitro* antibody secretion and serum autoantibody levels were monitored to determine if frequency changes in the B-1 cells correlate temporally with the appearance of autoimmunity.

5.2 Review of murine models.

5.2.1. MAIDS.

Murine acquired immunodeficiency syndrome (MAIDS) results from the infection of susceptible strains of mice with a murine leukemia virus (MuLV)

originally isolated by Laterjet and Duplan (Laterjet and Duplan, 1962) and later termed LP-BM5 MuLV by Mosier and colleagues (Mosier et al., 1985). Previous studies have shown both B cells and $CD4^+$ T cells, but not $CD8^+$ T cells are required for the development of MAIDS (Mosier et al., 1987; Yetter et al., 1988; Cerny et al., 1990). Murine AIDS is characterized by splenomegaly, lymphadenopathy, polyclonal B cell activation and hypergammaglobulinemia and severe immunosuppression (Mosier et al., 1985; Mosier, 1986). Although symptoms of disease can appear as early as one week post-infection (PI), the most critical time point for evidence of disease manifestations in MAIDS appears from two- to four weeks PI (Mosier et al., 1985; Mosier, 1986). By three weeks PI lymphadenopathy and splenomegaly are evident, as is the decline in *in vitro* immune responses (Mosier et al., 1985). The immunosuppression which begins at two weeks PI results in a total loss of *in vitro* responses to the T and B cell mitogens PHA, Con A, LPS and anti-IgM and loss of MLR activity by four weeks PI (Mosier et al., 1985; Mosier, 1986). In addition, responses to both T-cell-independent antigens (TNP-Ficoll) and T-cell-dependent antigens (SRBC) are lost by four weeks PI (Mosier et al., 1985, Mosier, 1986). By seven weeks PI, all *in vitro* immune responses are absent, followed by death at sixteen- to twenty weeks PI (Mosier, 1986).

5.2.2. cGvHD.

The induction of a murine chronic graft-versus-host disease (cGvHD) is dependent upon three factors: the injection of donor CD4+ alloreactive T cells into

a F_1 recipient (Gleichmann et al., 1982; Rolink and Gleichmann, 1983), incompatibility at the H-2 I region (IA/IE) between donor and F_1 recipient (Van Rappard et al., 1982; Van Rappard et al., 1983; Rolink and Gleichmann, 1983) and the presence of autoreactive B cells in the F1 recipient (Van Elven et al., 1981; Gleichmann et al., 1984). Characteristic symptoms of cGvHD include splenomegaly, lymphadenopathy, hypergammaglobulinemia, restricted polyclonal B cell activation, autoantibody production and immune complex glomerulonephritis (Portanova and Kotzin, 1988). As early as 1 week post cGvHD induction hypergammaglobulinemia is evident, lasting until 4 weeks post induction (Gleichmann et al., 1982). At 2 weeks post cGvHD induction there are detectable signs of splenomegaly and lymphadenopathy (Gleichmann et al., 1982). Serum IgG antibodies to erythrocytes, thymocytes, denatured DNA, and histones are detectable from 2 to 6 weeks post cGvHD induction (Gleichmann et al., 1982; Van Rappard et al., 1984; Portanova et al., 1985; Portanova et al., 1988). Immune complex glomerulonephritis proceeds peak autoantibody production occurring from 4 to 10 weeks post cGvHD induction (Portanova et al., 1988).

5.2.3. CIA.

Collagen-induced arthritis (CIA) is induced in susceptible male mice by intradermal immunization with native type II collagen (Courtenay et al., 1980). Symptoms of disease include redness and swelling of the limbs, serum autoantibodies reactive with collagen, gross joint deformation and, in severe cases, a total loss of joint mobility (Wooley et al., 1981). Two separate reports indicate

disease onset occurs between 5 and 7 weeks post-collagen immunization (Stuart et al., 1982; Wooley et al., 1981). Serum IgM antibodies reactive with collagen peak at two weeks post-immunization, whereas peak levels of serum IgG anti-collagen antibodies are found at 5 weeks post-immunization (Stuart et al., 1982).

5.3 Results.

5.3.1. $CD5^+$ ($Ly\text{-}1^+$) B cell frequency declines in three murine models of systemic autoimmune disease.

To determine if the frequency of the B-1 or conventional B cells changes during the course of murine systemic autoimmune diseases, two-color flow cytometric analysis of the splenic $Ly\text{-}1^+$, $Fc_\epsilon R^{dull}$ (equivalent of $Fc_\epsilon R^-$) and $Fc_\epsilon R^+$ B cell subsets was performed in MAIDS, cGvHD and CIA. The percent of $Ly\text{-}1^+$ splenic B cells showed a marginal decrease in all three models (Table 1). In both MAIDS and cGvHD the decrease in the percent of $Ly\text{-}1^+$ B cells occurred early, at 3 and 5 weeks following disease induction, respectively; whereas the decrease in CIA occurred later at 8 weeks post collagen injection. Data from FACS analysis of each model at 5 weeks post disease induction are shown in Figure 17.

In contrast to the percentage of splenic $Ly\text{-}1^+$ B cells, there was no obvious pattern of decline in the absolute number of splenic $Ly\text{-}1^+$ B cells in any of the three models examined (Table 2). As shown in Tables 3 and 4, the apparent decline in the splenic $Ly\text{-}1^+$ B cell frequency was the result of concurrent increases in splenic T cells and/or conventional B cells. For example, in the MAIDS model, mAb to CD4 (GK1.5) and CD8 (53.6.72) detected an increase in the total number

Table 1. Splenic Ly-1$^+$ B cell frequencies in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Percentage of Ly-1$^+$ B	SEM	(n)
MAIDS	0	3.1	0.322	16[a]
	1	2.3	0.509	4[b]
	2	2.5	0.706	4[b]
	3	1.8	0.381	4[b]
	5	2.0	0.330	4[b]
	7	1.8	0.250	2[b]
cGvHD	0	2.8	0.276	8[a]
	3	2.9	0.825	4[b]
	5	2.0	0.145	4[b]
	7	2.0	0.248	2[b]
	9	2.0	0.851	2[b]
CIA	0	3.2	0.217	4[a]
	3[c]	3.5		1[b]
	4[c]	3.4		1[b]
	5[c]	3.1		1[b]
	6[c]	2.8		1[b]
	8[c]	1.6		1[b]
	12[c]	1.9		1[b]

[a] n = age matched controls, with lymphocytes from two animals being pooled at each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

Figure 17. Two-color flow cytometric analysis of splenic Ly-1$^+$ and Fc$_\epsilon$R^{dull} B cells at 5 weeks following disease induction in MAIDS, cGvHD and CIA. Splenic lymphocytes from mice infected with LP-BM5 MuLV (panels b and d), injected with DBA2 donor cells (panels f and h), immunized with type II collagen (panels j and l) or age-matched controls (panels a, c, e, g, i and k) were washed and centrifuged through lympholyte M to remove dead cells. Viable spleen cells were stained with 2.4G2, biotin-53.7.313 and cyanine-b.7.6 (panels a, b, e, f, i and j) or 2.4G2, biotin-B3B4 and cyanine-b.7.6 (panels c, d, g, h, k and l) followed by PE-AV.

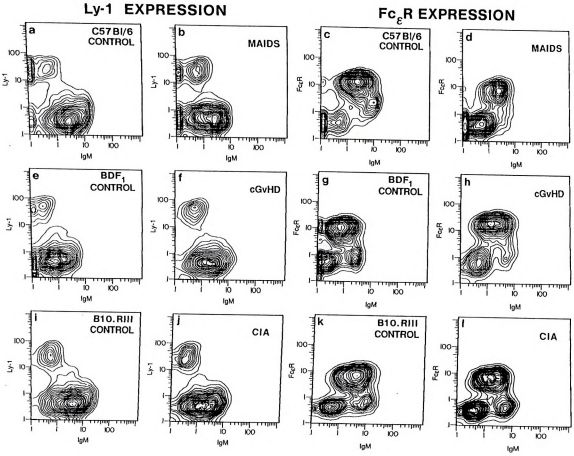
Ly-1 EXPRESSION
Fc$_\epsilon$R EXPRESSION
a
C57Bl/6
CONTROL
b
MAIDS
c
C57Bl/6
CONTROL
d
MAIDS
e
BDF$_1$
CONTROL
f
cGvHD
g
BDF$_1$
CONTROL
h
cGvHD
i
B10.RIII
CONTROL
j
CIA
k
B10.RIII
CONTROL
l
CIA
Ly-1
FcεR
IgM
1
10
100

Figure 17

Table 2. Absolute numbers of splenic Ly-1$^+$ B cells in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Number of Ly-1$^+$ B X 10^6	SEM	(n)
MAIDS	0	3.4	0.526	16[a]
	1	2.7	0.662	4[b]
	2	3.0	0.828	4[b]
	3	3.6	1.085	4[b]
	5	3.0	0.720	4[b]
	7	3.5	0.790	2[b]
cGvHD	0	2.7	0.271	8[a]
	3	3.4	0.680	4[b]
	5	2.3	0.244	4[b]
	7	2.6	0.145	2[b]
	9	2.9	1.545	2[b]
CIA	0	2.4	0.190	4[a]
	3[c]	3.1		1[b]
	4[c]	3.5		1[b]
	5[c]	2.6		1[b]
	6[c]	2.7		1[b]
	8[c]	2.7		1[b]
	12[c]	1.9		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled for each time point.

[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.

[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

Table 3. Absolute numbers of splenic T cells in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Number of T cells X 10^7	SEM	(n)
MAIDS	0	2.2	0.250	16[a]
	1	2.7	0.519	4[b]
	2	2.5	0.574	4[b]
	3	4.5	0.628	4[b]
	5	3.2	0.441	4[b]
	7	4.3	1.935	2[b]
cGvHD	0	1.4	0.171	7[a]
	3	2.2	0.116	4[b]
	5	3.0	0.800	4[b]
	7	1.6	0.185	2[b]
	9	2.3	0.845	2[b]
CIA	0	1.3	0.329	4[a]
	3[c]	1.7		1[b]
	4[c]	1.5		1[b]
	5[c]	1.3		1[b]
	6[c]	1.0		1[b]
	8[c]	2.2		1[b]
	12[c]	2.4		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled for each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

Table 4. Absolute numbers of splenic $Fc_\epsilon R^+$ B cells in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Number of $Fc_\epsilon R^+$ B X 10^7	SEM	(n)
MAIDS	0	6.5	0.645	15[a]
	1	6.6	1.158	4[b]
	2	6.0	1.311	3[b]
	3	8.8	2.790	4[b]
	5	5.8	0.396	4[b]
	7	8.1	2.185	2[b]
cGvHD	0	6.1	0.479	7[a]
	3	7.6	0.891	4[b]
	5	5.5	0.269	4[b]
	7	8.9	0.655	2[b]
	9	8.3	0.655	2[b]
CIA	0	4.4	0.399	4[a]
	3[c]	4.7		1[b]
	4[c]	6.2		1[b]
	5[c]	4.3		1[b]
	6[c]	6.1		1[b]
	8[c]	8.6		1[b]
	12[c]	5.6		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled for each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

of splenic T cells from 3-to 7-weeks post-infection, a period which coincided with declining percentages of splenic Ly-1$^+$ B cells. In the cGvHD model the decrease in the percentage of Ly-1$^+$ B cells was due to an increase in both splenic T cells (detected with mAb to CD4 and CD8) and conventional B cells (detected with mAb to B220 and $Fc_\epsilon R$). By 9 weeks post disease induction the T cell number increased in cGvHD, from a mean control of $1.35 \times 10^7 \pm 0.170$ SEM to $2.26 \times 10^7 \pm 0.851$ SEM and the conventional B cell number increased from a mean control of $6.09 \times 10^7 \pm 0.479$ SEM to $8.26 \times 10^7 \pm 0.657$ SEM. The decrease in the percentage of Ly-1$^+$ B cells at 8 and 12 weeks post collagen boost in the CIA model reflected an increase in the number of $Fc_\epsilon R^+$ B cells from a mean control of 4.37×10^7 to 8.63×10^7 and 5.58×10^7, respectively.

5.3.2. Decline in splenic $Fc_\epsilon R^{dull}$ B cell subset in cGvHD but not in MAIDS or CIA.

As shown in Table 5, the percentage of $Fc_\epsilon R^{dull}$ B cells declined in both the MAIDS and the cGvHD models, but not in the CIA model. However, when the absolute number of $Fc_\epsilon R^{dull}$ B cells was calculated in each of the three models, only the cGvHD model exhibited a loss in the absolute number of $Fc_\epsilon R^{dull}$ B (Table 6). In the cGvHD model the absolute number of splenic $Fc_\epsilon R^{dull}$ B cells declined from a mean control of 12×10^6 to 8×10^6, at 5- to 9-weeks post disease induction. Thus, in the spleen, the only decline in the B-1 cell population which represented both a decline in frequency and absolute cell number was the decline in $Fc_\epsilon R^{dull}$ B cells in the cGvHD model.

Table 5. Splenic $Fc_\epsilon R^{dull}$ B cell frequencies in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Percentage of $Fc_\epsilon R^{dull}$ B	SEM	(n)
MAIDS	0	11.2	0.693	15[a]
	1	8.6	0.867	4[b]
	2	10.9	0.670	3[b]
	3	8.3	0.907	4[b]
	5	6.0	1.103	4[b]
	7	7.2	2.257	2[b]
cGvHD	0	12.3	1.053	7[a]
	3	11.3	1.390	4[b]
	5	7.5	1.515	4[b]
	7	5.8	0.553	2[b]
	9	5.7	0.504	2[b]
CIA	0	13.5	2.075	4[a]
	3[c]	16.4		1[b]
	4[c]	13.7		1[b]
	5[c]	17.2		1[b]
	6[c]	14.5		1[b]
	8[c]	20.1		1[b]
	12[c]	13.4		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled at each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

Table 6. Absolute numbers of splenic $Fc_\epsilon R^{dull}$ B cells in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Number of $Fc_\epsilon R^{dull}$ B X 10^6	SEM	(n)
MAIDS	0	11.7	1.110	15[a]
	1	10.4	2.500	4[b]
	2	11.0	1.888	3[b]
	3	15.2	2.275	4[b]
	5	8.5	1.360	4[b]
	7	13.7	3.225	2[b]
cGvHD	0	12.1	1.362	7[a]
	3	15.0	3.475	4[b]
	5	8.1	0.947	4[b]
	7	7.8	0.150	2[b]
	9	8.0	1.750	2[b]
CIA	0	9.7	1.500	4[a]
	3[c]	14.6		1[b]
	4[c]	14.2		1[b]
	5[c]	14.4		1[b]
	6[c]	13.8		1[b]
	8[c]	33.4		1[b]
	12[c]	13.7		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled for each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

5.3.3. B cell subset frequencies in the peritoneal cavity.

Previously, Herzenberg and colleagues have shown that the peritoneal cavity is the anatomical site with the highest fraction of $CD5^+$ ($Ly\text{-}1^+$) B cells (Herzenberg et al., 1986), therefore two-color flow cytometric analysis was utilized to determine the frequency of the three phenotypic B cell subsets in the peritoneal cavity. Consistent with the results from the spleen, the frequency of $Ly\text{-}1^+$ B cells in the peritoneal cavity decreased in all three murine models (Table 7). Although the percentage of peritoneal $Ly\text{-}1^+$ B cells declined following disease induction in both the MAIDS and cGvHD models, a concomitant decrease in the absolute number of peritoneal $Ly\text{-}1^+$ B cells was not detected (Table 8). In contrast, in the CIA model there was a decrease in the absolute number of peritoneal $Ly\text{-}1^+$ B cells at 8 and 12 weeks post-collagen injection.

Consistent with the $Fc_\epsilon R^{dull}$ B cells in the spleen, the absolute number of $Fc_\epsilon R^{dull}$ B cells in the peritoneal cavity also decreased in the cGvHD model from a mean control of 3×10^6 to 2×10^6 at 9 weeks post infection (Table 9). This was reflected by a decline in $Fc_\epsilon R^{dull}$ B cells from 57% to 28% by 9 weeks post disease induction (Table 10). Contour plots of data representative of each model at 5 weeks are shown in Figure 18.

5.3.4. Isotype profiles.

Previous studies have indicated that B-1 cells predominantly differentiate into IgM and IgG_3 secreting cells (Sidman et al., 1986). In addition, Hayakawa and colleagues have shown that in NZB mice the IgM spontaneously secreted by

Table 7. Peritoneal cavity Ly-1$^+$ B cell frequencies in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Percentage of Ly-1$^+$ B	SEM	(n)
MAIDS	0	33.4	2.934	14[a]
	1	23.8	6.251	2[b]
	2	25.0	2.124	4[b]
	3	30.6	7.872	4[b]
	5	28.4	5.594	4[b]
	7	14.1	4.900	2[b]
cGvHD	0	30.5	3.715	8[a]
	3	16.6	1.627	4[b]
	5	18.7	4.661	4[b]
	7	19.3	4.201	2[b]
	9	24.7	0.500	2[b]
CIA	0	21.9	1.390	4[a]
	3[c]	18.0		1[b]
	4[c]	30.4		1[b]
	5[c]	14.3		1[b]
	6[c]	25.2		1[b]
	8[c]	4.8		1[b]
	12[c]	12.2		1[b]

[a] n = age matched controls, with lymphocytes from two animals being pooled at each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

Table 8. Absolute numbers of peritoneal Ly-1$^+$ B cells in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Number of Ly-1$^+$ B X 10^6	SEM	(n)
MAIDS	0	1.2	0.158	13[a]
	1	1.5	0.305	2[b]
	2	1.1	0.122	4[b]
	3	1.5	0.640	4[b]
	5	1.3	0.289	4[b]
	7	0.5	0.115	2[b]
cGvHD	0	1.6	0.180	8[a]
	3	1.4	0.171	4[b]
	5	1.9	0.304	4[b]
	7	1.0	0.175	2[b]
	9	1.9	0.690	2[b]
CIA	0	0.9	0.150	4[a]
	3[c]	0.8		1[b]
	4[c]	1.3		1[b]
	5[c]	0.3		1[b]
	6[c]	0.8		1[b]
	8[c]	0.2		1[b]
	12[c]	0.4		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled for each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two collagen injected mice, thus SEM is not indicated, (n=1).

Table 9. Absolute numbers of peritoneal $Fc_\epsilon R^{dull}$ B cells in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Number of $Fc_\epsilon R^{dull}$ B X 10^6	SEM	(n)
MAIDS	0	1.8	0.317	6[a]
	1[c]	1.2		1[b]
	2[c]	1.5		1[b]
	3[c]	1.3		1[b]
	5[c]	2.2		1[b]
	7[c]	0.9		1[b]
cGvHD	0	3.1	0.266	8[a]
	3	2.6	0.531	4[b]
	5	4.0	1.485	3[b]
	7	2.0	0.030	2[b]
	9	2.2	1.060	2[b]
CIA	0	1.4	0.218	4[a]
	3[c]	1.2		1[b]
	4[c]	2.1		1[b]
	5[c]	0.6		1[b]
	6[c]	1.3		1[b]
	8[c]	1.3		1[b]
	12[c]	0.9		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled for each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two experimental mice, thus SEM is not indicated, (n=1).

Table 10. Peritoneal $Fc_\epsilon R^{dull}$ B cell frequencies in MAIDS, cGvHD and CIA.

Model	Wk.	Mean Percentage of $Fc_\epsilon R^{dull}$ B	SEM	(n)
MAIDS	0	45.9	3.115	6[a]
	1[c]	27.0		1[b]
	2[c]	51.7		1[b]
	3[c]	42.1		1[b]
	5[c]	53.0		1[b]
	7[c]	21.1		1[b]
cGvHD	0	56.9	2.711	8[a]
	3	30.7	2.612	4[b]
	5	42.4	4.692	3[b]
	7	37.8	2.400	2[b]
	9	27.6	4.401	2[b]
CIA	0	36.6	3.869	4[a]
	3[c]	28.4		1[b]
	4[c]	49.3		1[b]
	5[c]	26.4		1[b]
	6[c]	42.6		1[b]
	8[c]	32.0		1[b]
	12[c]	29.6		1[b]

[a] n = age matched controls, with lymphocytes from two animals pooled at each time point.
[b] n = the number of experiments with lymphocytes from two animals pooled for each time point.
[c] Lymphocytes pooled from two experimental mice, thus SEM is not indicated, (n=1).

Figure 18. Two-color flow cytometric analysis of Ly-1$^+$ and Fc$_\epsilon$R^{dull} B cells in the peritoneal cavity following the induction of MAIDS, cGvHD and CIA. At 5 weeks peritoneal exudate cells were removed from mice infected with LP-BM5 MuLV (panels b and d), injected with donor DBA2 cells (panels f and h), immunized with type II porcine collagen (panels j and l) or age-matched controls (panels a, c, e, g, i, and k). Peritoneal exudate cells were stained with 2.4G2, biotin-53.7.313 and cyanine-b.7.6 (panels a, b, e, f, i, and j) or 2.4G2, biotin-B3B4 and cyanine-b.7.6 (panels c, d, g, h, k and l) followed by PE-AV.

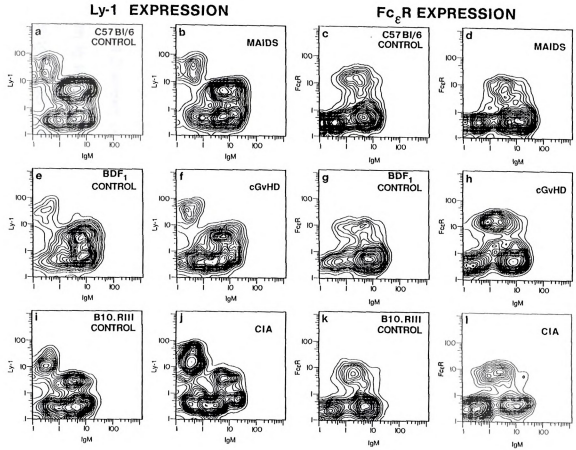
Ly-1 EXPRESSION
Fc$_\varepsilon$R EXPRESSION
a
C57Bl/6
CONTROL
b
MAIDS
c
C57Bl/6
CONTROL
d
MAIDS
e
BDF$_1$
CONTROL
f
cGvHD
g
BDF$_1$
CONTROL
h
cGvHD
i
B10.RIII
CONTROL
j
CIA
k
B10.RIII
CONTROL
l
CIA
Ly-1
FcεR
IgM
100
10
1

Figure 18

spleen cells is secreted by B-1 cells as opposed to conventional B cells (Hayakawa et al., 1984). Thus, to determine if the isotype profile of spontaneous antibody secretion in MAIDS, cGvHD and CIA correlates with the profile previously shown for CD5 B cells, IgM, IgG and IgA secretion was analyzed in splenic cells incubated for 1-3 days after harvest. In the MAIDS model a 7-fold increase in spontaneous IgM secretion (mean control 46.3 ng/ml ± 3.1 SEM to 418.2 ng/ml ± 34.7 SEM) and a 20-fold increase in IgG (mean control 8.4 ng/ml ± 1.9 SEM to 251.0 ng/ml ± 60.3 SEM) were evident at 3 and 5 weeks post-infection, respectively (Figure 19a). The cGvHD model showed a pattern of isotype secretion paralleling that observed in the MAIDS model with increases in both IgM and IgG at 3 and 5 weeks post cell transfer (Figure 19b). The level of spontaneously secreted IgA remained near control levels in both the MAIDS and cGvHD models (Figure 19 a, b). In contrast to MAIDS and cGvHD, the levels for spontaneously secreted IgM and IgG did not increase in the CIA model and there was a 13-fold increase in spontaneously secreted IgA (mean control 19.0 ng/ml ± 2.7 SEM to 252.7 ng/ml ± 20.2 SEM) at 8 weeks post-collagen boost (Figure 19c).

5.3.5. Serum Ig isotypes.

Serum IgM, IgG and IgA levels were also determined following disease induction in the MAIDS and cGvHD models. In the MAIDS model, there was a 6-fold increase in serum IgM levels (mean control of 0.9 ng/ml to 5.7 ng/ml) and a 10-fold increase in serum IgG levels (mean control of 3.8 ng/ml to 39.0 ng/ml) by 7 weeks post-infection (Figure 20a). Our observed increases in serum IgM and

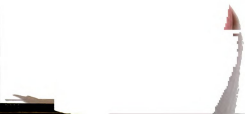

Figure 19. Spontaneous immunoglobulin isotype profile in MAIDS, cGvHD and CIA. Spontaneous Ig secretion was analyzed by culturing splenic lymphocytes from mice infected with LP-BM5 MuLV (panel a), injected with DBA2 donor cells (panel b) or immunized with porcine type II collagen (panel c) in RPMI 1640 supplemented media at 37°C, 10% CO_2 for 48-60 hours. Following *in vitro* culture the concentration of IgM (■), IgG (●) and IgA (▲) was determined with a RIA or an ELISA. The zero week time points represent the mean age-matched control levels. Error bars represent the standard error of the mean.

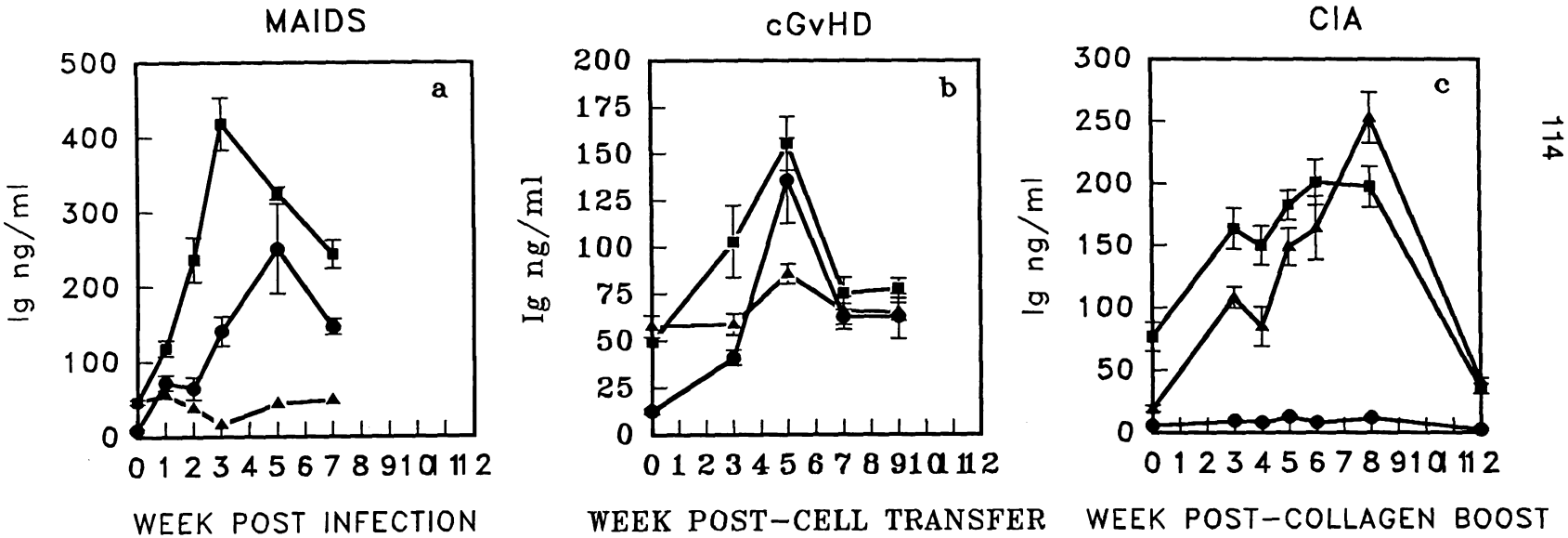
MAIDS
a
lg ng/ml
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEK POST INFECTION
cGvHD
b
lg ng/ml
200
175
150
125
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEK POST-CELL TRANSFER
CIA
c
lg ng/ml
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEK POST-COLLAGEN BOOST

Figure 19

Figure 20. Serum isotype profile in MAIDS and cGvHD. Serum samples were taken following the induction of MAIDS (panel a) or cGvHD (panel b). The levels of serum IgM (■), IgG (●) and IgA (▲) were determined with an ELISA. The zero week time point represents the mean age-matched control levels. Error bars represent the standard error of the mean.

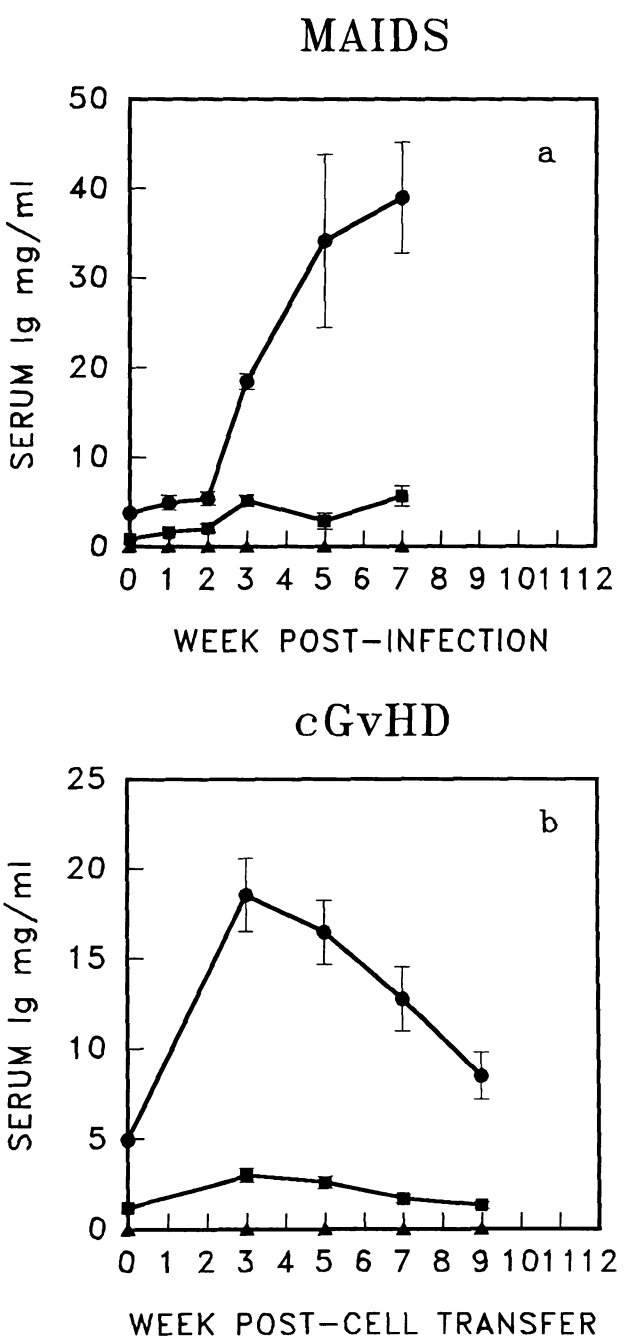
MAIDS
SERUM Ig mg/ml
50
40
30
20
10
0
a
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEK POST-INFECTION
cGvHD
SERUM Ig mg/ml
25
20
15
10
5
0
b
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEK POST-CELL TRANSFER

Figure 20

IgG during the progression of MAIDS are consistent with previous studies which report increases in both serum IgM and IgG with no marked changes in serum IgA (Pattengale et al., 1982; Mosier et al., 1985). As shown in Figure 20b, in the cGvHD model, both serum IgM and serum IgG peaked at 3 weeks post donor T cell injection with approximately a 3-fold increase in IgM (mean control of 1.2 ng/ml to 3.0 ng/ml) and a 4-fold increase in IgG (mean control of 5.0 ng/ml to 18.5 ng/ml). These results are consistent with previous reports of increases in serum IgG from 2- to 4-weeks post cGvHD induction (Gleichmann et al., 1982; Portanova and Kotzin, 1988). Due to a limited sample volume the serum isotype profile was not analyzed in the CIA model.

5.3.6. Specificity of enhanced Ig secretion.

Previously, it has been reported that $CD5^+$ B cells secrete autoantibodies to a host of autoantigens (Casali et al., 1987; Hardy et al., 1987; Kipps, 1989), thus, both supernatants from *in vitro* cultured cells and serum were tested for reactivity to ssDNA, IgG, TNP:BSA and type II collagen. The spontaneous Ig secreted by splenic B cells in both the cGvHD and the CIA models did not bind any of the four antigens tested (data not shown). However, in the MAIDS model approximately 65% of the IgM spontaneously secreted bound ssDNA, from a mean control of 42.5 ng/ml ± 2.5 SEM to 269.8 ng/ml ± 60.1 SEM at 3 weeks post-infection. Consistent with previous reports (Gleichmann et al., 1982; Portanova et al., 1985; Portanova and Kotzin, 1988), autoantibodies to ssDNA were found in the serum in both MAIDS and cGvHD (Figure 21). At 5 weeks post LP-BM5 MuLV

Figure 21. Increased levels of serum anti-ssDNA antibody in MAIDS and cGvHD. The levels of serum IgM anti-ssDNA (■) and serum IgG anti-ssDNA (●) were determined with an ELISA following the induction of MAIDS (solid lines) and cGvHD (dashed lines). The zero week time points represent the mean age-matched control levels. Error bars represent the standard error of the mean.

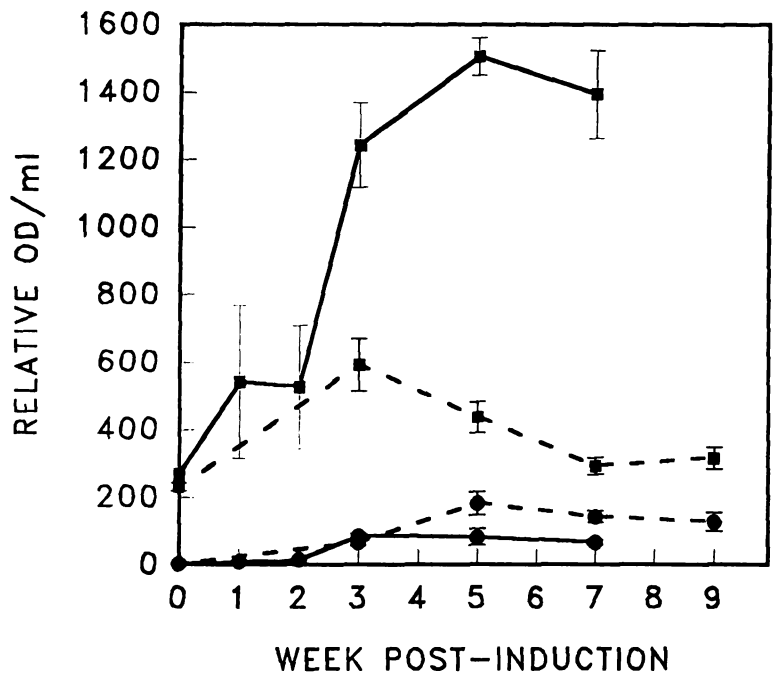
1600
1400
1200
1000
800
600
400
200
0
RELATIVE OD/ml
0 1 2 3 4 5 6 7 8 9
WEEK POST-INDUCTION

Figure 21

infection there was a 5-fold increase in IgM anti-ssDNA from a mean control of 268 relative OD/ml ± 19 SEM to 1506 relative OD/ml ± 56 SEM. In the cGvHD model the most marked changes were in the levels of IgG anti-ssDNA which increased from a mean control of 3 relative OD units/ml ± 0 SEM to 184 relative OD units/ml ± 34 SEM, at 5 weeks post donor T cell transfer. There were no marked changes in anti-IgG, anti-TNP:BSA or anti-collagen in the serum following MAIDS or cGvHD induction. In the CIA model there were marked changes in serum IgG anti-collagen levels from a mean control level of 0 relative OD units/ml to 149 relative OD units/ml at 5 weeks post-collagen boost (Figure 22).

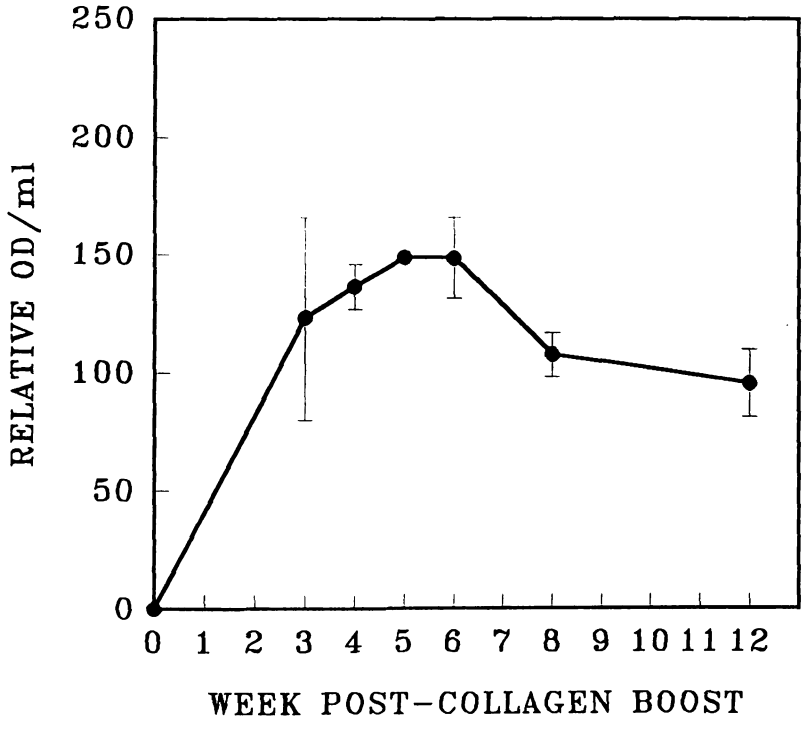

Figure 22. Increased levels of serum IgG anti-collagen antibody in CIA. The levels of serum IgG anti-collagen antibody were determined with an ELISA following the induction of CIA. The zero week time point represents the mean age-matched control levels. Error bars represent the standard error of the mean.

5.4 Summary.

The potential role of B-1 cells (*i.e.* the $CD5^+$ B cell and "sister" B cell subsets) in autoimmunity is controversial. $CD5^+$ B cells have been shown to secrete antibodies of similar specificity as those found in many systemic autoimmune diseases; in addition, increases in $CD5^+$ B cell frequency have been reported in patients suffering from rheumatoid arthritis, Sjögren's syndrome, myasthenia gravis, insulin-dependent diabetes mellitus and Hashimoto's thyroiditis. Whether these increases are due to expansion of B-1 lineage cells in the human or due to activation-induced expression of CD5 by conventional B cells is unclear. In the present study, we used three murine models of systemic autoimmunity: murine acquired immunodeficiency syndrome (MAIDS), chronic graft-versus-host disease (cGvHD), and collagen-induced arthritis (CIA) to determine whether increases in B-1 cell frequency are universally seen in models of autoimmunity which are mechanistically distinct. In contrast to the aforementioned human systemic autoimmune diseases which exhibit an increase in $CD5^+$ B cell frequency, the percentage of $CD5^+$ B cells declined in all three murine models of systemic autoimmune disease. Even though there was a decrease in the frequency of $CD5^+$ B cells there was no change in the actual number of $CD5^+$ B cells. Thus, the apparent decline in $CD5^+$ B cell frequency was due to increases in either T cells, conventional $Fc_\epsilon R^+$ B cells, or both. The only consistent decline in a B cell subset was the loss of IgM^+, $Fc_\epsilon R^{dull}$ cells in both the spleen and peritoneal cavity of mice undergoing a chronic graft-versus-host reaction. Therefore, our data

suggest that expansion of the B-1 subset does not occur as a general feature of murine systemic autoimmune disease. These observations, consistent with previous studies of *Ig* gene usage in autoreactive antibodies, support the view that expansion and differentiation of the $CD5^+$ B cell subset is not a central event leading to autoantibody production.

6.0 DISCUSSION.

$CD5^+$ B-cell-mediated Help.

With the identification of CD72 (murine Lyb-2) as the ligand for CD5 (Luo et al., 1992), the possibility arose that these molecules may play a role in contact-dependent, intercellular communication between B cells. Thus, the preceding studies were undertaken to determine if $CD5^+$ B cells are capable of providing an activating signal to splenic B cells, which express CD72. The presence of irradiated $CD5^+$ neoplastic BCL_1-3B3 B cells significantly enhanced proliferation and differentiation of normal splenic B cells. Proliferation of both resting and *in vivo*-activated cells was totally dependent upon the addition of IL-2; whereas, differentiation of both splenic and peritoneal B cells required IL-5, in addition to IL-2. The help provided by BCL_1-3B3 cells was contact-dependent. Although the helper capacity of a series of B cell lines corresponded to their level of CD5 expression and, in all cases was contact-dependent; anti-CD5 mAb did not block BCL_1-3B3-mediated help.

The experiments on B-cell-mediated help presented in this dissertation were patterned after classic studies of T-cell-mediated help. Current models for T-B collaboration suggest a bidirectional communication involving multiple ligand/receptor pairs resulting in subsequent cytokine release (Parker, 1993). One of the results of the contact-dependent signal provided to B cells during T-B interactions is an enhanced responsiveness to exogenous lymphokines (Parker, 1993). A similar acquisition of interleukin responsiveness may be occurring with B-cell-mediated help. However, in contrast to previous reports which indicate IL-4

is necessary for progression into S phase following T-B collaborations (Noelle et al., 1989; Bartlett et al., 1990; Noelle et al., 1991), $CD5^+$ B-cell-mediated help requires the presence of IL-2 for cell division. This suggests that the B cell-mediated signal specifically affects either IL-2R expression or IL-2-induced intracellular signalling. In order to more fully understand the mechanisms involved in BCL_1-3B3-mediated help, further investigations of both of these possibilities are necessary.

The IL-2 necessity noted for splenic B cell proliferation was also seen in the differentiative response. Although the addition of irradiated $CD5^+$ B cells modestly enhanced the IL-5 induced Ig secretion, the greatest augmentation of both IgM and IgG secretion occurred when irradiated BCL_1-3B3 cells were added in the presence of the IL-2/IL-5 combination. Once again these findings are in contrast to previous studies which have shown either IL-4/IL-5 or IL-2/IL-4 combinations are necessary for enhanced Ig secretion following T-B interactions (Hodgkin et al., 1990; Croft and Swain, 1991; Noelle et al., 1991). Thus, there appears to be a novel activation pathway involved in B-cell-mediated help.

It is possible that the dependency of BCL_1-3B3-mediated help upon the addition of exogenous IL-2 indicates an induction of IL-2R in response to the contact-mediated signal(s) delivered by BCL_1-3B3 cells. Alternatively, IL-2 could provide a first signal to splenic B cells which allows them to proliferate in response to BCL_1-3B3-mediated signals. In order to determine the sequence of signalling events a series of experiments was performed in which the signals were

sequentially provided. Based on the results of these studies, BCL_1-3B3 cells provide the first signal, rendering the splenic B cells responsive to IL-2, resulting in proliferation. Furthermore, the addition of exogenous IL-2 appears to be most critical within the first six hours following the BCL_1-3B3-mediated signal. Thus, further evaluations of IL-2R expression and IL-2 intracellular signalling pathways should be undertaken to determine which activation pathway(s) is affected. In order to more clearly understand the stimulatory capacity of the BCL_1-3B3-mediated signal it was important to determine which populations of B cells could respond to B-cell-mediated help. The BCL_1-3B3-mediated signal did not synergize with IL-4 + GAMIg or IL-5 + DXS signals. In addition both resting and *in vivo*-activated splenic B cells showed proliferative responses in the presence of irradiated, $CD5^+$ B cells and IL-2. Thus, neither primary *in vitro* nor primary *in vivo* activation signals are required for IL-2-dependent, BCL_1-3B3-mediated help. In contrast to splenic B cells, B cells from the peritoneal cavity (PEC B cells) responded minimally. This suggests that some activation parameters are critical for responses to B-cell-mediated help.

Previous studies have shown there is a higher frequency of $CD5^+$ B cells in the peritoneum than in the spleen (Hayakawa et al., 1986). Studies by Wortis and colleagues (Ying-zi et al., 1991) suggest that these $CD5^+$ B cells represent a B cell population which has been activated by a thymus-independent type 2 (TI-2) antigen. Consistent with these findings acridine orange analysis of RNA and DNA content indicated that the PEC B cells have, on average, considerable more RNA

than splenic B cells found in the 55% Percoll fraction. Thus, the minimal proliferative responses obtained from PEC B cells could be the result of an *in vivo* activation event. However, it should be noted that the PEC B cells did not spontaneously proliferate *in vitro* at a rate greater than the 55% Percoll splenic B cell fraction. In addition, the PEC B cells did not respond to IL-2 without the presence of irradiated, $CD5^+$ B cells. Thus, there is no evidence that the PEC B cells have already received a signal equivalent to that mediated by the irradiated BCL_1-3B3 cells. In fact, the enhanced Ig secretion by PEC B cells in response to irradiated BCL_1-3B3 cells and the IL-2/IL-5 lymphokine combination indicates PEC B cells retain the ability to respond to the contact-dependent signal provided by the $CD5^+$ B cells. Therefore, the primary difference between splenic B cells and PEC B cells appears to lie in the regulation of proliferation.

The original impetus for studying the helper activity of $CD5^+$ B cells was the possible involvement of CD5/CD72 in contact-dependent signalling. The help provided by $CD5^+$, BCL_1-3B3 B cells was contact-dependent. In addition, analysis of a panel of neoplastic B cell clones revealed that the level of helper activity corresponded to the level of CD5 expression and was in all cases contact-dependent, providing further support that the CD5 molecule is involved. However, anti-CD5 monoclonal antibody did not block BCL_1-3B3-mediated help. It is possible that the epitope recognized by the CD5 mAb does not block binding of CD72. Alternatively, the concentrations of antibody used may have been insufficient to block CD5/CD72 interactions. Thus, in order to rule out an

involvement for the CD5 molecule in B-cell-mediated help further investigations are necessary.

If the CD5/CD72 ligand/receptor pair is solely involved in B-cell-mediated help, then the stimulation provided by $CD5^+$ B cells should parallel the stimulation provided by anti-CD72 (Lyb-2) monoclonal antibodies. Three previous studies have shown that stimulation of splenic B cells with anti-Lyb-2 mAb results in B cell proliferation (Subbarao and Mosier, 1983; Laurindo et al., 1987; Subbarao et al. 1988). These findings are in contrast to the studies presented in this dissertation, as the addition of irradiated $CD5^+$ B cells alone did not induce B cell proliferation. Two previous studies have also indicated that mAb to Lyb-2 synergizes with anti-IgM antibody to enhance splenic B cells proliferation (Yakura et al., 1986; Subbarao et al., 1988). Once again these findings are in contrast to the results presented in this dissertation and provide support that molecules other than CD5/CD72 may be involved in B-cell-mediated help. In addition, although three neoplastic B cell lines which expressed CD5 provided help, in a preliminary study irradiated B cells from the peritoneal cavity of a BALB/c mouse did not provide a similar stimulation of splenic B cell proliferation. Thus, it is possible that the expression of CD5 by the B cell lines which possess helper activity is coincidental. Therefore, the potential involvement of other ligand/receptor pairs in B-cell-mediated help was investigated.

Previous studies have shown that the CD40L/CD40, LFA-1/ICAM-1 and TCR/MHC class II ligand/receptor pairs play a role in T-cell-mediated help for

humoral responses (Parker, 1993). In addition, a preliminary report by Grammer and Lipsky showed that a human B cell line 626.1 increased DNA synthesis by purified human peripheral blood B cells in the presence of IL-2 and *Staphylococcus aureus* (Grammer and Lipsky, 1993). A soluble fusion protein which contained the extracellular domain of human CD40 blocked this increase in DNA synthesis. This study suggests there is a protein on activated B cells which is capable of interacting with CD40 on B cells. Thus, antibodies to the CD40L, MHC class II molecules, LFA-1 and ICAM-1 were included in the evaluation of potential interaction molecules. In the studies presented in this dissertation, concentrations of the anti-CD40L antibody MR1 which have previously been shown to inhibit T-B interactions (Foy et al., 1993) did not inhibit the B-cell-mediated help. Antibodies to the MHC class II molecules did not inhibit the B-cell-mediated help, but instead increased the proliferation of IL-2 stimulated splenic B cells. This noted increase in proliferation is consistent with previous studies which have shown antibodies to MHC class II molecules mediate: increases in B cell proliferation, Ig secretion, and intracellular cAMP and PKC translocation (Cambier and Lehmann, 1989; Lane el al., 1990; Bishop, 1991; Cambier et al., 1987). In contrast to CD40L and MHC class II antibodies, antibodies to the adhesion molecules LFA-1 (CDlla, CD18) and ICAM-1 did inhibit up to 49% of the B-cell-mediated help. The percent inhibition seen with the addition of these antibodies is within the range previously noted in two separate reports on contact-dependent T-cell-mediated B cell help (Tohma et al., 1991; Owens, 1991) and is consistent with a role for these

molecules in cell adhesion.

If $CD5^+$ B-cell-mediated help is solely dependent upon surface molecule interactions, then paraformaldehyde-fixed BCL_1-3B3 cells should provide stimulation equivalent to the stimulation provided by irradiated BCL_1-3B3 cells. Although paraformaldehyde-fixed BCL_1-3B3 cells stimulated splenic B cell proliferation in the presence of IL-2, the stimulation was reduced when compared to the stimulation provided by irradiated BCL_1-3B3 B cells. Thus it is possible that irradiated BCL_1-3B3 cells secrete a soluble factor(s) which enhances $CD5^+$ B-cell-mediated help. O'Garra and colleagues have previously shown that a number of $CD5^+$ B cell lymphomas secrete both IL-6 and IL-10 (O'Garra et al., 1990). Consistent with O'Garra's findings, IL-10 has been found at concentrations up to 10U/ml in supernatants from the BCL_1-3B3 cells used in my studies (H. Dehghani, unpublished observations). Therefore, the ability of exogenous IL-6 and IL-10 to enhance the stimulation provided by paraformaldehyde-fixed BCL_1-3B3 cells was examined. In the presence of exogenous IL-10 the paraformaldehyde-fixed BCL_1-3B3 cells provided stimulation equivalent to irradiated BCL_1-3B3 cells. In contrast, the addition of exogenous IL-6 only minimally enhanced the stimulation provided by paraformaldehyde-fixed BCL_1-3B3 cells. In addition, anti-IL-10 mAb inhibited up to 42% of the stimulation provided by irradiated BCL_1-3B3 cells. Surprisingly, although exogenous IL-6 did not enhance the helper capacity of paraformaldehyde-fixed BCL_1-3B3 cells, anti-IL-6 mAb inhibited up to 56% of the stimulation provided by irradiated BCL_1-3B3 cells. Klinman and colleagues have

previously shown that splenic B cells from BALB/c mice secrete IL-6 (Shirai et al., 1993). Thus, if the splenic B cells are secreting optimal levels of IL-6, then one would not expect the addition of exogenous IL-6 to increase stimulation. Alternatively, it is possible that the BCL_1-3B3 cells express a membrane form of IL-6 and that this molecule is directly involved in mediating B-cell help.

Physiological Relevance.

It has been nearly two decades since Jerne proposed the network theory of immune regulation (Jerne, 1974). The network theory proposes that there is an established equilibrium within the immune system. Foreign antigens perturb the normal equilibrium resulting in the production of antibodies. A key point of the network theory is the production of autoantibodies reactive with Ig. Foreign antigens disrupt the equilibrium between Ig and anti-idiotypic antibodies by enhancing the production of Ig and its auto anti-idiotypic antibody. The auto anti-idiotypes then provide a feedback mechanism for dampening the response once the foreign antigen is removed. Thus, Jerne's network theory proposes that autoantibodies play a physiological role in normal immune functions. Consistent with this theory, autoantibodies have been found in the serum of normal mice and normal humans(Dighiero et al., 1985; Ternynck and Avrameas, 1986; Huetz et al., 1988). In addition, murine hybridomas secrete antibodies reactive with self antigens (Dighiero et al., 1983). However, these findings are somewhat inconsistent with thymic deletion theories which propose that autoreactive T cells are deleted in the thymus. Current understanding of T-cell-mediated help suggests T cells

expressing CD40L interact with antigen stimulated $CD40^+$ B cells resulting in proliferation and antibody production (Parker, 1993). If T-B interactions are necessary for antibody production and autoreactive T cells are deleted in the thymus then how does one account for the production of autoantibodies in normal mice?

The studies presented in this dissertation provide support for $CD5^+$ B-cell-mediated help which somewhat parallels T-cell-mediated help; however, in contrast to T-cell-mediated help which utilizes IL-4, a T_H2 derived lymphokine, $CD5^+$ B-cell-mediated help is dependent upon IL-2, a T_H1 derived lymphokine. Previous studies by Gajewski and colleagues have shown that initial antigen challenge of murine splenic cells results in the activation of a "naive" T cell (T_H0 cell) which secretes IL-2 but does not secrete IFNγ or IL-4 (Gajewski et al., 1989). Upon further antigen stimulation both T_H1 and T_H2 clones are established which secrete IL-2 and IFNγ or IL-4 and IL-5, respectively. When combined with the data presented in this dissertation these findings suggest a model whereby initial antigen stimulation induces T_H0 cells to secrete IL-2. In response to the IL-2 secreted by T_H0 cells $CD5^+$ B cells could stimulate both the proliferation and differentiation of splenic B cells. In addition, because the $CD5^+$ B-cell-mediated help does not directly involve T-B contact-mediated signalling, thymic deletion would not play a role and autoreactive B cells could be stimulated to produce autoantibodies. The splenic B cells secrete both IgM and IgG in response to $CD5^+$ B-cell-mediated help, thus this help may also stimulate memory B cells. Within the

confines of Jerne's network theory the stimulation of autoreactive B cells would actually play a key role in eliciting normal B cell responses to foreign antigens. However, if the $CD5^+$ B-cell-mediated help is not controlled either through anatomical compartmentalization or mechanisms which control the surface molecules involved in providing the contact-dependent signal, the stimulation of autoreactive B cells could have pathological effects.

$CD5^+$ B Cell Frequencies in MAIDS, cGvHD and CIA.

Previous studies in both genetically autoimmune mice and humans have shown there is a correlation between increases in $CD5^+$ B cell frequencies and systemic autoimmune pathogenesis (Hayakawa et al., 1983, 1984; Sidman et al., 1986; Scribner et al., 1987; Hardy et al., 1987; Becker et al., 1990). Although there is an increase in $CD5^+$ B cell frequency it is unclear whether this increase contributes to autoimmune pathogenesis or is a result of the induction of autoimmunity. In the studies presented in this dissertation I have examined one possible mechanism whereby $CD5^+$ B cells could contribute to autoimmune pathogenesis in mice. Based on the results of these studies, $CD5^+$ neoplastic B cells stimulate both splenic B cell proliferation and differentiation. If this stimulation plays a role in autoimmunity by stimulating the proliferation of autoreactive conventional or $CD5^+$ B cells, then increases in $CD5^+$ B cell frequency may correlate with disease progression. Furthermore, if the expansion of $CD5^+$ B cells plays a critical role in the induction of systemic autoimmunity, then increases in $CD5^+$ B cell frequencies should occur within mechanistically distinct models of

autoimmunity. Thus, the changing dynamics of B cell subsets was analyzed in three induced models of murine systemic autoimmunity: murine acquired immunodeficiency (MAIDS), chronic graft-versus-host disease (cGvHD), and collagen-induced arthritis (CIA). The percentage of splenic $CD5^+$ ($Ly\text{-}1^+$) B cells declined in MAIDS, cGvHD and CIA. It should be noted that this decline in splenic $CD5^+$ B cell frequency was a reflection of increases in other cell populations, rather than a decline in the absolute number of splenic $CD5^+$ B cells. In each of the three models studied, a portion of the decrease in $CD5^+$ B cell frequency was due to an increase in the absolute number of splenic T cells. In addition to increases in T cell number, there was also an increase in the absolute number of conventional, $Fc_\epsilon R^+$, splenic B cells in MAIDS, cGvHD, and CIA. Based on the differences when comparing frequencies and actual numbers for $CD5^+$ B cells it may be more informative in future human and animal studies to report both the actual cell numbers and the frequencies for $CD5^+$ B cells.

The frequency of $CD5^+$ B cells in the peritoneal cavity also declined in each of the three models studied. In parallel to splenic B cells frequencies the decline in peritoneal $CD5^+$ B cells in both the MAIDS and cGvHD models represented an increase in the absolute number of T and/or conventional B cells, as opposed to a decrease in the absolute number of peritoneal $CD5^+$ B cells. In contrast to MAIDS and cGvHD, the absolute number of $CD5^+$ B cells decreased in the CIA model. However, it should be noted that only one series of animals were used for these studies; thus, further evaluations are necessary to determine if this loss

represents a consistent pattern or an isolated event.

The most marked change in a B cell subset occurred in the cGvHD model. In both the spleen and peritoneal cavity of cGvHD mice, there was a progressive decline in the frequency and absolute number of $Fc_\epsilon R^{dull}$ B cells. This $Fc_\epsilon R^{dull}$ population includes $CD5^+$, IgM^{bright} B cells as well as $CD5^-$, IgM^{bright}, B cells. There are several possible explanations for the loss of the IgM^+, $Fc_\epsilon R^{dull}$ B cells which occurred in cGvHD. In the first scenario, the $Fc_\epsilon R^{dull}$ B cells may be activated and terminally differentiate or undergo an isotype switch. Consistent with this scenario, the decline in $Fc_\epsilon R^{dull}$ B cells occurred concurrently with increases in serum IgM and IgG and increases in the *in vitro* secretion of IgM and IgG in splenic B cell cultures. However, it should be noted that the decline in $Fc_\epsilon R^{dull}$ B cells continued even after serum and *in vitro* spontaneously secreted Ig levels declined, suggesting terminal differentiation cannot be a complete explanation for the progressive loss of $Fc_\epsilon R^{dull}$ B cells. Alternatively, the $Fc_\epsilon R^{dull}$ B cell population may be undergoing some cytolytic event due to either apoptosis or the action of cytolytic T cells. In addition, the B-1 frequencies were not examined in the lymph nodes from mice with MAIDS, cGvHD and CIA because lymph nodes have been reported to lack $CD5^+$ B cells (Hayakawa et al., 1983). However, a recent study by Hitoshi et al. showed that there were $Ly\text{-}1^+$ B cells in the inguinal lymph nodes of mice with MAIDS and that these $Ly\text{-}1^+$ B cells were infected with the LP-BM5 MuLV (Hitoshi et al., 1993). Thus, the $Fc_\epsilon R^{dull}$ B cells could have been sequestered to an atypical anatomical location such as the lymph node.

The B cell subset responsible for secretion of pathogenic autoantibodies in systemic autoimmune disease is a controversial issue. In humans, $CD5^+$ B cells are a source of low-affinity polyreactive IgM autoantibodies in both healthy individuals and autoimmune patients (Burastero et al., 1988). However, Klinman and Steinberg concluded that the hypergammaglobulinemia seen in both NZB and MRL *lpr/lpr* spontaneously autoimmune mice is due to generalized polyclonal B cell activation as opposed to preferential stimulation of a minor autoreactive B cell population (Klinman and Steinberg, 1987). Further support for the view that conventional B cells may be involved in production of pathogenic autoantibodies has been provided by Reap and colleagues using radiation chimeras (Reap et al., 1992). These investigators showed that the conventional B cells derived from the bone marrow were responsible for all of the anti-chromatin and most rheumatoid factor (RF) antibody production in these mice. The $CD5^+$ B cells derived from the peritoneal cavity produced a minor proportion of the RF. The conclusion that conventional B cells are critically involved in systemic autoimmune processes is consistent with the increases in the conventional B cell population observed in all three of the induced models of autoimmunity examined.

In contrast to previous studies in genetically autoimmune mice and human systemic autoimmune disease, there was not a correlation between increases in $CD5^+$ B cell frequencies and disease progression in MAIDS, cGvHD and CIA. However, there was an increase in conventional splenic B cells in each of the three models studied. These findings are consistent with $CD5^+$ B cell studies which

show $CD5^+$ B cells stimulate the proliferation of splenic B cells. Thus, a role for $CD5^+$ B cells in the pathogenesis cannot be ruled out without a further understanding of the molecules involved in $CD5^+$ B-cell-mediated help. Once the molecules involved in mediating B cell help are defined it may be possible to go back to *in vivo* models and perform antibody blocking studies to determine whether blocking B:B interactions has an effect on autoimmune pathogenesis. Of the three models studied, the MAIDS model is of special interest because $CD5^+$ B cells have been shown to be infected with the disease inducing agent LP-BM5 MuLV (Hitoshi et al., 1993) and murine strains which do not have $CD5^+$ B cells are less susceptible to disease induction.

Models for $CD5^+$ B-cell-mediated help.

In summary of the findings presented in this dissertation, two models for $CD5^+$ B-cell-mediated help are diagrammed in Figure 23. In the first model for $CD5^+$ B-cell-mediated help, an unidentified molecule(s) expressed on the neoplastic $CD5^+$ B cell interacts with a receptor(s) expressed on splenic B cells. As a result of this ligand/receptor interaction there is a transient upregulation in the expression of receptors for IL-2, IL-5 and IL-6. Thus, the splenic B cells become responsive to lymphokines resulting in significant increases in proliferation in the presence of exogenous IL-2, and differentiation into IgM and IgG secreting cells in the presence of exogenous IL-2 plus IL-5. In addition to upregulating lymphokine receptor expression, the contact-mediated signal enhances interleukin 6 secretion from the splenic B cells. The IL-6 secreted by splenic B cells has an

Figure 23. Models for $CD5^+$ B-cell-mediated help.

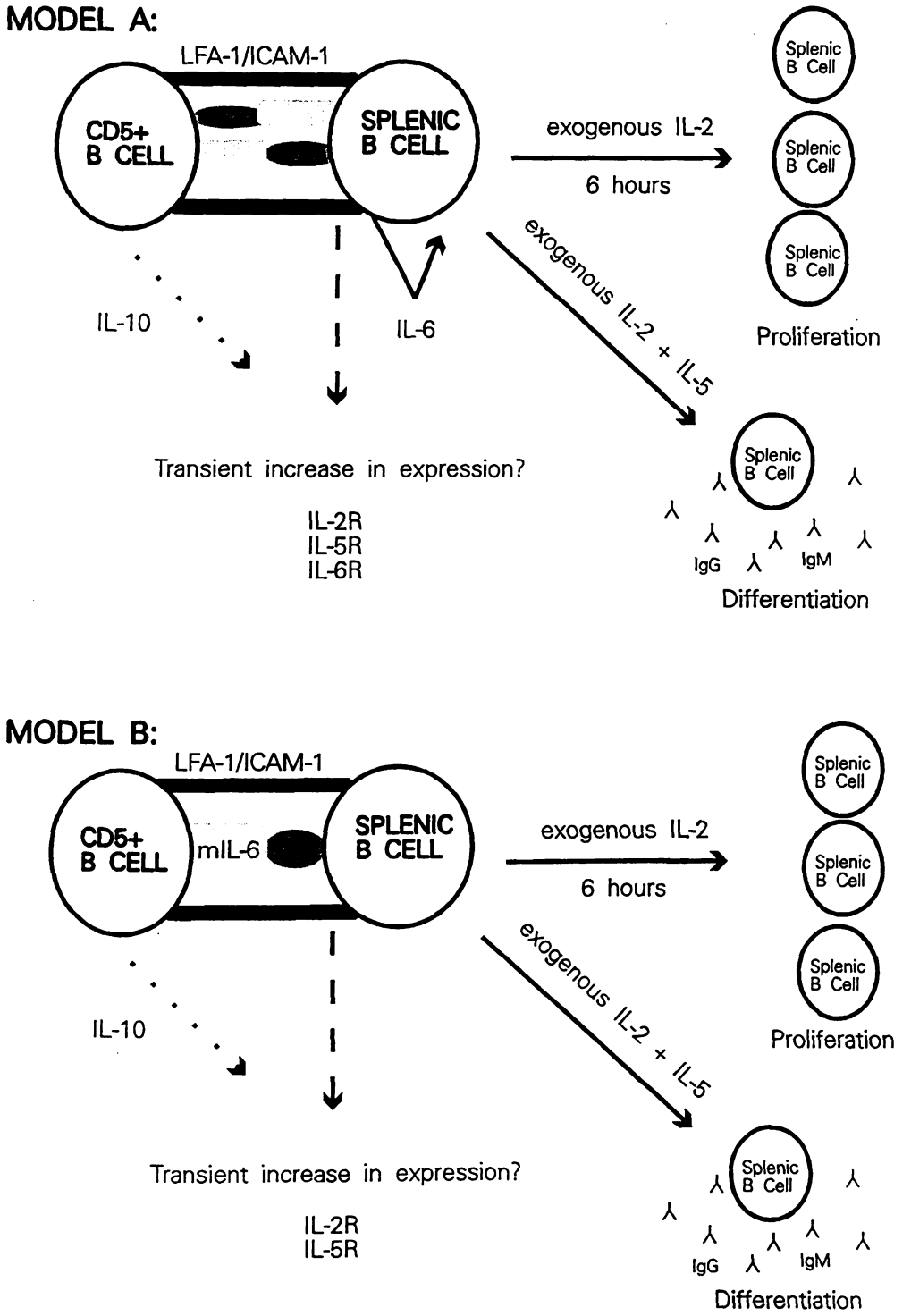
MODEL A:
LFA-1/ICAM-1
CD5+ B CELL
SPLENIC B CELL
exogenous IL-2
6 hours
Splenic B Cell
Splenic B Cell
Splenic B Cell
Proliferation
exogenous IL-2 + IL-5
IL-10
IL-6
Transient increase in expression?
IL-2R
IL-5R
IL-6R
Splenic B Cell
IgG
IgM
Differentiation
MODEL B:
LFA-1/ICAM-1
CD5+ B CELL
mIL-6
SPLENIC B CELL
exogenous IL-2
6 hours
Splenic B Cell
Splenic B Cell
Splenic B Cell
Proliferation
exogenous IL-2 + IL-5
IL-10
Transient increase in expression?
IL-2R
IL-5R
Splenic B Cell
IgG
IgM
Differentiation

Figure 23

autocrine effect and further enhances splenic B cell proliferation either via modulation of IL-2R expression or direct effects on intracellular signalling pathways. A previous study by Fluckiger et al., has shown that IL-10 induces high affinity IL-2 receptors on anti-CD40 activated human B cells rendering these cells responsive to IL-2 for both proliferation and differentiation (Fluckiger et al., 1993). In parallel to these findings, interleukin 10 which is secreted by the $CD5^+$ B cell further enhances B cell proliferative responses by upregulating IL-2R expression on the splenic B cells. The adhesion molecules LFA-1 and ICAM-1 tether the $CD5^+$ B cell to the splenic B cell, further stabilizing ligand/receptor interactions.

In the second model for $CD5^+$ B-cell-mediated help, membrane IL-6 which is present on $CD5^+$ B cells interacts with a receptor on splenic B cells. As a result of this interaction there is a transient upregulation in the expression of receptors for IL-2 and IL-5. Thus, in the presence of exogenous IL-2 the splenic B cells proliferate. In addition, in the presence of IL-2 and IL-5 the splenic B cells differentiate into IgM and IgG secreting cells. Interleukin 10 secreted by the $CD5^+$ B cells further upregulates the expression of IL-2R on the splenic B cells and thus, enhances B cell proliferation in the presence of exogenous IL-2. Consistent with model A, LFA-1 and ICAM-1 function as adhesion molecules and stabilize $CD5^+$ B:splenic B ligand/receptor interactions.

The first model for $CD5^+$ B-cell-mediated help predicts that a contact-mediated signal between $CD5^+$ B cells and splenic B cells results in a transient upregulation of lymphokine receptors. To further evaluate this prediction, splenic

B cells can be stained with mAb to the IL-2R, IL-5R and IL-6R, followed by FACS analysis. To further distinguish between the induction of low affinity and high affinity lymphokine receptors, interleukin binding assays can be performed. By varying the signals provided to the splenic B cells the contributions of the contact-mediated signal, IL-6 and IL-10 can further be evaluated. For example, stimulating the splenic B cells with paraformaldehyde-fixed BCL_1-3B3 cells plus anti-IL-6, would evaluate the effects of the contact-dependent signal. Utilizing paraformaldehyde-fixed BCL_1-3B3 cells without the addition of anti-IL-6 mAb would allow a comparison which addresses the contributions of IL-6. Furthermore, utilizing irradiated BCL_1-3B3 B cells plus anti-IL-6 would address the role of both the contact-mediated signal and IL-10. In addition to enhancement of interleukin receptor expression, this model predicts that the contact-mediated signal upregulates IL-6 secretion from the splenic B cells. This prediction can further be evaluated by stimulating splenic B cells with paraformaldehyde-fixed BCL_1-3B3 cells, followed by ELISA analysis of supernatants for the presence of IL-6. In addition, IL-2 can be added, to determine if the enhanced IL-6 secretion requires both a contact-mediated signal and IL-2. As the predicted role of IL-6 is as an autocrine factor these analyses should be performed in the presence of anti-IL-6 receptor antibody.

In order to address the physiological relevance of the $CD5^+$ B-cell-mediated help proposed by model A, it is critical to determine the molecules involved in mediating the contact-dependent signal. Previous studies utilizing antibodies reactive with surface proteins expressed on splenic B cells have shown that a

number of molecules can mediate activation signals including CD19, CD20, CD21 (CR2), CD22, CD23 ($Fc_{\epsilon}RII$), CD40 and CD72 (Clark and Lane, 1991). One approach which could be undertaken to address the involvement of these molecules in $CD5^+$ B-cell-mediated help is to use mAb reactive with the ligands for these molecules. However, this approach may be problematic because the blocking antibody may not be specific for the epitope on the ligand which interacts with the receptor on the splenic B cell. In addition, it is possible that the target molecule on the splenic B cell may have multiple ligands; thus, blocking only one ligand on the $CD5^+$ B cell may not inhibit the contact-dependent interactions. As an alternative approach, soluble chimeric protein constructs should be utilized to address this question. For example, currently there is a soluble CD40-Ig construct, the ability of this construct should be evaluated for its ability to block $CD5^+$ B-cell-mediated help. Furthermore, this type of an approach would allow for the evaluation of signals mediated by splenic B cell proteins such as CD19 and CD20, for which ligands have not yet been identified.

The second model for $CD5^+$ B-cell-mediated help predicts that membrane associated IL-6 present on the $CD5^+$ B cell is directly involved in mediating the contact-dependent signal. Preliminary staining analysis of BCL_1-3B3 cells has revealed the presence of surface IL-6. If, as model B predicts, this surface IL-6 is directly involved in contact-dependent signalling, then the formation of BCL_1-3B3:splenic B conjugates should be blocked by mAb reactive with IL-6. In addition, model B predicts that this membrane IL-6 mediated signal induces a

transient upregulation in the expression of receptors for IL-2 and IL-5. This prediction can further be assessed by staining the splenic B cells with antibodies directed to the IL-2R and the IL-5R, followed by FACS analysis. If staining analysis indicates there is an increase in receptor expression, the affinities of these receptors can further be evaluated by performing lymphokine binding assays. To evaluate the predicted role of IL-10 in IL-2R expression enhancement, paraformaldehyde-fixed BCL_1-3B3 cells plus IL-10 can be utilized to stimulate splenic B cells and the IL-2R expression can be monitored with staining analysis.

Although BCL_1-3B3 staining analysis has revealed the presence of surface IL-6, it is not clear whether this IL-6 is a membrane form of IL-6, or IL-6 which has been secreted by the BCL_1-3B3 cells and has either absorbed to the surface or has bound to an IL-6R on the BCL_1-3B3 cells. Previously, membrane IL-1 has been demonstrated on macrophages by the ability of plasma membranes to stimulate IL-1-dependent responses (Kurt-Jones et al., 1985). In parallel to these studies, plasma membranes from BCL_1-3B3 cells should be assessed for their ability to stimulate a contact-dependent signal equivalent to the signal provided by paraformaldehyde-fixed BCL_1-3B3 cells. Furthermore, in Kurt-Jones study, the IL-1 could not be eluted from the macrophage membranes by EDTA, high salt or low pH treatment of the membranes. Thus, the ability of BCL_1-3B3 membranes which have been similarly treated to provide contact-mediated help can be assessed.

One possible functional role for the $CD5^+$ B-cell-mediated help is the generation of memory B cells. Although the phenotype of memory B cells has

been an ongoing debate (Gray, 1993), the J11D^{lo} phenotype appears to be one of the most promising phenotypic markers for memory B cells. Thus, to further address the role of CD5$^+$ B cells in the generation of memory B cells, splenic B cells can be stimulated with paraformaldehyde-fixed BCL$_1$-3B3 cells and stained with antibodies to J11D, followed by FACS analysis. In addition, once the ligand/receptor pair(s) directly involved in mediating the contact-dependent signal are defined, ligand-receptor interactions can be blocked with antibody or soluble chimeric protein constructs to determine if there is a reduction in the generation of B cells which express the memory phenotype.

Successful identification of the ligand/receptors involved in mediating the contact-dependent signal will pave the way for studies to address the physiological relevance of CD5$^+$ B-cell-mediated help. If the ligand(s) on the CD5$^+$ B cell involved in the contact dependent interactions can be identified it may be possible to generate gene knockout mice which do not express this ligand. These mice can be utilized to address the physiological relevance of CD5$^+$ B-cell-mediated help. For example, if this help is important to the development of natural autoantibodies then one would expect these knockout mice would have little or no serum autoantibodies. In addition, if these autoantibodies play a normal role in the physiology of the immune system as Jerne's network theory proposes, then the knockout mice may exhibit altered primary and secondary humoral responses to foreign antigens. Furthermore, if the CD5$^+$ B-cell-mediated help plays a role in the generation or maintenance of memory B cells, then memory responses may be

inhibited. In addition to questions which address the normal physiology of the immune system, these mice could be utilized to determine if disruptions in $CD5^+$ B-cell-mediated help effects the induction of autoimmunity. Of particular interest for this evaluation is the murine AIDS model where CD5 B cells have been shown to be infected with the disease inducing agent.

The $CD5^+$ B cell line BCL_1-3B3 stimulates the proliferation and differentiation of splenic B cells in the presence of IL-2. The help mediated by $CD5^+$ B cells parallels T-cell-mediated help in that it involves contact-dependent signalling, enhances lymphokine responsiveness, and results in B cell proliferation and differentiation. However, B-cell-mediated help is unique from T-cell-mediated help because it requires IL-2, a T_H1 derived lymphokine as opposed to IL-4, a T_H2 derived lymphokine. The ability of $CD5^+$ B cells to enhance proliferation and differentiation of other B cells could play numerous roles in the immune system. The $CD5^+$ B cells are the first B cell subset to appear during ontogeny and have been suggested to play a role in repertoire development, possibly through anti-idiotype antibody secretion. The demonstration of $CD5^+$ B-cell-mediated help supporting the IL-2-mediated proliferation provides another mechanism by which these cells could support expansion of developing B cells. $CD5^+$ B cells may also play a role in autoimmune disease. Previous studies indicate $CD5^+$ B cells are present at increased frequencies in a number of systemic autoimmune diseases (Hardy, et al., 1987; Hara et al., 1988, Youinou et al., 1988; Ragheb and Lisak, 1990; Nicoletti et al., 1990). Through contact-dependent B-B interactions, the

$CD5^+$ B cells may be playing a regulatory role in these diseases, by enhancing Ig secretion of other B cells, in the presence of IL-2. Developing a better understanding of the molecules directly involved in $CD5^+$ B-cell-mediated signalling will provide a tool to further dissect the physiological and perhaps pathological consequences of $CD5^+$ B-cell-mediated help. Furthermore, future studies may provide some exciting new insight about the functional role of $CD5^+$ B cells.

LIST OF REFERENCES

7.0 LIST OF REFERENCES

Alberola-Ila, J., Places, L., Cantrell, D. A., Vives, J., and Lozano, F. (1992). Intracellular events involved in CD5-induced human T cell activation and proliferation. *J. Immunol.* **148**, 1287.

Ahmed, S. A., Dauphinée, M. J., Montoya, A. I., and Talal, N. (1989). Estrogen induces normal murine $CD5^+$ B cells to produce autoantibodies. *J. Immunol.* **142**, 2647.

Antin, J. H., Emerson, S. G., Martin, P., Gadol, N., and Ault, K. A. (1986). Leu-1^+ ($CD5^+$) B cells. A major lymphoid subpopulation in human fetal spleen: phenotypic and functional studies. *J. Immunol.* **136**, 505.

Armitage, R. J., and Goff, L. K. (1988). Functional interaction between B cell subpopulations defined by CD23 expression. *Eur. J. Immunol.* **18**, 1753.

Bartlett, W. C., McCann, J., Shepherd, D. M., Roy, M., and Noelle, R. J. (1990). Cognate interactions between helper T cells and B cells. IV. Requirements for the expression of effector phase activity by helper T cells. *J. Immunol.* **145**, 3956.

Becker, H., Weber, C., Storch, S. and Federlin, K. (1990). Relationship between $CD5^+$ B lymphocytes and the activity of systemic autoimmunity. *Clin. Immunol. Immunopath.* **56**, 219.

Bishop, G. A. (1991). Requirements of class II-mediated B cell differentiation for class II cross-linking and cyclic AMP. *J. Immunol.* **147**, 1107.

Bofill, M., Janossy, G., Janossa, M., Burford, G. D., Seymour, G. J., Wernet, P., and Kelemen, E. (1985). Human B cell development. II. Subpopulations in the human fetus. *J. Immunol.* **134**, 1531.

Bonagura, V. R., Pernis, B., Agostino, N., and Hatam, L. (1992). The major rheumatoid factor cross-reactive idiotype is dominantly expressed by *Staphylococcus aureus* Cowan Strain I-activated $CD5^+$ control B cells. *Cell. Immunol.* **140**, 381.

Brennan, F., Plater-Zyberk, C., Maini, R. N., and Feldman, M. (1989). Coordinate

expansion of "fetal type" lymphocytes (TCRγ T and $CD5^+$ B) in rheumatoid arthritis and primary Sjögren's syndrome. *Clin. Exp. Immunol.* **77**, 175.

Brooks, K. H., Uhr, J. W., and Vitetta, E. S. (1984). A B cell growth factor-like activity is secreted by cloned, neoplastic B cells. *J. Immunol.* **133**, 3133.

Burastero, S. E., Casali, P., Wilder, R. L., and Notkins, A. L. (1988). Monoreactive high affinity and polyreactive low affinity rheumatoid factors are produced by $CD5^+$ B cells from patients with rheumatoid arthritis. *J. Exp. Med.* **168**, 1979.

Caligaris-Cappio, F., Gobbi, M., Bofill, M., and Janossy, G. (1982). Infrequent normal B lymphocytes express features of B-chronic lymphocytic leukemia. *J. Exp. Med.* **155**, 623.

Cambier, J. C., Newell, M. K., Justement, L. B., McGuire, J. C., Leach, K. L., and Chen, Z. Z. (1987). Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. *Nature* **327**, 629.

Cambier, J. C., and Lehmann, K. R. (1989). Ia-mediated signal transduction leads to proliferation of primed B lymphocytes. *J. Exp. Med.* **170**, 877.

Cantor, H., and Boyse, E. A. (1975). Functional subclasses of T-lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **141**, 1376.

Carmack, C. E., Shinton, S. A., Hayakawa, K., and Hardy, R. R. (1990). Rearrangement and selection of V_H11 in the Ly-1 B cell lineage. *J. Exp. Med.* **172**, 371.

Casali, P., Burastero, S. E., Nakamura, M., Inghirami, G., and Notkins, A. L. (1987). Human lymphocytes making rheumatoid factor antibody to ssDNA belong to Leu-1^+ B-cell subset. *Science* **236**, 77.

Cerny, A., Hügin, A. W., Hardy, R. R., Hayakawa, K., Zinkernagel, R. M., Makino, M., and Morse, H. C. (1990). B cells are required for induction of T cell abnormalities in a murine retrovirus-induced immunodeficiency syndrome. *J. Exp. Med.* **171**, 315.

Ceuppens, J. L., and Baroja, M. L. (1986). Monoclonal antibodies to the CD5 antigen can provide the necessary second signal for activation of isolated resting T cells by solid-phase-bound OKT3. *J. Immunol.* **137**, 1816.

Clark, E. A., and Lane, P. J. (1991). Regulation of human B-cell activation and

adhesion. *Ann. Rev. Immunol.* **9**, 97.

Courtenay, J. S., Dallman, M. J., Dayan, A. D., Martin, A., and Mosedale, B. (1980). Immunization against heterologous type II collagen induces arthritis in mice. *Nature* **283**, 666.

Croft, M., and Swain S. (1991). B cell responses to fresh and effector T helper cells. *J. Immunol.* **146**, 4055.

Dauphinée, M., Tovar, Z., and Talal, N. (1988). B cells expressing CD5 are increased in Sjögren's syndrome. *Arthritis Rheumatol.* **31**, 642.

Davidson, W. F., Fredrickson, T. N., Rudikoff, E. K., Coffman, R. L., Hartley, J. W., and Morse, H. C. (1984). A unique series of lymphomas related to the Ly-1$^+$ lineage of B lymphocyte differentiation. *J. Immunol.* **133**, 744.

Davies, A. A., Ley, S. C., and Crumpton, J. J. (1992). CD5 is phosphorylated on tyrosine after stimulation of the T-cell antigen receptor complex. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6368.

Dexter, C. M., and Corley, R. B. (1987). Expression of the Fc gamma receptor on Ly-1$^+$ B lymphocytes. *Eur. J. Immunol.* **17**, 867.

Dighiero, G., Lymberi, P., Mazie, C., Rouyre, S., Butler-Browne, S., Whalen, R. G., and Avrameas, S. (1983). Murine hybridomas secreting natural monoclonal antibodies reacting with self antigen. *J. Immunol.* **131**, 2267.

Dighiero, G., Lymberi, P., Holmberg, D., Lundkvist, I., Coutinho, A., and Avrameas, S., (1985). High frequency of natural auto-antibodies in normal newborn mice. *J. Immunol.* **134**, 765.

Fluckiger, A.-C., Garrone, P., Durand, I., Galizzi, J.-P., and Banchereau, J. (1993). Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. *J. Exp. Med.* **178**, 1473.

Fox, R. I., Harlow, D., Royston, I., and Elder, J. (1982). Structural characterization of the human T cell surface antigen (p67) isolated from normal and neoplastic lymphocytes. *J. Immunol.* **129**, 401.

Foy, T. M., Shepherd, D. M., Durie, F. H., Aruffo, A., Ledbetter, J. A., and Noelle, R. J. (1993). In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by antibody to the ligand for CD40, gp39. *J. Exp. Med.* **178**, 1567.

Freedman, A. S., Boyd, A. W., Bieber, F. R., Daley, J., Rosen, K., Horowitz, J. C., Levy, D. N., and Nadler, L. M. (1987). *Blood* **70**, 418.

Freedman, A. S., Freeman, G., Whitman, J., Segil, J., Daley, J., and Nadler, L. M. (1989). Studies of *in vitro* activated $CD5^+$ B cells. *Blood* **73**, 202.

Gadol, N., and Ault, K. A. (1986). Phenotypic and functional characterization of human Leu1 (CD5) cells. *Immunol. Rev.* **93**, 23.

Gajewski, T. F., Schell, S. R., Nau, G., and Fitch, F. W. (1989). Regulation of T-cell activation: differences among T-cell subsets. *Immunol. Rev.* **111**, 79.

Gleichmann, E., VanElvan, E. H., and Van der Veen J. P. W. (1982). A systemic lupus erythematosus (SLE)-like disease in mice induced by abnormal T-B cell cooperation. Preferential formation of autoantibodies characteristic of SLE. *Eur. J. Immunol.* **12**, 152.

Gleichmann, E., Pals, S. T., Rolink, A. G., Radaszkiewicz, T., and Gleichmann, H. (1984). An integrated circuit of T-cell subsets, MHC structures, and immunological diseases. *Immunol. Today* **5**, 324.

Grammer, A., and Lipsky, P. (1993). Evidence for CD40 involvement in homotypic B cell signalling. *J. Immunol.* **150**, 255A.

Gray, D. (1993). Immunological memory. *Ann. Rev. Immunol.* **11**, 49.

Griffiths, M. M., Eichwald, E. J., Martin, J. H., Smith, C. B., and DeWitt, C. W. (1981). Immunogenetic control of experimental type II collagen-induced arthritis. I. Susceptibility and resistance among inbred strains of rats. *Arthritis Rheum.* **24**, 781.

Gu, H., Forster, I., and Rajewsky, K. (1990). Sequence homologies, N sequence insertion and J_H gene utilization in V_HDJ_H joining: implications for the joining mechanism and the ontogenetic timing of Ly-1 B and B-CLL progenitor generation. *EMBO J.* **9**, 2133.

Hara, M., Kitani, A., Hirose, T., Norioka, K., Harigai, M., Suzuki, K., Tabata, H., Kawagoe, M., and Nakamura, H. (1988). Stimulatory effect of CD5 antibody on B cells from patients with rheumatoid arthritis. *Clin. Immunol. Immunopathol.* **49**, 223.

Hardy, R. R., Hayakawa, K., Parks, D. R., and Herzenberg, L. A. (1983). Demonstration of B-cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence. *Nature* **306**, 270.

Hardy, R. R., Hayakawa, K., Herzenberg, L. A., Morse, H. C., and Davidson, W. F. (1984). Ly-1 as a differentiation antigen on normal and neoplastic B cells. *Curr. Top. Microbiol. Immunol.* **113**, 231.

Hardy, R. R., and Hayakawa, K. (1986). Development and physiology of Ly-1 B and its human homolog, Leu-1 B. *Immunol. Rev.* **93**, 53.

Hardy, R. R., Dangl, J. L., Hayakawa, K., Jager, G., Herzenberg, L. A., and Herzenberg, L. A. (1986). Frequent λ light chain gene rearrangement and expression in a Ly-1 B lymphoma with a productive κ chain allele. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1438.

Hardy, R. R., Hayakawa, K., Shimizu, M., Yamasaki, K., and Kishimoto, T. (1987). Rheumatoid factor secretion from human Leu-1$^+$ B cells. *Science* **236**, 81.

Hardy, R. R., and Hayakawa, K. (1992). Generation of Ly-1 B cells from developmentally distinct precursors. *Ann. N. Y. Acad. Sci.* **651**, 99.

Hayakawa, K., Hardy, R. R., Parks, D. R., and Herzenberg, L. A. (1983). The "Ly-1 B" cell subpopulation in normal, immunodefective, and autoimmune mice. *J. Exp. Med.* **157**, 202.

Hayakawa, K., Hardy, R. R., Honda, M., Herzenberg, L. A., Steinberg, A. D., and Herzenberg, L. A. (1984). Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2494.

Hayakawa, K., Hardy, R. R., Herzenberg, L. A., and Herzenberg, L. A. (1985). Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J. Exp. Med.* **161**, 1554.

Hayakawa, K., Hardy, R. R., and Herzenberg, L. A. (1986). Peritoneal Ly-1 B cells: genetic control, autoantibody production, increased lambda light chain expression. *Eur. J. Immunol.* **16**, 450.

Herzenberg, L. A., Stall, A. M., Lalor, P. A., Sidman, C., Moore, W. A., Parks, D. R., and Herzenberg, L. A. (1986). The Ly-1 B cell lineage. *Immunol. Rev.* **93**, 81.

Hitoshi, Y., Okada, Y., Sonoda, E., Tominaga, A., Makino, M., Suzuki, K., Kinoshita, J., Komuro, K., Mizuochi, T., and Takatsu, K. (1993). Delayed progression of a murine retrovirus-induced acquired immunodeficiency syndrome in X-linked immunodeficient mice. *J. Exp. Med.* **177**, 621.

Hodgkin, P. D., Yamashita, L. C., Coffman, R. L., and Kehry, M. R. (1990). Separation of events mediating B cell proliferation and Ig production by using T cell

membranes and lymphokines. *J. Immunol.* **145**, 2025.

Holmdahl, R., Andersson, M. E., Goldschmidt, T. J., Jansson, L., Karlsson, M., Malmström, V., and Mo, J. (1989). Collagen induced arthritis as an experimental model for rheumatoid arthritis. *AMPIS* **97**, 575.

Hügin, A. W., Vacchio, M. S., and Morse, H. C. (1991). A virus-encoded "superantigen" in a retrovirus-induced immunodeficiency syndrome of mice. *Science* **252**, 424.

Ishida, H., Hastings, R., Kearney, J., and Howard, M. (1992). Continuous anti-IL-10 antibody administration depletes mice of CD5 B cells but not conventional B cells. *J. Exp. Med.* **175**, 1213.

Ishida, H., Hastings, R., Thompson-Snipes, L., and Howard, M. (1993). Modified immunological status of anti-IL-10 treated mice. *Cell. Immunol.* **148**, 371.

Jerne, N. K. (1974). Towards a network theory of the immune system. *Ann. Immunol. (Inst. Pasteur).* **125**, 373.

Kamal, M., Katira, A., and Gordon, J. (1991). Stimulation of B lymphocytes via CD72 (human Lyb-2). *Eur. J. Immunol.* **21**, 1419.

Kantor, A. B. (1991). The development and repertoire of B-1 cells (CD5 B cells). *Immunol. Today.* **12**, 389.

Kantor, A. B., Stall, A. M., Adams, S., Herzenberg, L. A., and Herzenberg, L. A. (1992). Adoptive transfer of murine B-cell lineages. *Ann. N. Y. Acad. Sci.* **651**, 168.

Katira, A., Kamal, M., and Gordon, J. (1992). Occupancy of CD72 (the CD5 counterstructure) enhances interleukin-4-dependent CD23 expression in resting B lymphocytes. *Immunol.* **76**, 422.

Kazbay, K., and Osterland, C. K. (1990). The frequency of Leu1$^+$ B cells in autoantibody positive and negative autoimmune diseases in neonatal cord blood. *Clin. Exp. Rheumatol.* **8**, 231.

Kipps, T. J., and Vaughan, J. H., (1987). Genetic influence on the levels of circulating CD5 B lymphocytes. *J. Immunol.* **139**, 1060.

Kipps, T. J. (1989). The CD5 B cell. *Adv. Immunol.* **47**, 117.

Klinken, S. P., Fredrickson, T. N., Hartley, J. W., Yetter, R. A., and Morse, H. C.

(1988). Evolution of B cell lineage lymphomas in mice with a retrovirus-induced immunodeficiency syndrome, MAIDS. *J. Immunol.* **140**, 1123.

Klinman, D. M., and Steinberg, A. D. (1987). Systemic autoimmune disease arises from polyclonal B cell activation. *J. Exp. Med.* **165**, 1755.

Kurt-Jones, E. A., Beller, D. I., Mizel, S. B., and Unanue, E. R. (1985). Identification of a membrane-associated interleukin 1 in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1204.

Lane, P. J. L., McConnell, F. M., Schieven, G. L., Clark, E. A., and Ledbetter, J. A. (1990). The role of class II molecules in human B cell activation. Association with phosphatidyl inositol turnover, protein tyrosine phosphorylation and proliferation. *J. Immunol.* **144**, 3684.

Lalor, P. A., Herzenberg, L. A., Adams, S., and Stall, A. M. (1989). Feedback regulation of murine Ly-1 B cell development. *Eur. J. Immunol.* **19**, 507.

Lanier, L. L., Warner, N. L., Ledbetter, J. A., and Herzenberg, L. A. (1981). Expression of Lyt-1 antigen on certain murine B cell lymphomas. *J. Exp. Med.* **153**, 998.

Lanier, L. L., Arnold, L. W., Raybourne, R. B., Russel, S., Lynes, M. A., Warner, N. L., and Haughton, G. (1982). Transplantable B-cell lymphomas in B10.H-2^{a}H-4^{b} p/Wts mice. *Immunogenetics* **16**, 367.

Laterjet, R., and Duplan, J. F. (1962). Experiment and discussion on leukaemogenesis by cell-free extracts of radiation-induced leukaemia in mice. *Ind. J. Rad. Biol.* **5**, 339.

Laurindo, I., Finkelman, F. D., Mizuguchi, J., and Mond, J. J. (1987). B-cell activation stimulated by monoclonal anti-Lyb2.1 antibody is mediated through a receptor distinct from the BSF-1 receptor. *Cell. Immunol.* **108**, 473.

Ledbetter, J. A., Rouse, R. V., Micklem, H. S., and Herzenberg, L. A. (1980). T cell subsets defined by expression of Lyt-1, 2, 3 and Thy-1 antigens. *J. Exp. Med.* **152**, 280.

Ledbetter, J. A., June, C. H., Grosmaire, L. S., and Rabinovitch, P. S. (1987). Crosslinking of surface antigens causes mobilization of intracellular calcium in T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1384.

Lögdberg, L., and Shevach, E. M. (1985). Role of the Ly 1 antigen in interleukin 1-induced thymocyte activation. *Eur. J. Immunol.* **15**, 1007.

Luo, W., Van De Velde, H., Von Hoegen, I., Parnes, J. R., and Thielemans, K. (1992). Ly-1 (CD5), a membrane glycoprotein of mouse T lymphocytes and a subset of B cells, is a natural ligand of the B cell surface protein Lyb-2 (CD72). *J. Immunol.* **148**, 1630.

Manohar, V., Brown, E., Leiserson, M., and Chused, T. M. (1982). Expression of Lyt-1 by a subset of B lymphocytes. *J. Immunol.* **129**, 532.

Miller, R. A., and Gralow, J. (1984). The induction of Leu-1 antigen expression in human malignant and normal B cells by phorbol myristic acetate (PMA). *J. Immunol.* **133**, 3408.

Mix, E., Olsson, T., Correale, J., Baig, S., Kostulas, V., Olsson, O., and Link, H. (1990). B cells expressing CD5 are increased in cerebrospinal fluid of patients with multiple sclerosis. *Clin. Exp. Immunol.* **79**, 21.

Miyama-inaba, M., Kuma, S., Inaba, K., Ogata, H., Iwai, H., Yasumizu, R., Muramatsu, S., Steinman, R. M., and Ikehara, S. (1988). Unusual phenotype of B cells in the thymus of normal mice. *J. Exp. Med.* **168**, 811.

Mosier, D., Yetter, R. A., and Morse, H. C. (1985). Retroviral induction of acute lymphoproliferative disease and profound immunosuppression in adult C57BL/6 mice. *J. Exp. Med.* **161**, 766.

Mosier, D. E. (1986). Animal models for retrovirus-induced immunodeficiency disease. *Immunol. Invest.* **15**, 233.

Mosier, D. E., Yetter, R. A., and Morse, H. C. (1987). Functional T lymphocytes are required for a murine retrovirus-induced immunodeficiency disease (MAIDS). *J. Exp. Med.* **165**, 1737.

Nakayama, E., Von Hoegen, I., and Parnes, J. R. (1989). Sequence of the Lyb-2 B-cell differentiation antigen defines a gene superfamily of receptors with inverted membrane orientation. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1352.

Nicoletti, F., Maroni, P. L., Barcellini, W., Steensvag, P. O., Mauro, M., Lunetta, M., Pagano, M., Consoli, U., and Zanussi, C. (1990). Enhanced percentages of $CD5^+$ B lymphocytes in newly diagnosed IDDM patients. *Immunol. Lett.* **23**, 211.

Noelle, R. J., McCann, J., Marshall, L., and Bartlett, W. C. (1989). Cognate interactions between helper T cells and B cells. III. Contact-dependent, lymphokine-independent induction of B cell cycle entry by activated helper T cells. *J. Immunol.* **143**, 1807.

Noelle, R. J., Daum, J., Bartlett, W. C., McCann, J., and Shepherd, D. M. (1991). Cognate interactions between helper T and B cells. V. Reconstitution of T helper cell function using purified plasma membranes from activated Th1 and Th2 T helper cells and lymphokines. *J. Immunol.* **146**, 1118.

O'Garra, A., Stapleton, G., Dhar, V., Pearce, M., Schumacher, J., Rugo, H., Barbis, D., Stall, A., Cupp, J., Moore, K., Vieeira, P., Mosmann, T., Whitmore, A., Arnold, L., Haughton, G., and Howard, M. (1990). Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Int. Immunol.* **2**, 821.

O'Garra, A., Chang, R., Go, N., Hastings, R., Haughton, G., and Howard, M. (1992). Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur. J. Immunol.* **22**, 711.

Osman, N., Ley, S. C., and Crumpton, M. J. (1992). Evidence for an association between the T cell receptor/CD3 antigen complex and the CD5 antigen in human T lymphocytes. *Eur. J. Immunol.* **22**, 2995.

Owens, T. (1991). A role for adhesion molecules in contact-dependent T help for B cells. *Eur. J. Immunol.* **21**, 979.

Parker, D. C. (1993). T cell-dependent B cell activation. *Ann. Rev. Immunol.* **11**, 331.

Pattengale, P. K., Taylor, C. R., Twomey, P., Hill, S., Jonasson, J., Beardsley, T., and Haas, M. (1982). Immunopathology of B-cell lymphomas induced in C57BL/6 mice by dualtropic murine leukemia virus (MuLV). *Am. J. Pathol.* **107**, 362.

Pennell, C. A., Arnold, L. W., Haughton, G., and Clarke, S. H. (1988). Restricted Ig variable retion gene expression among Ly-1$^+$ B cell lymphomas. *J. Immunol.* **141**, 2788.

Pennell, C. A., Mercolino, T. J., Grdina, T. A., Arnold, L.W., Haughton, G., and Clarke, S. H. (1989). Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. *Eur. J. Immunol.* **19**, 1289.

Pennell, C. A., Maynard, E., Arnold, L. W., Haughton, G., and Clarke, S. H. (1990). High frequency expression of S107 V_H genes by peritoneal B cells of the B10.H-2^{a}H-4^{b}p/wts mice. *J. Immunol.* **145**, 1592.

Plater-Zyberk, C., and Maini, R. N. (1988). Phenotypic and functional features of CD5$^+$ B lymphocytes in rheumatoid arthritis. *Scand. J. Rheumatol.* **75**, 76.

Portanova, J. P., Claman, H. N., and Kotzin, B. L. (1985). Autoimmunization in murine graft-vs-host disease. I. Selective production of antibodies to histones and DNA. *J. Immunol.* **135**, 3850.

Portanova, J. P., and Kotzin, B. L. (1988). Lupus-like autoimmunity in murine graft-versus-host disease. *Concepts Immunopathol.* **6**, 119.

Ragheb, S., and Lisak, R. P. (1990). The frequency of $CD5^+$ B lymphocytes in the peripheral blood of patients with myasthenia gravis. *Neurology* **40**, 1120.

Reap, E. A., Sobel, E. S., Cohen, P. L., and Eisenberg, R. A. (1992). The role of $CD5^+$ B cells in the production of autoantibodies in murine systemic lupus erythematosus. *Ann. N. Y. Acad. Sci.* **651**, 588.

Robinson, W. H., Ying, H., Miceli, M. C., and Parnes, J. R. (1992). Extensive polymorphism in the extracellular domain of the mouse B cell differentiation antigen Lyb-2/CD72. *J. Immunol.* **149**, 880.

Robinson, W. H., Tutt-Landolfi, M. M., Schäfer, H., and Parnes, J. R. (1993). Biochemical identity of the mouse Ly-19.2 and Ly-32.2 alloantigens with the B cell differentiation antigen Lyb-2/CD72. *J. Immunol.* **151**, 4764.

Royston, I., Majda, J. A., Baird, S. M., Meserve, B. L., and Griffiths, J. C. (1980). Human T cell antigens defined by monoclonal antibodies: The 65,000-dalton antigen of T cells (T65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulin. *J. Immunol.* **125**, 725.

Rolink, A. G., and Gleichmann, E. (1983). Allosuppressor and allohelper T cells in acute and chronic graft-vs-host (GVH) disease. III. Different Lyt subsets or T cells induce different pathological lesions. *J. Exp. Med.* **158**, 546.

Saito, K., Tanaka, Y., Ota, T., Eto, S., and Yamashita, U. (1991). B-B cell interactions in the spontaneous activation of B cells in autoimmune NZB mice. *Microbiol. Immunol.* **35**, 741.

Sato, H., and Boyse, E. A. (1976). A new alloantigen expressed selectively on B cells: the Lyb-2 system. *Immunogenetics* **3**, 565.

Schwartz, R. C., Stanton, L. W., Riley, S. C., Marcu, K. B., and Witte, O. N. (1986). Synergism of v-*myc* and v-Ha-*ras* in the *in vitro* neoplastic progression of murine lymphoid cells. *Mol. Cell. Bio.* **6**, 3221.

Scribner, C. L., Hansen, C. T., Klinman, D. M., and Steinberg, A. D. (1987). The interaction of the *xid* and *me* genes. *J. Immunol.* **138**, 3611.

Shen, F., Spanondis, M., and Boyse, E. A. (1977). Multiple alleles of the Lyb-2 locus. *Immunogenetics* **4**, 481.

Sherr, D. H., Braun, J., and Dorf, M. E. (1987). Idiotype-specific Ly-1 B cell-mediated helper activity: hybridomas that produce anti-idiotype antibody and nonimmunoglobulin lymphokine(s). *J. Immunol.* **138**, 2057.

Shirai, A., Holmes, K., and Klinman, D. (1993). Detection and quantitation of cells secreting IL-6 under physiologic conditions in BALB/c mice. *J. Immunol.* **150**, 793.

Sidman, C. L., Marshall, J. D., Masiello, N. C., Roths, J. B., and Shultz, L. D. (1984). Novel B-cell maturation factor from spontaneously autoimmune viable motheaten mice. *Immunol.* **81**, 7199.

Sidman, C. L., Schultz, L. D., and Evans, R. (1985). A serum-derived molecule from autoimmune viable motheaten mice potentiates the action of a B cell maturation factor. *J. Immunol.* **135**, 870.

Sidman, C. L., Shultz, L. D., Hardy, R. R., Hayakawa, K., and Herzenberg, L. A. (1986). Production of immunoglobulin isotypes by Ly-1$^+$ B cells in viable motheaten and normal mice. *Science* **232**, 1423.

Slack, J. H., Hang, L., Amundson, C., Clevinger, B. L., and Dixon, F. J. (1989). Autoimmune mice and stimulated normal mice make λ1. with increased V region diversity. *J. Immunol.* **142**, 894.

Smith, H. R., and Olson, R. R. (1990). CD5$^+$ B lymphocytes in systemic lupus erythematosus and rheumatoid arthritis. *J. Rheumatol.* **17**, 833.

Snow, E. C., Mond, J. J., and Subbarao, B. (1986). Enhancement by monoclonal anti-Lyb-2 antibody of antigen-specific B lymphocyte expansion stimulated by TNP-Ficoll and T lymphocyte-derived factors. *J. Immunol.* **137**, 1793.

Solvason, N., Lehuen, A., and Kearney, J. F. (1991). An embryonic source of Ly1 but not conventional B cells. *Int. Immunol.* **3**, 543.

Solvason, N., Chen, X., Shu, F., and Kearney, J. F. (1992). The fetal omentum in mice and humans. A site enriched for precursors of CD5 B cells early in development. *Ann. N. Y. Acad. Sci.* **651**, 10.

Spertini, F., Stohl, W., Ramesh, N., Moody, C., and Geha, R. S. (1991). Induction of human T cell proliferation by a monoclonal antibody to CD5. *J. Immunol.* **146**, 47.

Stall, A. M., Adams, S., Herzenberg, L. A., and Kantor, A. B. (1992). Characteristics and development of the murine B-1b (Ly-1 B sister) cell population. *Ann. N. Y. Acad. Sci.* **651**, 33.

Stanton, T., Stevens, T. L., Ledbetter, J. A., and Wofsy, D. (1986). Anti-Ly-1 antibody induces interleukin 2 release from T cells. *J. Immunol.* **136**, 1734.

Sthoeger, Z. M., Wakai, M., Tse, D. B., Vinciguerra, V. P., Allen, S. L., Budman, D. R., Lichtman, S. M., Schulman, P., Weiselberg, L. R., and Chiorazzi, N. (1989). Production of autoantibodies by CD5-expressing B lymphocytes from patients with chronic lymphocytic leukemia. *J. Exp. Med.* **169**, 255.

Stuart, J. M., Townes, A. S., and Kang, A. H. (1982). Nature and specificity of the immune response to collagen in type II collagen-induced arthritis in mice. *J. Clin. Invest.* **69**, 673.

Subbarao, B., and Mosier, D. E. (1983). Induction of B lymphocyte proliferation by monoclonal anti-Lyb 2 antibody. *J. Immunol.* **130**, 2033.

Subbarao, B., Morris, J., and Baluyut, A. R. (1988). Properties of anti-Lyb-2-mediated B-cell activation and the relationship between Lyb-2 molecules and receptors for B-cell stimulatory factor-1 on murine B lymphocytes. *Cell. Immunol.* **112**, 329.

Suranyi, P., Szegedi, G., Damianovich, S., Juhasz, F., Stenszky, V., and Farid, N. R. (1989). B lymphocyte subsets in Hashimoto's thyroiditis. *Immunol. Lett.* **22**, 147.

Suzuki, N., and Sakane, T. (1989). Induction of excessive B cell proliferation and differentiation by an *in vitro* stimulus in culture in human systemic lupus erythematosus. *J. Clin. Invest.* **83**, 937.

Ternynck, T., and Avrameas, S. (1986). Murine natural monoclonal antibodies-a study of their polyspecificities and their affinities. *Immunol. Rev.* **94**, 99.

Tohma, S., Hirohata, S., and Lipsky, P. E. (1991). The role of CD11a/CD18-CD54 interactions in human T cell-dependent B cell activation. *J. Immunol.* **146**, 492.

Traganos, F., Darzynkiewicz, Z., and Sharpless, T. (1977). Simultaneous staining of ribonucleic and deoxyribonucleic acids in unfixed cells using acridine orange in a flow cytofluorometric system. *J. Histochem. Cytochem.* **25**, 46.

Tung, J., Michaelson, J., Sato, H., Vitetta, E. S., and Boyse, E. A. (1977). Properties of the Lyb-2 molecule. *Immunogenetics* **5**, 485.

Uher, F., and Dickler, H. B. (1988). Interactions between B lymphocyte subpopulations. I. Augmentation of the responses of resting B lymphocytes by activated B lymphocytes. *J. Immunol.* **140**, 1442.

Van De Velde, H., Von Hoegen, I., Luo, W., Parnes, J. R., and Thielemans, K. (1991). The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. *Nature.* **351**, 662.

Van Elven, E. H., Van der Veen, F. M., Rolink, A. G., Issa, P., Duin, T., and Gleichmann, E. (1981). Diseases caused by reactions of T cells to incompatible structures of the major histocompatibility complex. V. High titers of IgG autoantibodies to double stranded DNA. *J. Immunol.* **127**, 2435.

Van Rappard, F. M., Van der Veen, F. M., Rolink, A. G., and Gleichmann, E. (1982). Diseases caused by structures of the major histocompatibility complex. VI. Autoantibodies characteristic of systemic lupus erythematosus induced by abnormal T-B cell cooperation across I-E. *J. Exp. Med.* **155**, 1555.

Van Rappard, F. M., Van der Veen, F. M., Radaszkiewicz, T. L., and Gleichmann, E. (1983). Attempts at standardization of lupus-like graft-vs-host disease: inadvertent repopulation by DBA/2 spleen cells of H-2 different nonirradiated F_1 mice. *J. Immunol.* **130**, 2693.

Van Rappard, F. M., Van der Veen, F. M., Kiesel, U., Poels, L., Schuler, W, Melief, C. J. M., Landegent, J., and Gleichmann, E. (1984). Further evidence against random polyclonal antibody formation in mice with lupus-like graft-vs-host disease. *J. Immunol.* **132**, 1814.

Vandenberghe, P., and Ceuppens, J. L. (1991). Immobilized anti-CD5 together with prolonged activation of protein kinase C induce interleukin 2-dependent T cell growth: evidence for signal transduction through CD5. *Eur. J. Immunol.* **21**, 251.

Verwilghen, J., Vandesande, R., Vandenberghe, P., and Ceuppens, J. L. (1990). Crosslinking of the CD5 antigen on human T cells induces functional IL2 receptors. *Cell. Immunol.* **131**, 109.

Von Hoegen, I., Nakayama, E., and Parnes, J. R. (1990). Identification of a human protein homologous to the mouse Lyb-2 B cell differentiation antigen and sequence of the corresponding cDNA. *J. Immunol.* **144**, 4870.

Waldschmidt, T. J., Kroese, F. G. M., Tygrett, L. T., Conrad, D. H., and Lynch, R. G. (1991). The expression of B cell surface receptors. III. The murine low-affinity IgE Fc receptor is not expressed on Ly-1 or "Ly-1 like" B cells. *Int. Immunol.* **3**, 305.

Wang, C. Y., Good, R. A., Ammirati, P., Dymbort, G., and Evans, R. L. (1980). Identification of a p69,71 complex expressed on human T cells sharing determinants with B-type chronic lymphatic leukemic cells. *J. Exp. Med.* **151**, 1539.

Whitlock, C. A., and Witte, O. N. (1982). Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3608.

Whitlock, C. A., Ziegler, S. F., Treiman, L. J., Stafford, J. I., and O. N. Witte. (1983). Differentiation of cloned populations of immature B cells after transformation with Abelson murine leukemia virus. *Cell* **32**, 903.

Wooley, P. H., Luthra, H. S., Stuart, J. M., and David, C. S. (1981). Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates. *J. Exp. Med.* **154**, 688.

Yakura, H., Shen, F.-W., Boyse, E. A., and Tang, L. (1980). The Lyb-2 phenotype of hemolytic PFC. *Immunogenetics* **10**, 603.

Yakura, H., Kawabata, I., Ashida, T., Shen, F., and Katagiri, M. (1986). A role for Lyb-2 in B cell activation mediated by a B cell stimulatory factor. *J. Immunol.* **137**, 1475.

Yetter, R. A., Buller, R. M., Lee, J. S., Elkins, K. L., Mosier, D. E., Fredrickson, T. N., and Morse, H. C. (1988). $CD4^+$ T cells are required for development of murine retrovirus-induced immunodeficiency syndrome (MAIDS). *J. Exp. Med.* **168**, 623.

Ying-zi, C., Rabin, E., and Wortis, H. H. (1991). Treatment of murine $CD5^-$ B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. *Int. Immunol.* **3**, 467.

Youinou, P., Mackenzie, L., Jouquan, J., LeGoff, P., and Lydyard, P. M. (1987). CD5 positive B cells in patients with rheumatoid arthritis: phorbol ester mediated enhancement of detection. *Ann. Rheum. Dis.* **46**, 17.

Youinou, P., Mackenzie, L., Broker, B. M., Isenberg, D. I., Drougou-Lelong, A., Gentric, A., and Lydyard, P. M. (1988). The importance of CD5-positive B cells in non-organ-specific autoimmune diseases. *Scand. J. Rheumatol. suppl.* **76**, 243.