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THE NEGATIVE EFFECT OF CORTICOSTERONE ON MURINE BONE MARROW B LYMPHOCYTES: MODULATION BY IL-7 AND STROMAL CELLS

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ABSTRACT

THE NEGATIVE EFFECT OF CORTICOSTERONE ON MURINE BONE MARROW B LYMPHOCYTES: MODULATION BY IL-7 AND STROMAL CELLS

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B cells are critical to many facets of host defense making their development and production in the marrow of vital importance. Glucocorticoids (Gc), especially those produced endogenously in trauma patients, burn victims, the malnourished, etc., where the neuroendocrine stress axis is activated, can greatly impair lymphopoiesis. This reduces the numbers of circulating B cells and compromises immune defense. Considering the importance of B cell development, surprisingly little was known about the mechanisms involved in the downregulation of lymphopoiesis by Gc. The experiments performed in this thesis were designed to address the following questions: 1) How do increased concentrations of the natural glucocorticoid, corticosterone (Cs), effect the various stages of development of cells of the B lineage in murine bone marrow? 2) Can stromal cells that support hematopoiesis modulate the *in vitro* response of precursor B cells to corticosterone? 3) Can the cytokines interleukin-7 and/or stem cell factor, which are essential to B cell development, modulate the adverse effects of corticosterone on precursor B cells *in vitro*? Using a Cs implantation system, circulating Cs was elevated to concentrations analogous to that seen during normal physiological stress in the mouse model system. Flow cytometric identification of the subpopulations of developing B lymphocytes indicate that Cs had adverse effects within 12 hours especially on pre B cells. By 36 hours nearly all (70-90%) of early-pro, late-pro, pre and immature

cells were lost from the bone marrow. Only mature B cells and the earliest defined progenitor, the pre-pro B cell, showed resistance to Cs. Phenotypic and DNA analysis via flow cytometry showed that Cs induced apoptosis in pro, pre and IgM^+ B cells from murine bone marrow and decreased cell cycling in pro and pre B cells *in vitro*. Surprisingly, interleukin-7 which promotes lymphopoiesis, but not stem cell factor, completely inhibited Cs-induced apoptosis and cell cycle arrest in the earliest B lymphocytes, the pro B cells. IL-7 also had a modest protective effect on pre B cells, reducing apoptosis by 30%, but it did not protect them from the Cs-induced decrease in cell cycling. It provided only limited protection to immature IgM^+ bearing cells. Stromal cells also significantly reduced Cs-induced apoptosis (30-50%) for all stages of B lymphocyte development *in vitro* although they did not restore cell cycling to normal levels. Clearly these experiments have shown that Cs can have a rapid and adverse effect on the development of cells of the B lineage in mouse BM *in vivo* and *in vitro*. Nevertheless, substantial protection afforded the precursor B cells by interleukin-7 and stromal cells *in vitro* suggest that with further investigations this cytokine and other factors produced by stromal cells could potentially promote immune recovery from stress and Cs induced damage.

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LIST OF ABBREVIATIONS

BM	bone marrow
Cs	corticosterone
Gc	glucocorticoid
Dex	dexamethasone
IL	interleukin
SCF	stem cell factor
Ig	immunoglobulin
BCR	B cell receptor
TCR	T cell receptor
HSA	heat stable antigen
SCID	severe combined immunodeficiency
RAG	recombination activation gene
tdt	terminal deoxynucleotidal transferase
BSAP-5	B-cell-specific activator protein-5
NFκB	nuclear factor kappa B
IκB	inhibitor of nuclear factor kappa B
LTBMC	long term bone marrow culture
VCAM	vascular cellular adhesion molecule
ECM	extracellular matrix
SDF-1	stromal derived factor-1
TSLP	thymic stromal lymphopoietin
CSF	colony stimulated factor
VLA	very late antigen
PI3K	phosphatidylinositol 3-kinase
γc	common gamma chain
ACTH	adrenal corticotrophin hormone
CRH	corticotrophin releasing hormone
HPA	hypothalamic-pituitary-adrenal
LDL	low density lipoprotein
HDL	high density lipoprotein
CBG	corticosterone binding globulin
GcR	glucocorticoid receptor
SHR	steroid hormone receptor
hsp	heat shock protein
GRE	glucocorticoid response element
SCR	steroid receptor coactivator
GRIP	glucocorticoid receptor interacting protein
HAT	histone acetyltransferase
AIDS	aquired immunodeficiency syndrome
PS	phosphatidylserine
TNFR	tumor necrosis factor receptor
ICE	interleukin 1-β converting enzyme

APAF	apoptotic protease activating factor
DD	death domain
STAT	signal transducers and activators of transcription
Jak	janus kinase
Smase	sphingomyelinase
PI-PLC	phosphatidylinositol phospholipase C

INTRODUCTION

The successful generation of mature B cells (B cell lymphopoiesis) is critical to maintenance of the immune system. Aberrations in lymphopoiesis can result in a variety of diseases and/or failure to adequately respond to pathogens. Research in this area of immunology has been limited due to the complexity of the development process, the difficulty in identification of developing B lymphocytes and the very heterogeneous nature of the bone marrow where B cells develop in adult mammals. Therefore much of the research on B lymphocytes has been performed using immortalized cell lines, rather than natural cells. However, over the past decade methodology has been developed to successfully identify B lymphocytes from the other cell types that exist in the bone marrow (BM) (Coffman and Weissman, 1981). In addition, distinct stages in B lymphocyte development have been defined based on the differential expression of surface proteins (Hardy et al., 1991). These stages have also been defined functionally based on Ig gene rearrangement. This thesis utilizes this sophisticated methodology to study, in depth, the effect of glucocorticoid (Gc) elevation on different substages of cells of the B lineage.

Chronic elevation of Gc, due to either physiological stress or pharmacological administration, can result in immunosuppression. *In vivo* research from our lab has shown that Gc elevation reduces the development of B cells in mice (Garvy et al., 1993). It is likely that induction of apoptosis in these developing cells is at least one mechanism whereby Gc elevation adversely affects lymphopoiesis. However *in vivo* studies of apoptosis are severely limited due to the rapid phagocytosis of cells early in the apoptotic

process. Evidence that Gc can induce significant amounts of apoptosis in these populations have come from *in vitro* studies; the synthetic Gc, dexamethasone (dex), has been used for decades as a classic inducer of apoptosis in thymocytes (developing T lymphocytes). Although fewer studies have been performed using normal developing B lymphocytes, some research has shown that glucocorticoids can also induce apoptosis in these cells, mostly IgM^- B cells, *in vitro* (Garvy et al., 1993; Griffiths et al., 1994; Merino et al., 1994). Very few *in vitro* studies of the effect of Gc on B or T lymphocytes have been performed using the natural Gc (corticosterone [Cs] in rodents). Natural and synthetic Gc have been reported to have different affinities for the Gc receptor and different biological efficacies. To focus on physiological rather than pharmacological effects of Gc, the effect of the natural steroid hormone, Cs, rather than synthetic Gc has been investigated here. *In vivo* studies were performed herein to determine the effect of increased concentrations of circulating Cs on each stage in B lymphocyte development to determine if losses induced by Cs correspond with populations thought to be more susceptible to apoptosis. *In vitro* studies were also performed to thoroughly determine the apoptotic effect of Cs on developing B lymphocytes in culture.

Additionally, this thesis investigates potential modulation of Cs-induced apoptosis in developing B lymphocytes *in vitro* by cells and factors that would normally be found in the BM microenvironment. B cell lymphopoiesis depends on contact with, and factors secreted from, large fibroblast-like cells found in the bone marrow called stromal cells. Stromal cell lines have been utilized here to determine if, in addition to promoting B cell lymphopoiesis, these cells could modulate the B lymphocyte response to Cs. A cytokine derived from stromal cells, interleukin-7 (IL-7), has been shown to support B cell

lymphopoiesis, when added to B lymphocyte cultures not containing stromal cells (Namen et al., 1988). This cytokine has been shown to be specific for early stages of B and T cell lymphopoiesis and clinical interest in this cytokine for immunotherapy has recently developed (Maeurer et al., 2000; Westermann et al., 1998). Therefore we have investigated the direct effect of IL-7 addition on B lymphocyte response to Cs *in vitro*. The cytokine stem cell factor (SCF) promotes the commitment of early blood cell progenitors to the B lineage in combination with IL-7 (McNiece et al., 1991). SCF also amplifies the lymphopoietic effect of IL-7 on early stages during B cell development, so the affect of this cytokine on B lymphocyte induced apoptosis, alone and in combination with IL-7 has also been investigated.

In Chapter 1 of this document the literature on B cell lymphopoiesis, glucocorticoids and apoptosis will be reviewed. This will be followed by three data chapters covering 1) the *in vivo* effect of Cs elevation on developing B lymphocytes 2) stromal cell mediated modulation of the affect of Gc on B lymphocytes *in vitro* and 3) the *in vitro* effect of IL-7 and/or SCF on Gc-induced apoptosis and cell cycle arrest in bone marrow B lymphocytes.

CHAPTER 1: LITERATURE REVIEW

B CELL LYMPHOPOIESIS

Background

B cell lymphopoiesis occurs in the bone marrow of adult mammals and is a highly complex, multi-stage process that results in the generation of mature B cells that are involved in host defense. Membrane bound immunoglobulins (Ig) are the molecules responsible for B cell recognition of antigen and are composed of two heavy chains and two light chains. The generation of the immense diversity required for antigen recognition is via the recombination of the heavy and light chain genes (Rast and Litman, 1998). Briefly, recombination is achieved by rearrangement of several variable gene segments to the constant domain. This occurs for both the heavy and light chains. In addition to diversity derived from recombination, there are five heavy chain isotypes that result in various effector functions. The genes are mu, delta, gamma, alpha and epsilon which result in five isoforms, IgM, IgD, IgG, IgA and IgE, respectively. On the cell surface Ig molecules are associated with Igα and Igβ coreceptors that are part of the B cell receptor (BCR) complex (Hombach et al., 1990; Kashiwamura et al., 1990; Wienands, 2000). Ligation of Ig causes an intracellular phosphorylation cascade through the cytoplasmic domains of Igα and Igβ that results in increased proliferation and differentiation to plasma cells that produce large quantities of soluble Ig (antibodies) (Justement, 2000; Matsuo et al., 1993; Nomura et al., 1991). Antibodies mediate neutralization of toxins, initiate the complement cascade and elicit T cell responses in defense of foreign pathogens.

The dysregulation of B cell lymphopoiesis can result in several diseases. Multiple disorders such as systemic lupus erythematosis, asthma and rheumatoid arthritis can result from B cells with faulty or "anti-self" Igs produced via dysregulated lymphopoiesis. This thesis is concerned with the down-regulation of lymphopoiesis, which can occur during physiological stress and can result in compromised immune responses. In fact, this lab has extensively studied on the affects of zinc deficiency (a common example of physiological stress) on lymphopoiesis (DePasquale-Jardieu and Fraker, 1984; Fraker et al., 1977; King et al., 1995; Osati-Ashtiani et al., 1998). Zinc deficiency results in massive losses of precursor ($B220^{+}IgM^{-}$) B lymphocytes in the bone marrow, suggesting that downregulation of B lymphopoiesis may be the major mechanism whereby peripheral B lymphocytes are decreased, leading to a compromised immune system. During zinc deficiency and other conditions of chronic stress, a steroid hormone, glucocorticoid (Gc), is believed to induce many of the physiological responses. In fact this lab has also shown that chronic elevation of Gc decreased BM B cell in mice (Garvy et al., 1993). The mechanism by which lymphopoiesis is downregulated by glucocorticoids is not well defined and this is the phenomenon that this thesis will focus on.

Model Systems

There are currently few treatments available for the devastating, sometimes fatal, health scenarios presented above and there are often no reliable cures. Lack of progress in this field can, at least in part, be attributed to a lack of understanding of the mechanisms whereby B lymphocytes develop into functional mature cells and how

homeostasis is maintained. A multi-stage, complex maturation process in the highly heterogeneous bone marrow (BM) compartment can be concluded to be one main reason for our lack of advancement. The majority of our knowledge about B cell lymphopoiesis has been attained from cell lines, short-term whole bone marrow cultures or long-term bone marrow cultures. The murine (mouse) model has proven to be very useful in studying lymphopoiesis, and while not identical to the human system has shown fundamental similarities (Akashi et al., 2000; LeBien, 1998). The use of immortalized cell lines to study B lymphocytes has proven to be useful for determining important intracellular processes such as transcription and signaling in specific stages of B cell development. However, a limitation of cell lines is that they are not always a reliable model for determining cellular functions such as differentiation, cell cycle and cell death responses, since these are the systems often modified from normal by immortalization. Short-term bone marrow cultures have been valuable for studying some responses of primary developing B cells, although within a few days the majority of B cells will die due to the absence of microenvironmental support factors. The advent of a long term culture system developed by Whitlock and Witte (Whitlock et al., 1984), which will be revisited later, has resulted in a reliable *in vitro* system to specifically grow and regenerate B lymphocytes for long periods of time. This review addresses the murine system and the experiments presented in this thesis were performed on primary mouse cells or stromal cell lines unless otherwise specified.

Hematopoiesis

Figure 1.1 is a diagram of the generation of the various lineages of blood cells from a hematopoietic stem cell. In the BM of adult mammals all lineages of blood cells are generated and, for the most part, go through multiple stages of development there. T cells are a notable exception; they are generated in the BM and then migrate to the thymus for maturation. Overall, there are at least eight different blood cell lineages present in various stages of maturation in the BM. Developing and mature B lymphocytes compose approximately 30% of the total cells of the marrow. The B cells are a branch of the lymphoid lineage along with T and natural killer (NK) cells. In fact many parallels are seen between the development of B and T cells, including differentiation, proliferation and selection based on the generation of a functional BCR or T cell receptor (TCR), respectively. The study of T cell lymphopoiesis has been aided by the nearly homogeneous tissue that the majority of their development takes place in, the thymus. On the other hand, to study primary B lymphocytes there was a vital need for methodology to identify specific subsets of cells within the very heterogeneous population of cells found in the marrow.

Flow Cytometry: Phenotypic Markers for Monitoring B Cell Lymphopoiesis

Flow cytometry has proven to be of great use in analysis of heterogeneous tissues, such as the BM. Fluorochrome conjugated antibodies to surface proteins can be used to identify various cell types. Multi-parameter flow cytometry can allow for the analysis of several fluorochromes simultaneously, thus the expression of several cellular proteins can be analyzed simultaneously. This "multi-color" labeling has been useful in not only

identifying the various developmental stages of B lymphocytes in BM, but also in identifying distinct developmental subsets within the population. For many years mature B lymphocytes were identified and purified based on surface Ig expression. Precursor cells, committed to the B lineage but lacking surface Ig, could not be identified from other cell types found in the marrow. By immunizing rats with murine B cells and neoplastic pre B cell lines, a monoclonal antibody was produced which could identify Ig expressing B cells and precursor B cells (Coffman and Weissman, 1981). The antibody recognized a 220,000 MW glycoprotein (B220, CD45RA); it was determined that this antibody was specific for the B lineage. B220 is a phosphotyrosine phosphatase that dephosphorylates and inactivates src tyrosine kinases such as lyn (Katagiri et al., 1999; Satterthwaite and Witte, 1996). The precursor cells have been further differentiated since then based on stage-specific expression of various proteins.

Several labs have developed murine B lymphocyte identification schemes independently and the incongruent nomenclature and differing phenotypic markers used in identification has added confusion to the field, although each supports the same order of development based on the progression of Ig recombination (Lu et al., 1998; Osmond et al., 1998). This lab has adopted the established phenotypic nomenclature developed by Hardy et al (Hardy et al., 1991). Figure 1.2 is a detailed representation of the ordered development of B lymphocytes showing surface proteins, relative size and expression of significant proteins in each defined stage in development. It incorporates information that will be presented throughout this review on B lymphopoiesis. Pro B cells can be identified by the surface expression of the molecule recognized by the S7 monoclonal antibody (leukosialin, CD43) (Hardy et al., 1989). S7 is the clone designation and it is

also used as the nomenclature for the antigen it recognizes. Although it is expressed on many cell types in the marrow, it is specific for the pro B cell stage of development in $B220^+$ B lymphocytes of the bone marrow. S7 is a highly sialylated integral membrane protein which has been shown to play a role in adhesion (Laferte and Dennis, 1988, Walker, 1999 #195). The pro B cells can be subdivided further based on expression of heat stable antigen (HSA) (CD24) and a membrane bound aminopeptidase, BP-1 (Ly-51, 6C3) (Wu et al., 1991). HSA (CD24) is a highly glycosylated protein which is involved in adhesion and in the regulation of lymphopoiesis. In transgenic mice that overexpress HSA and in HSA knockout mice, the generation of early B lymphocyte precursors is downregulated (Hough et al., 1996; Nielsen et al., 1997). Although BP-1 expression is tightly regulated in B cell development a functional role has not been assigned, and lymphopoiesis is normal in BP-1 deficient mice (Lin et al., 1998; Wang et al., 1998). The earliest pro B cells, pre-pro, do not express either HSA or BP-1 ($B220^+S7^+HSA^-BP\text{-}1^-$). The expression of HSA on the surface identifies the next stage of development, the early-pro B cells ($B220^+S7^+HSA^+BP\text{-}1^-$). Late-pro B cells are identified by the expression of BP-1 ($B220^+S7^+HSA^+BP\text{-}1^+$) as development proceeds toward the pre B cell stage the expression of S7 is lost ($B220^+S7^-HSA^+BP\text{-}1^+$). Immature B cells have IgM on their surface, and maturity of this lineage is marked by the coexpression of surface IgM and IgD. The orderly progression through these stages to maturity is based on the rearrangement of Ig heavy and light chain genes and the expression of various proteins that play a role in the rearrangements, differentiation, proliferation and apoptosis. This will be discussed more in-depth below and is addressed throughout the experimental sections.

Immunoglobulin Gene Rearrangement

In general pro B cells are defined as cells committed to the B lineage prior to expression of cytoplasmic μ heavy chain. The identification of a variety of other distinctive features of these cells has led to a better functional understanding of their development and maturation. Using reverse transcriptase polymerase chain reaction (RT-PCR) amplification techniques, the ordered progression of heavy chain gene rearrangements was elucidated (Hardy et al., 1991). The earliest pro B cells (pre-pro as defined by Hardy) have Ig genes in a germline configuration and the cells are small and noncycling. Although evidence exists which suggests that this population may not be entirely composed of cells committed to the B lineage, a substantial fraction of these cells retain the ability to differentiate into mature B cells. The next defined stage, the early-pro B cells, are a large, proliferating cell type undergoing recombination of the μ heavy chain diversity regions (D_H) to joining (J_H) regions. In the late pro B cells, DJ_H recombination is completed and joined to one of several variable gene segments (V_H). These cells are also large and proliferating. Upon completion of μ heavy chain rearrangement, and subsequent cytoplasmic expression, the cells are considered pre B cells. There exists a stage where the pre B cells are large and actively cycling followed by a stage of non-proliferation and smaller size (Coffman, 1982, Osmond, 1986 #132) (see Figure 2). At this stage the pre B cells undergo recombination of the joining region to either a kappa or lambda light chain variable region (Vκ or Vλ, respectively). Completed IgM rearrangement results in export to the cell surface, defining the cells as

immature B lymphocytes. Maturity is accomplished following production and surface expression of IgD.

B Lymphocyte Homeostasis and Selection

The complexity of this recombination process suggests a high potential for faulty rearrangements, yet its importance suggests a need for quality control. In fact studies on the population dynamics of B lymphopoiesis indicated that the production of BM B lymphocytes far exceeds the number of mature cells generated (Osmond, 1990; Osmond, 1986). Using tritiated thymidine incorporation to study *in vivo* proliferation it was discovered that certain precursor B cells actively cycle. These were identified as being large cells in the late pro B cell stage and in the pre B cell stages of development. Further experiments using vincristine to block cells in metaphase were performed to determine the rate of proliferation of the various B cell subsets (Opstelten and Osmond, 1983). Together, these experiments led to the estimation that approximately fifty million B cells are generated per day in the bone marrow, but only around ten percent of those are exported from the marrow. The major losses appear to occur in transition from the pro B to pre B and from pre B to immature B cells (Lu and Osmond, 1997). This suggested that, indeed, many B lymphocytes were somehow being eliminated and this was consistent with the probability of the generation of faulty and anti-self Ig gene rearrangements. Therefore, it is widely accepted that B lymphocytes with nonsense and anti-self Ig gene rearrangements are eliminated before reaching maturity.

The mechanism by which cells identify faulty or functional heavy chains began to be elucidated by the identification of the expression of a surrogate light chain in early

stages of B cell development. The surrogate light chain is composed of two non-variable molecules, termed λ5 and VpreB (Kudo and Melchers, 1987; Sakaguchi and Melchers, 1986). Surrogate light chain associates with rearranged μ heavy chains via a disulfide bond and allows for its transient surface expression (Karasuyama et al., 1990). These associated molecules along with Igα and Igβ are termed the pre-B cell receptor (pre-BCR). The pre-BCR appears to be used as a checkpoint molecule for either further differentiation or elimination of the cell (Karasuyama et al., 1994; Rolink et al., 2000). Transgenic mice with a defective pre-BCR, due to a disrupted λ5 gene, show a block in the progression of pro B cells into pre B cells (Kitamura et al., 1992; Rolink et al., 1993). In addition, the SCID defect in mice results in the inability to rearrange Ig genes and therefore B lymphopoiesis is halted early in the pro B cell stage (Bosma et al., 1999; Bosma et al., 1983; Schuler et al., 1986). Insertion of a completed μ heavy chain and pre-BCR expression in SCID mice allows the progression to the pre B cell stage (Chang et al., 1995). Another suggested checkpoint in B lymphopoiesis occurs immediately following surface expression of the completed IgM molecule. This can again be demonstrated using the SCID model. After insertion of a completed μ and progression to the pre B cell stage, maturity only occurs with insertion of a recombined light chain (Reichman-Fried et al., 1990). The transgenic expression of a kappa light chain allowed pre B cells to differentiate into mature B cells, although at a two to three fold lower number of cells. These experiments have given strong evidence for the stages in B cell development where cells with functional Ig gene rearrangements are positively selected.

It is now widely accepted that elimination of developing B lymphocytes during lymphopoiesis occurs via apoptosis. Several lines of evidence support this hypothesis.

In vivo detection of apoptotic cells is minimal due to rapid phagocytosis of cells at early stages of death (Fadok and Henson, 1998; Fadok et al., 1992), but *in vitro* culturing techniques have given some insight into death in developing B lymphocytes. Isolation of BM and short term culture have shown that B lymphocytes undergo apoptosis. Pre, late pro and immature B cells undergo a higher rate of apoptosis, relative to the early pro and mature B cells, when removed from the BM microenvironment (Lu and Osmond, 1997). In addition, it has been shown that early pro B cells and mature B cells have elevated levels of Bcl-2, a protooncogene with anti-apoptotic potential (Li et al., 1993; Merino et al., 1994). Cursory studies have also shown that precursor B cells, not expressing surface IgM, are more susceptible to apoptosis induced by the synthetic glucocorticoid, dexamethasone (Garvy et al., 1993; Lu and Osmond, 1997; Merino et al., 1994). In transgenic mice with elevated expression of Bcl-2 in B lymphocytes, cell survival is heightened (Strasser et al., 1991). In Bcl-2 deficient mice, B lymphopoiesis is normal through birth, but soon thereafter B lymphocytes are absent in the bone marrow, spleen and periphery (Nakayama et al., 1993; Veis et al., 1993). These observations are fundamental to this thesis, since we are thoroughly studying Gc induced apoptosis in B cell lymphopoiesis. An in depth review of the mechanisms of apoptosis and glucocorticoid affects on immune cells will be covered later.

Stage Specific Gene Expression

The expression of some important genes, whose products are involved in B lymphopoiesis, have also been determined. RT-PCR was used to detect these genes from sorted primary cell populations (Li et al., 1993). The recombinase activating genes,

recombination activating genes 1 and 2 (RAG-1 and RAG-2), code for proteins involved in the recombination machinery (Schatz et al., 1989); their gene expression pattern correlates specifically with the B cell populations undergoing active rearrangement. They are upregulated in pro B cells, transiently down regulated in large, proliferating pre B cells and again are expressed in small pre B cells, undergoing light chain rearrangements. Mice lacking functional RAG-1 or 2 are unable to undergo Ig gene rearrangements and B lymphopoiesis is blocked in the pro B cell stage (Mombaerts et al., 1992; Shinkai et al., 1992). Terminal deoxynucleotidal transferase (tdt) is an enzyme involved in adding random nucleotides to the heavy chain joining regions to increase diversity (Desiderio et al., 1984; Landau et al., 1987). It is expressed in the pro B cell populations, but it is not detectable in the pre B cells that have completed heavy chain rearrangements (Opstelten et al., 1986). These expression patterns correlate with the stages in which their functions are critical, therefore in addition to surface phenotype and status of Ig rearrangement, the presence of certain intracellular proteins can define the various stages of development.

Recently several transcription factors have been identified which appear to be critical in the differentiation of developing B lymphocytes. PU.1 and Ikaros are two such factors. Transgenic PU.1 -/- or Ikaros -/- mice, have arrested B cell development at a stage prior to the generation of pro B cells (McKercher et al., 1996; Scott et al., 1994; Wang et al., 1996). Another transcription factor indicated in development of B lymphocytes are the E2A gene products E12 and E47. Mice with a targeted null E2A mutation fail to develop B cells early in development, before DJ rearrangements (Bain et al., 1994; Bain and Murre, 1998). B-cell-specific activator protein (BSAP-5) is a

transcription factor that has been shown to be involved in heavy chain recombination (Hagman et al., 2000). It is expressed in B cells through the pro B cell stage of development and its elimination blocks the development of cells after DJ_H rearrangements (Nutt et al., 1997; Urbanek et al., 1994). Nuclear factor kappa B (NFκB) is a molecule that was originally identified as a transcription factor that binds to an enhancer element for the κ light chain (Sen and Baltimore, 1986). Several family members have been identified and appear to have redundant function in B lymphopoiesis, since eliminating one of these factors does not halt lymphopoiesis (Burkly et al., 1995; Sha et al., 1995). Although, the constitutive expression of a dominant negative form of the NFκB inhibitor (IκB) does result in inhibition of VκJκ rearrangement and transcription of the κ light chain (Scherer et al., 1996). These findings demonstrate that specific transcription factors are involved in stage-specific differentiation during B lymphopoiesis. This underscores the complex and tightly regulated nature of the development of B lymphocytes through various differentiation stages.

Microenvironmental Effects on B Lymphopoiesis

The BM microenvironment is critical for B cell lymphopoiesis. In the BM, precursor B lymphocytes develop in close association with the cytoplasmic projections of reticular-like cells. Figure 1.3 is a diagram depicting the development of B cells within the context of the matrix of the microenvironment. *In vivo* injection of radiolabeled antibodies to B lymphocytes demonstrated that the earliest progenitors are found near the bone cortex and as the cells mature they progress towards the central core of the marrow for exportation to the periphery (Jacobsen and Osmond, 1990; Jacobsen et al., 1990;

Osmond, 1990; Osmond et al., 1992). The microenvironment plays an integral role in lymphopoiesis as is demonstrated by high losses of B lymphocytes following their removal from the BM. It was believed that the reticular cells that the lymphocytes were in contact with were supporting lymphopoiesis by providing direct contact and soluble factors for the developing cells. Cells that provide support during the development of other cell types are called stromal or nurse cells. Here we use the term stroma as the nomenclature for the cells of the bone marrow that support B cell lymphopoiesis

Long term bone marrow cultures (LTBMC) developed by Whitlock and Witte (Whitlock et al., 1984) provided a system by which B lymphopoiesis could be generated and sustained for months or even years. Whole bone marrow is placed in culture and after approximately 2 weeks an adherent layer of cells, that contains stromal cells, is established and following 3-4 weeks after initiation lymphoid cells are constitutively generated. In young cultures, through 8 weeks, the predominant lymphocytes in culture are IgM^- B cells with few IgM^+ cells. These precursor cells retain the ability to fully recombine Ig genes and differentiate into mature B cells. In older cultures the predominant cell type is IgM^+. This culture system has provided a means to reliably elucidate many key players in B lymphopoiesis. Prior to the development of the B lymphopoietic culture system a culture system that promoted myelopoiesis was developed (Dexter et al., 1977). Interestingly, a major difference between these culture systems was the addition of glucocorticoids to the medium, which promoted the development of myeloid cells. This suggested that perhaps glucocorticoids might suppress lymphopoiesis and/or promote myelopoiesis. The work presented in this thesis addresses the negative effect of glucocorticoids on B lymphopoiesis, thereby providing

insight into the mechanism whereby these two different culture systems can lead to the development of two different types of blood cells. Additionally, the affect of increased concentrations of Cs on myelopoiesis will be addressed in the experimental portion of this dissertation.

Morphological analysis of the adherent layer of LTBMC showed two distinct cell types, a large, cytoplasmically spread, fibroblast-like cell and a macrophage-like cell (Witte et al., 1987). The fibroblast-like cells were termed stromal cells, since the B precursors developed in close association specifically with this cell type (Witte et al., 1987). Although stromal cells have not been shown to be identical in nature, some general characteristics of the cell type were established. For example, stromal cells from LTBMC express surface vascular cellular adhesion molecule (VCAM1) and CD44, and various components of the extracellular matrix (ECM) such as collagen IV, fibronectin and laminin are also expressed (Witte et al., 1993; Witte et al., 1987). More importantly, stromal cells are responsible for the production of various cytokines that support lymphopoiesis (Funk et al., 1995). In fact, two cytokines that will be discussed thoroughly later in this review, interleukin 7 (IL-7) and stem cell factor (SCF, kit ligand, steel factor), are produced specifically by stromal cells in LTBMC. In addition, stromal cells produce a pre B cell growth stimulating factor (stromal derived factor-1, SDF-1) and thymic stromal lymphopoietin (TSLP) (Levin et al., 1999; Nagasawa et al., 1994). The production of macrophage colony stimulating factor (M-CSF) is also specific to stromal cells, whereas the macrophages produce the interleukin 1 beta (IL-1β) cytokine. Stromal cell lines have also been established from LTBMC, many of which can support both myelopoiesis and lymphopoiesis depending on the culture conditions used (Collins and

Dorshkind, 1987; Dorshkind et al., 1986). Some variation in expression of proteins can be observed with different clones and the degree of support provided to B lymphocytes can also vary (Henderson et al., 1990). In fact, some BM stromal cell lines have been shown to have a negative affect on lymphopoiesis (Borghesi et al., 1997). This suggests that stromal cells have the ability to both support or inhibit B cell development, yet little is known about the mechanism by which these cells can affect the lymphocyte so differently.

It has also been noted that stromal cell function can be modulated by exogenous factors that in turn can affect lymphopoiesis. For example, the addition of the aryl hydrocarbon 7,12-dimethylbenzanthracene to cultures containing B lymphocytes and bone marrow stromal cells resulted in apoptosis of the B cells (Yamaguchi et al., 1997). This induction of death was due to either the stromal cells or stromal cell derived factors since the B lymphocytes were shown to contain very few aryl hydrocarbon receptors and, in cultures without stromal cells, the B lymphocytes did not undergo apoptosis. Estrogen also appears to down regulate B cell lymphopoiesis in long term cultures via modulating stromal cell function rather than by directly affecting the B cells (Smithson et al., 1995). Treatment of B lymphocytes alone, did not induce cell death or inhibit proliferation, whereas a reduction in B cell lymphopoiesis occurred when B cells were cultured on stromal cells that were pretreated with estrogen. Specific cytokines have also been shown to modulate the expression of surface proteins on stromal cells. IL-1β addition to stromal cell clones resulted in the upregulation of both CD54 and VCAM-1 on the cell surface, whereas IL-4 upregulated only VCAM-1 and TGF-β downregulated expression of VCAM-1 on stromal cells (Dittel et al., 1993). Both CD54 and VCAM-1 have been

indicated in providing direct cell-cell contacts with B lymphocytes that promote B cell lymphopoiesis. Clearly various molecules that would normally be found in the bone marrow microenvironment can cause modification in stromal cell function, thereby potentially modifying their affect on the development of blood cells. Although the normal effects of stromal cells on B cell development have been studied in depth, little is known about the effect that Cs might have on these cells. Considering what is known of Cs negative long-term effects on B lymphocyte development this thesis addressed the potential of stromal cells to modify B cell responses to Cs and the potential for Cs to directly modify the production of cytokines known to be involved in lymphopoiesis.

Cell-Cell and Cell-Extracellular Matrix Contact

Considering the close association of the precursor B lymphocytes with stromal cells both in LTBMC and *in situ*, the role of direct contact appeared to be important in lymphopoiesis. One hypothesis regarding the close association between B lymphocytes and stroma was simply the physical retention of developing cells in the marrow. However, it was observed that suspending lymphocytes over stromal cells by using a porous membrane resulted in decreased cell viability and loss of cell differentiation, thus arguing for a more intricate role for cell-cell and cell-extracellular matrix (ECM) adhesion in lymphopoiesis (Borghesi et al., 1997; Kierney and Dorshkind, 1987; Manabe et al., 1994). Contact between pro B cells and stromal cells enhanced survival of the pro B cells and resulted in an upregulation in the anti-apoptotic Bcl-2 protein and a concomitant decrease in pro-apoptotic Bax protein levels (Gibson et al., 1996). In addition to adhesion playing a role in supporting B cell lymphopoiesis it has also been

suggested that lymphocytes can initiate signaling cascades in stromal cells (Jarvis and LeBien, 1995; Jarvis et al., 1997). The incubation of B lymphocytes directly on a stromal cell line, resulted in tyrosine phosphorylation at focal adhesion points in stromal cells, indicating the initiation of adhesion mediated signaling. Therefore the interactions between stromal cells and lymphocytes appear to be more complex than originally thought with each cell type potentially able to affect the other in a positive or negative manner.

There are multiple potential adhesive interactions that could play a role in stromal cell supported B lymphopoiesis. Miyake et al (Miyake et al., 1991) attempted to identify if adhesion molecules on bone marrow stromal cells could directly affect B cell development. This was done by creating monoclonal antibodies against cell surface proteins. Two antibodies, M/K-1 and M/K-2, had specific affinity to a surface molecule on stromal cells present in LTBMC and in the bone marrow. Addition of the antibodies to newly initiated LTBMC resulted in the ablation of lymphopoiesis in preestablished cultures and also resulted in the rapid detachment of lymphoid cells. This antibody recognized a molecule with sequence homology to human VCAM-1. One ligand of VCAM-1 is the $\alpha 4\beta 1$ integrin (VLA-4), therefore an antibody to the α subunit of VLA-4 was made to determine if it would have similar effects on lymphopoiesis (Miyake et al., 1991). In fact, it did cause rapid detachment of lymphocytes in LTBMC and its addition to newly established cultures completely blocked lymphopoiesis. This indicated that VCAM-1 on stroma and VLA-4 on lymphocytes interact and facilitate lymphopoiesis. VLA-4 is also known to use fibronectin as a ligand, but blocking peptides that disrupted this interaction did not disrupt adhesion or lymphopoiesis. Additionally, immunostaining

showed that VCAM-1 and VLA-4 are located together in areas of contact between stromal cells and precursor B lymphocytes (Murti et al., 1996, Jacobsen, 1996 #78). It has also been demonstrated that adhesion of human precursor B cells to stromal cells is mediated to a great extent by the interactions of VLA-4 and VCAM-1 (Dittel et al., 1993). Thus, direct cell-cell interactions between stromal cells and B lymphocytes are important in the normal development of B lymphocytes in the bone marrow.

Stromal Cell Derived Cytokine Support

In addition to the important role of contact in stromal cell support of lymphopoiesis another critical role of stromal cells is the production of cytokines, although cytokine effects and close association between stromal cells and lymphocytes can be interrelated. Many cytokines are produced in membrane bound forms as well as soluble forms. Although close association with the stroma may augment these effects it has also been shown that soluble support via the stroma can also increase B lymphocyte survival and proliferation, although not to the extent of direct association (Borghesi et al., 1997). In 1988 a stromal cell derived factor, IL-7, was identified and purified, that supports proliferation of pre B lymphocytes (Namen et al., 1988; Namen et al., 1988). Biochemical analysis identified it as a 25-kilodalton glycoprotein and its structure of four alpha helical bundles placed it in the Type-I class of cytokines. In culture, without stromal cell support, recombinant IL-7 can support prolonged proliferation of IgM^- cells (Lee et al., 1988). Initially the responding cells are consistent with late pro B cells and pre B cells: cytoplasmic μ^+, $BP\text{-}1^+$ and IgM^- (Lee et al., 1989). Extended culture periods with IL-7 ultimately resulted in an accumulation of cytoplasmic μ^- B lymphocytes,

corresponding with the early pro B cell stage. Subsequently it was discovered that IL-7 also played a similar role in thymocyte development (Okazaki et al., 1989; Watson et al., 1989). It induced proliferation in early $CD8^-CD4^-$ thymocytes. Since then, T cells and thymocytes have been used extensively in the study of IL-7 and the IL-7 receptor (IL-7R) and relatively little work has been done using B lymphocytes. It can be surmised that, at least in part, the complexity of the B cell system has hindered research efforts in this area.

In addition to inducing proliferation, IL-7 could promote survival of early B and T cells. One mechanism whereby IL-7 might promote survival is by the activation of the phosphatidyl inositol 3-kinase (PI3K) pathway (Dadi et al., 1993; Pallard et al., 1999). This survival pathway will be discussed in greater detail later in this thesis, but briefly PI3K phosphorylates AKT (protein kinase B) thereby inactivating the pro-apoptotic Bad protein. It has also been shown that IL-7 can promote survival by maintaining or upregulating anti-apoptotic Bcl-2 levels and decreasing pro-apoptotic Bax levels (Lu et al., 1999). IL-7 upregulated Bcl-2 protein levels and increased cell survival in early precursor thymocytes and in a T lymphoma cell line (Kim et al., 1998; Lee et al., 1996; von Freeden-Jeffry et al., 1997). Although Bcl-2 may play a role in IL-7 induced survival it may not be the exclusive survival mechanism. In mice deficient for the IL-7 receptor, overexpression of Bcl-2 did not rescue B lymphopoiesis, but did promote the survival of circulating (mature) B cells (Maraskovsky et al., 1998). It should be noted that IL-7 receptor deficiency also resulted in a lack of proliferation in the developing B lymphocytes, therefore Bcl-2 could potentially promote the survival of B lymphocytes but this effect would not be seen due to the lack of expansion of the cells. Therefore, the role of Bcl-2 in IL-7 mediated survival has not been completely elucidated. This thesis

will provide further insight into the effect of IL-7 on apoptosis and cell cycle arrest in B lymphocytes induced by Cs.

In 1990 the receptor for human and murine IL-7 was cloned (Goodwin et al., 1990). Binding studies revealed that the receptor exists in both a high and low affinity form. The receptor is observed on many murine cell types, both myeloid and lymphoid. The high affinity form of the receptor is found on IgM^- B lymphocytes and in $CD4^-CD8^-$ or single positive thymocytes. Injection of a blocking antibody to the high affinity form of the IL-7R resulted in a dramatic decrease in B cell precursors and thymocytes, although peripheral lymphocytes remained for two weeks (Sudo et al., 1993). These data supported an important role for IL-7 in lymphopoiesis. Later studies, utilizing transgenic mice, showed that elimination of IL-7 or IL-7R resulted in ablation of B lymphopoiesis, except for an odd population of immature B cells found in the spleen (Corcoran et al., 1998; von Freeden-Jeffry et al., 1995). The exact role of IL-7 in B lymphopoiesis is still being investigated. It was originally thought that the cytokine was involved strictly in proliferation and survival of precursor B cells. More recently support is growing for a role of IL-7 in differentiation as well. It was shown that IL-7Rα knockout mice have precursor B lymphocytes with normal D-J recombination of the heavy chain but there is a marked reduction in V gene recombination, correlating with the physical distance of the V gene from the recombination site (Corcoran et al., 1998). In addition, the expression of the pax-5 gene, whose product, BSAP-5, binds the heavy chain and stimulates recombination is significantly reduced. To determine if signaling through the IL7R could result in Ig gene rearrangement, a mutation was made on the cytoplasmic domain of the receptor (Corcoran et al., 1996). This mutation caused elimination of the proliferative

effects of IL-7 but did not affect Ig heavy chain rearrangement. The IL-7R, therefore, has been shown to promote several distinct intracellular responses through its cytoplasmic domain.

Crosslinking studies showed that the IL-7Rα subunit was associated with the common γ chain (γc) of the IL-2 receptor (Noguchi et al., 1993). Association with γc enhanced the binding affinity of IL-7Rα and increased receptor internalization upon ligand binding. In addition, blocking antibodies to γc inhibited IL-7 induced proliferation of a B cell line (Kondo et al., 1994). IL-4, IL-9 and IL-15 also contain the γc as part of their receptors. The cytoplasmic tail γc has been shown to associate with janus kinase family member Jak3 and stimulate STAT signaling resulting in proliferation (Malabarba et al., 1995; Miyazaki et al., 1994). Src kinases and phosphatidyl inositol 3-kinases (PI3K) have also been shown to be activated in response to IL-7.

Another cytokine that appears to be involved in B cell lymphopoiesis is stem cell factor (SCF). SCF is a glycosylated homodimer that plays a broad role in aiding the commitment of hematopoietic progenitors to the various lineages when present in combination with specific growth factors for those specific cell types (Witte, 1990). To date, the only cytokine that has been shown to be critical in B lymphopoiesis is IL-7, but SCF synergizes with IL-7 to increase proliferation of pro and pre B cells (McNiece et al., 1991). In addition, this combination of cytokines results in differentiation of $B220^-$ murine bone marrow cells into $B220^+$ B lymphocytes, whereas neither cytokine, alone, can promote the commitment to the B lineage. SCF can be produced in both soluble and membrane bound forms. Experiments whereby only the soluble isoform of SCF was

produced indicated that the membrane bound form likely is the active form of the cytokine (Miyazawa et al., 1995).

The receptor for SCF is c-kit which is a receptor tyrosine kinase expressed on the surface of many hematopoietic cells; on B cells its expression is restricted to the pro B cell stage (Rico-Vargas et al., 1994). Like IL-7, SCF can initiate multiple signaling cascades in the cell and it has shown some ability to promote survival. It may, in part, promote survival through the PI3K induced activation of AKT (Blume-Jensen et al., 1998). Other activating cell signaling pathways that have been shown to be activated by SCF binding of c-kit are the JAK/STAT, MAPK and Src family transduction pathways (Linnekin, 1999). Another tyrosine kinase receptor, flt-3, appears to parallel c-kit in its function on B lymphocytes. Like c-kit, it is expressed on pro B lymphocytes and its ligand, FL, can induce proliferation in pre-pro B cells (Hirayama et al., 1995; Matthews et al., 1991). Considering the similarities and the differences between the biological effects and signaling pathways of IL-7 and SCF this thesis investigates the effect of both of these cytokines on background and Cs-induced apoptosis to determine whether they have similar, different or additive effects on B lymphocytes.

Since the discovery of the above-mentioned factors, other stromal cell derived molecules that may play a role in lymphopoiesis have been discovered. Two such factors are thymic stromal lymphopoietin (TSLP) and stromal derived factor-1 (SDF-1). TSLP has been shown to act as a costimulatory factor with IL-7 and promotes proliferation through the immature stage of development (Friend et al., 1994). Interestingly, it was shown that the receptor for TSLP is composed of the IL-7Rα chain and the novel TSLP receptor (Levin et al., 1999). Whereas TSLP appears to affect later stages in B cell

development, SDF-1 appears to elicit its effect on earlier B cells. SDF-1 can induce proliferation in pre B cells and has also been shown to be a chemoattractant factor for various progenitor populations (Aiuti et al., 1997; Nagasawa et al., 1994). Mice deficient in SDF-1 or its receptor, CXCR4, have a block in fetal lymphopoiesis before the pro B cell stage. It is becoming clear that many factors are involved in the successful generation of mature B lymphocytes and that they may elicit their effects at different developmental stages.

Summary

The generation and development of B lymphocytes in adult mammals is a tightly regulated, multi-stage process that occurs in the highly heterogeneous bone marrow. It is the complex and critical rearrangement of Ig genes that appears to drive the development of B cells. Two advances have aided in progress in this field, flow cytometry and a long term culturing technique that promotes B cell lymphopoiesis. Flow cytometry has provided a powerful tool for analysis of the B lymphocytes in their various stages of development and LTBMC has allowed for the discovery of several microenvironmental support factor critical in lymphopoiesis. IL-7 is a stromal cell derived factor that, in particular, has been shown to be critical in early stages of B lymphocyte development in mice. Without IL-7 pro and pre B lymphocytes cannot survive or proliferate long-term in culture. In addition to LTBMC, transgenic mice have provided a system for defining the *in vivo* effects of many of these lymphopoietic molecules. Taken together, it is becoming clear that during the development of B lymphocytes a myriad of factors work together to promote cell survival, proliferation and differentiation and that response to these factors depends on the stage of development. Each developmental stage can be defined by selective expression of relevant proteins and surface markers and proper recombination of Ig is critical for advancement to maturity. Even with the experimental advances that have been made in the last two decades, much work remains in eliciting the mechanism of B lymphopoiesis, especially in determining how normal physiological changes may modulate this process. This lab has a unique advantage in studying normal bone marrow B lymphocytes in various stages of development in part due to access to a multi-parameter flow cytometer. Considering this advantage we have chosen to investigate the

effect of Gc on normal developing B lymphocytes from progenitor through maturity. As will be further discussed in the next portion of this literature review, Gc can have dramatic negative effects on B cells, yet little is known of this phenomenon. Here we will determine *in vivo* and *in vitro* responses of normal B lymphocytes to Gc, as well as the potential modification of these responses by stromal cells, IL-7 or SCF.

GLUCOCORTICOIDS AND THE IMMUNE SYSTEM

Background

Glucocorticoids (Gc) are steroid hormones that are produced in the cortex of the adrenal gland. Their basal production is critical to mammals, since the lack of Gc can result in death. Natural variations in serum levels of Gc occur in a diurnal fashion with levels in humans being highest in the morning hours and in nocturnal animals, such as rodents, levels are highest at night (Nichols and Tyler, 1967). Levels of Gc are regulated by adrenal corticotrophin hormone (ACTH; corticotrophin) produced in the anterior pituitary gland. ACTH is upregulated by vasopressin and corticotrophin releasing hormone (CRH), whereas glucocorticoids cause its down-regulation (Engler et al., 1999). Together this cascade of hormones link together neurobiology and endocrinology and is referred to as the hypothalamic-pituitary-adrenal (HPA) axis. The major external effector of the HPA axis is stress. Stress can be defined as anything that disturbs homeostasis. Some stressors, such as exercise, are thought of as positive and elicit a transient increase in Gc. Other stressors induce a chronic increase in the production and circulation of Gc. Classic examples of negative stress are seen in burn patients, trauma victims, malnourishment and depression. It is chronic exposure to Gc that can have a variety of negative effects including compromised immune function. Therefore it is this phenomenon that is of interest here.

As early as 1947 the relationship between stress and Gc elevation was suggested (Selye, 1947). Just 2 years later, it was proposed by Hench (Hench, 1949) that Gc mediate anti-inflammatory action. In fact, for decades synthetic Gc such as prednisone

and dexamethasone have been used as anti-inflammatory drugs in diseases such as arthritis, autoimmunity and asthma. This provides both pharmacological as well as physiological reasons for studying the effects of glucocorticoids. Along with the noted anti-inflammatory effects of glucocorticoids, immunosuppression is evident. In patients with the aforementioned stressors, there is often a marked decrease in peripheral lymphocytes (lymphopenia) and ultimately compromised immune defense against infection.

This lab became interested in glucocorticoid induced immunosuppression while studying zinc deficiency in mice. It was observed that zinc deficiency resulted in chronic elevation of the naturally occurring Gc, Corticosterone (Cs) (in humans the major circulating glucocorticoid is cortisol) (DePasquale-Jardieu and Fraker, 1980). Adrenalectomy of these mice resulted in protection of the immune system during zinc deficiency (DePasquale-Jardieu and Fraker, 1980). There is evidence that suppression of lymphopoiesis is at least partially the mechanism whereby Gc induces lymphopenia. Thymic atrophy has been a hallmark of elevated Gc, indicating that it may decrease the numbers of developing T lymphocytes. It was later determined that indeed developing T lymphocytes were being lost, most notably the double positive ($CD4^+CD8^+$) precursor thymocytes (Cohen, 1992). The effect of elevated Gc on B cell lymphopoiesis was not investigated until relatively recently, when our lab and others analyzed the BM compartment in mice with elevated levels of the steroid. Garvy et al (Garvy et al., 1993) demonstrated that chronic elevation of Cs in mice resulted in selective depletion of the developing B lymphocytes in the BM. It was also noted that IgM^- precursor B cells were more sensitive to these Cs induced losses and suggested that the mechanism may be due

to Gc mediated cell cycle arrest and apoptosis in these cells. The role of glucocorticoid induced apoptosis and cell cycle arrest will be thoroughly discussed in the apoptosis section of this review.

In addition to the negative regulatory effect of elevated Gc on developing B and T lymphocytes, it appears that Gc can also play a role in normal lymphocyte selection and even survival under some conditions. In the thymus it was recently shown that corticosterone could be produced by epithelial cells (Vacchio et al., 1994). In experiments using radiolabeled steroid precursors, thymic tissue was capable of complete glucocorticoid metabolism, suggesting that the thymus contains all of the necessary enzymes for steroid biosynthesis (Lechner et al., 2000). In thymic organ cultures, the elimination of Cs production resulted in the loss of cells that would normally be positively selected by crosslinkage of the TCR (Vacchio and Ashwell, 1997; Vacchio et al., 1998). In addition to promoting survival of "selected" T cells, it was observed that glucocorticoids could inhibit apoptosis induced by serum depletion in a Bcl-2 expressing T lymphoma cell line (Huang and Cidlowski, 1999). To date, there does not appear to be any evidence for the role of Gc in the positive selection of B lymphocytes, but given the parallels between the two processes it seems likely that Gc may play a similar role in B lymphopoiesis. These observations underscore the complex role that Gc plays in the immune system and suggest that the steroid may act as an effector for cell survival or cell death depending on the context of the cell. This thesis addresses Gc induced downregulation of B lymphopoiesis, induction of apoptosis and cell cycle arrest on normal B lymphocytes, thus defining conditions whereby Gc has a negative effect on B cell development.

Glucocorticoid Biochemistry

As previously stated the synthesis of glucocorticoids occurs in the cortex of the adrenal gland. The precursor for all steroid hormones is cholesterol and the majority of cholesterol used in steroid hormone biosynthesis is trafficked into the adrenal gland by either low density lipoprotein (LDL) in humans or high density lipoprotein (HDL) in mice. In the adrenal the majority of cholesterol is stored in lipid vesicles until stimulation of synthesis (Vinson et al., 1992). Figure 1.4 is a diagram of the pathways of steroid biosynthesis and the enzymes involved. The first step is the generation of pregnenalone by the cytochrome P450 side chain cleavage enzyme. This is followed by 3β-hydroxysteroid dehydrogenase Δ 4,5-isomerase reaction resulting in progesterone. In humans a 17α-hydroxylase followed by a 21- and 11β- hydroxylase reaction generates cortisol. It has been long believed that the 17α-hydroxylase is absent in rodents, therefore the 21-hydroxylase and 11β-hydroxylase reactions result in the production of corticosterone, which differs from cortisol only in lacking a hydroxyl group at carbon 17. More recently though, evidence for 11β-hydroxylase activity in murine *in vitro* adrenal cultures has been demonstrated (Touitou et al., 1990). In addition to synthesis of glucocorticoids, mineral corticoids and sex hormones are also derived from cholesterol in the adrenal gland.

Following synthesis and release from the adrenal gland, Gc are transported through the system bound to the corticosteroid binding globulin (CBG, transcortin) (Westphal and Devenuto, 1966). However, it is believed that the biologically active form of Gc is not bound to CBG, but exists in a free state (Bright, 1995; Mendel et al., 1989). A dynamic equilibrium between free and bound Gc is apparent, therefore suggesting that

regulation of Gc binding to CBG might result in partial control of Gc activity. In fact, CBG levels have been shown to decrease in response to stress such as surgery in humans (Vogeser et al., 1999). These decreases have also been observed in rodents exposed to classical stressors (Tannenbaum et al., 1997). It is believed that the downregulation of CBG results in an upregulation of free Gc. In addition, at sites of inflammation it has been shown that elastase can cleave CBG, drastically reducing its affinity for Gc (Pemberton et al., 1988). These results suggest that not only are Gc actions controlled by upregulated production of the steroid but also by modulation of its availability.

The Glucocorticoid Receptor

Following transport throughout the system, Gc elicit their effects by binding to the glucocorticoid receptor (GcR). The GcR belongs to the steroid hormone receptor (SHR) superfamily of proteins and is present in nearly all mammalian tissues at thousands of molecules per cell (Ballard et al., 1974). This family includes receptors for the sex hormones, thyroid hormones and arylhydrocarbons (such as dioxin). In 1985 the human GcR was cloned, demonstrating the existence of both α and β isoforms (Hollenberg et al., 1985). The α isoform was shown to be the ligand binding form composed of 777 amino acids whereas the non-ligand binding β form was missing 15 amino acids at the carboxyl terminus. It was recently shown that the GcRβ is expressed at modest yet varying levels and may act as a ligand independent negative regulator of GcR (Bamberger et al., 1995). The α form of GcR has several functional domains, which are consistent throughout the SHR family. The protein contains a ligand binding domain, a DNA binding domain and two transactivating domains (AF-1 and AF-2). A diagram of

the GcR is shown in Figure 1.5. Sequence analysis and crystallography of the DNA binding domain revealed the presence of two zinc finger motifs (Freedman and Luisi, 1993). The loop structure formed by zinc binding between four cysteine residues is a common feature of DNA binding proteins. The ligand binding domain is located at the carboxyl terminal end of the protein. Radiolabeled ligand affinity studies have shown that dexamethasone binds the receptor with high affinity. The natural steroids, cortisol or corticosterone, bind with modest affinity and sex hormones bind with only low affinity. The transcription activating domain, AF-1, is located at the amino-terminal end of the receptor and its activity is independent of ligand binding (Almlof et al., 1997). In contrast the AF-2 transactivating domain is located in the ligand binding region and is dependent on steroid binding (Danielian et al., 1992). Steroid binding is thought to cause a transformation of the AF-2 region, allowing coactivating proteins, which mediate transcription, to bind the SHR (Feng et al., 1998; Wagner et al., 1995).

Early studies showed that under hormone free conditions a large form of GcR was located in the cytosolic fraction of cell extracts (Baxter and Tomkins, 1971). Interestingly, the addition of steroid resulted in a lower mass receptor that could be isolated from nuclear fractions (Higgins et al., 1973; Schmidt et al., 1982). This led to the hypothesis that Gc binds to a large inactive receptor complex in the cytosolic compartment resulting in release of associated factors and translocation to the nucleus. It was later determined by immunohistochemical studies that indeed Gc binding to the GcR resulted in translocation of the receptor from the cytosol to the nucleus. In pursuit of identification of the putative associated proteins of the inactive GcR complex, cytosolic receptor was isolated and purified (Gustafsson et al., 1989; Rexin et al., 1992). A 90-

kilodalton protein was isolated and further immunolabeling and sequence analysis showed the protein to be heat shock protein 90 (hsp90). Point mutations in the ligand binding domain of the GcR resulted in loss of hsp90 binding. This suggested that hsp90 bound the ligand binding domain of the GcR (Chakraborti et al., 1992). Further coprecipitation studies of the GcR suggested that there were a variety of associated proteins. To date the cytosolic form of the GcR has been shown to have two hsp90 molecules, hsp70, hsp56 and a small immunophillin p23 molecule associated with it. Upon binding of steroid to the receptor, this protein complex dissociates allowing bound receptor to translocate to the nucleus. GcR are phosphorylated proteins that undergo hyperphosphorylation upon agonist ligand binding (Bodwell et al., 1998). The hyperphosphorylation appears to be critical in GcR regulation of transcription, since receptors mutated at certain phosphorylation sites have severely impaired transcriptional transactivation. Additionally, phosphorylation occurs in regions of association with cell cycle dependent kinases. Interestingly, phosphorylation of the GcR appears to be dependent on the cell cycle status. In the experiments performed thus far, ligand binding induced hyperphosphorylation in the S phase of the cycle, but not during G_2/M (Hu et al., 1997). Collectively these data suggest that the regulation of GcR activity is modulated by more than just ligand binding.

Glucocorticoid Receptor Signaling

In 1974 it was observed that the insect glucocorticoid, ecdysteroid, caused puffing on polytene chromosomes of *Drosophila melanogaster*, suggesting that Gc may elicit its effect by modulating transcription (Ashburner et al., 1974). It was later determined that,

in fact, GcR was affecting transcription of certain genes via binding to a DNA consensus sequence, 5'-GGTACAnnnTGTTCT-3' (Beato et al., 1989; Chandler et al., 1983; Karin et al., 1984). The GcR DNA binding site was termed the glucocorticoid response element (GRE). Exactly how receptor binding to DNA regulates the transcription machinery has not been determined. There is evidence for direct interactions between GcR and the transcription initiation complex and for association of the GcRs with coactivator proteins (Tsai and O'Malley, 1994). Members of the p160 family of proteins (steroid receptor coactivator 1 [SCR1], GcR-interacting protein 1 [GRIP1]) were found to associate with the AF-2 region of nuclear receptors and act as coactivators of transcription following ligand binding (Hong et al., 1996; Onate et al., 1995). Recently these coactivators have been found to promote transcription activation by binding the histone acetyltransferases (HAT) CBP or p300 (Torchia et al., 1997). The HAT activity promotes transcription by acetylating histone H3 of chromatin, thus modifying chromatin structure to allow transcription machinery access to the DNA (Kuo and Allis, 1998). In addition to HAT activity CBP and p300 have been shown to directly bind the basal transcription machinery. Therefore, GcR, may play a direct role in stimulating transcription by controlling chromatin structure and perhaps by directly interacting with the transcription initiation complex.

Active ligand-bound GcR has also been implicated in cross-talk with other signaling cascades. Interestingly, it was long believed that steroid hormones mediated transcription activation, but recently it was shown that SHR can also negatively regulate transcription of certain genes. Gc appears to negatively regulate transcription of AP-1 and NF-kB regulated genes. AP-1 promotes transcription of immediate early genes

stimulated by growth factors and NF-kB stimulates transcription of genes involved in immune cell activation and inflammatory cytokines. *In vitro* experiments suggested that GcR negatively regulates AP-1 transcription by directly interacting with the fos/jun heterodimer (Heck et al., 1994; Lucibello et al., 1990). This interaction is ligand dependent, but does not require GcR binding to the DNA. In contrast, the repressive effects of GcR on NF-kB activities do not appear to be mediated by direct interactions between the two proteins. NF-kB is bound to IkB in its inactive cytoplasmic form [reviewed in Hatada, 2000 #323]. Activation of NF-kB requires the degradation of IkB followed by nuclear translocation and transcription initiation. It appears that GcR inhibits NF-kB activity by upregulating IkB gene transcription, thus rendering NF-kB to its inactive form (Auphan et al., 1995). Cross-talk between nuclear receptors and transcription factors is an active area of study and is proving to be important in the understanding of how Gc affects cells.

In addition to the direct effect of GcR on cellular processes, it is also becoming apparent that hsp90 may also mediate signaling following its dissociation from the GcR. The creation of a fusion protein containing the GcR hormone binding domain (also the hsp90 binding domain) and the adenovirus E1A protein, resulted in steroid responsiveness by the otherwise innocuous E1A (Picard et al., 1988). The results suggested that release of the inhibitory protein complex may result in at least a partial steroid effect. In addition, hsp90 also interacts with $pp60^{v\text{-}src}$ kinase and has been shown to diminish its kinase activity (Xu and Lindquist, 1993). It is becoming apparent that signal transduction via the GcR is a multi-faceted process that can influence or be influenced by various other cellular signals.

Another mechanism for regulating GcR mediated signaling may be via the direct transcriptional upregulation or downregulation of the receptor. It has been shown that Gc itself can downregulate the transcription of GcR, ultimately resulting in decreased levels of the receptor protein (Burnstein et al., 1994; Cidlowski and Cidlowski, 1981). In addition to the negative effect on transcription, Gc binding to the GcR results in destabilization of the receptor. Recently, the phosphorylation state of the GcR had been implicated in the stability of the receptor, with hyperphosphorylation induced by ligand binding correlating with decreased receptor stability (Bodwell et al., 1998). Abolishment of the eight phosphorylation sites, either individually or in combination, has shown that decreased levels of phosphorylation correlates with decreased transactivation of responsive genes and increased receptor half-life (Webster et al., 1997). The exact mechanism of GcR stability and modification is not well understood, although it is becoming clear that GcR mediated pathways are tightly regulated.

Summary

Glucocorticoids are hormones that have a diverse range of effects on many physiological functions in mammals. Here we have focussed on the effect of Gc on the immune system, specifically the development of B lymphocytes. At basal concentrations Gc appear to play a positive role in normal homeostasis during thymocyte development, yet the effect of basal levels of Gc on B lymphocyte development has not been confirmed but can be surmised to likely play a similar role. A chronic increase in Gc production and circulation, due to the chronic activation of the HPA stress axis, clearly has a negative affect on the immune system. This lab has had an integral role in determining how the induction of the stress axis via zinc deficiency has had a negative affect on the immune system and B lymphocyte development and the specific downregulation of BM B lymphocytes by chronic Cs elevation. Since then identification techniques and methodologies have advanced dramatically in this field, allowing for further investigation into the effects that Gc have on developing B lymphocytes *in vivo* and *in vitro*. The downregulation of developing B lymphocytes by Gc likely occurs by both a decrease in cell survival (apoptosis) and a downregulation in proliferation. These phenomenon will be discussed in the next section of this review. Additionally, many *in vitro* experiments studying B lymphocyte responses to Gc have utilized the synthetic Gc, dex, yet little is known of the effects of the naturally occurring Cs on B lymphocytes. This thesis will thoroughly investigate the effects of Cs on normal BM B lymphocytes from mice to provide new insights into the mechanism of Gc negative actions on these developing immune cells.

APOPTOSIS

History

In 1972 Kerr and Wyllie (Kerr et al., 1972) observed that in normal tissue development, certain cells underwent cell death in an ordered manner with distinct morphological characteristics. This form of death was subsequently termed apoptosis and varied from necrotic cell death in that cytoplasmic contents were not released to the extracellular environment, thus avoiding an uncontrolled immune response. Nearly a decade passed before a modest interest in apoptosis was scientifically revived. It wasn't until the 1990's that the fundamental importance of apoptosis became widely recognized and sparked intensive research in this area. It is now clear that cell death is germane to the homeostasis of multicellular organisms and dysregulation of it can have serious health consequences.

Excessive apoptosis has been indicated as playing a central role in several diseases. A classic example is apparent in the etiology of acquired immunodeficiency syndrome (AIDS). Central to the manifestation of AIDS is the excessive elimination of helper T lymphocytes by apoptosis (Meyaard et al., 1992; Wang et al., 1999). The faulty induction of apoptosis is also apparent in neurodegenerative diseases, such as Parkinson's and Alzheimer's (Waggie et al., 1999). Along with excessive apoptosis, the failure to undergo appropriate cell death also can contribute to disease. In B cell follicular lymphoma, a genetic translocation results in the upregulation of the anti-apoptotic protein Bcl-2 (this protein will be discussed in detail later in this chapter) (Bakhshi et al., 1985; Cleary et al., 1986). The malignant cells in this type of cancer did not exhibit increased

proliferation as was thought to be a hallmark of malignancies, rather they failed to die normally. Apoptosis is also thought to play a significant role in the proper development of B and T lymphocytes with death being induced in cells with faulty Ig or T cell receptor rearrangements, respectively. The failure of B or T cells with anti-self antigen receptors to undergo proper elimination can lead to autoimmune diseases such as Systemic Lupus Erythematosis, asthma and rheumatoid arthritis (O'Reilly and Strasser, 1999). Relative to this thesis, our lab has implicated apoptosis in the mechanism of downregulation of developing B lymphocytes in response to Gc elevation (Garvy et al., 1993). A downregulation in the development of B and/or T lymphocyte can result in decreased circulating cells (lymphopenia) that ultimately results in a compromised immune response. Clearly the past ten years have brought apoptosis to the forefront in the study of the etiology of many diseases and thus further study of this process could significantly contribute in the understanding, the control, and potentially the cure of sometimes fatal diseases.

Apoptotic Morphology and Detection

Morphologically most apoptotic cells undergo a decrease in cell size and volume, nuclear condensation, blebbing of the plasma membrane and eventual formation of membrane bound apoptotic bodies. During early research on apoptosis a seminal observation was made in thymocytes induced to undergo apoptosis with dexamethasone (Wyllie, 1980). It was shown that in the apoptotic nucleus an endonuclease is activated that caused DNA fragmentation, consistent with cleavage at the internucleosomal linker regions of chromatin. As previously stated, apoptotic cell death varies from necrotic cell

death in that the cytosolic contents remain membrane bound whereas during necrosis the dying cell swell and ultimately releases its contents to the surrounding milieu. During apoptosis the plasma membrane undergoes changes in fluidity, resulting in a transition of phosphatidylserine (PS) from the inner to the outer leaflet of the phospholipid bilayer (Fadok et al., 1992). The exposure of PS then triggers rapid engulfment by phagocytic cells. Recently, a protein kinase C delta mediated scramblase has been proposed as the mechanism whereby PS is exposed on the outer leaflet (Frasch et al., 2000). Another fundamental difference between apoptosis and necrosis is the lack of internucleosomal DNA cleavage in necrotic cells. Late in necrosis DNA cleavage can occur, but results in indistinct fragmentation, in contrast to the nucleosomal cleavage pattern seen in apoptosis (Bicknell and Cohen, 1995).

Early studies in apoptosis depended on the morphologic analysis of the dying cells, therefore more sophisticated research such as the analysis of cell death in heterogeneous tissue was difficult if not impossible. Later an electrophoretic method was developed that could determine the degree of apoptosis in cell populations by the presence of a DNA "ladder", indicative of internucleosomal DNA cleavage. This methodology aided somewhat in studying apoptosis, but it only provided quantitative data and not qualitative identification of dying cells. This lab made a significant contribution in methodology for the identification of apoptosis in whole cells (Telford et al., 1991; Telford et al., 1994). Cells could be fixed in ethanol and the DNA stained with propidium iodide whereby cells containing hypodiploid DNA were determined to be apoptotic. This method allowed for the analysis of heterogeneous tissues, since mixed populations could be phenotyped for specific cell types and analyzed on an individual

cell basis. Prior to this, apoptosis in bone marrow B lymphocytes, the major topic of this thesis, could not be adequately studied unless complex purification protocols or cell lines were utilized.

Apoptosis Activation (Caspases and Mitochondria)

In the 1990's intensive research efforts were applied to the identification of a central biochemical pathways that lead to the commitment to and execution of apoptosis. Many intracellular processes were implicated as central death effectors, but further investigation showed apoptosis initiation could occur independent of the proposed effectors. Calcium influx, generation of reactive oxygen species and endonuclease activation were just a few processes implicated as central to the activation of apoptosis (reviewed in Schwartzman and Cidlowski, 1993). Each of these were later shown to be common features of apoptosis under many circumstances but not necessary for death. In recent years with the effort of many laboratories a model of the central pathway of apoptosis has been generated (Figure 1.6). It integrates both mitochondrial changes and the activation of a class of proteases called caspases. The model also defines two distinct types of apoptosis, receptor-mediated death that is initiated in healthy cells (caspase dependent) and death that occurs in damaged or neglected cells (mitochondria dependent). A brief overview of receptor-mediated apoptosis verses apoptosis in damaged cells will be presented below, followed by a detailed overview of the central death pathway. Briefly, a classic example of cells that are healthy but eliminated is evident in the immune response. This type of autoregulatory death occurs via the crosslinking of death receptors of the tumor necrosis factor receptor (TNFR) superfamily

(Nagata, 1997). This results in the activation of the caspase cascade and ultimately apoptosis. Mitochondrial depolarization and the release of cytochrome c is a common feature seen in this type of death, but it is not necessary for it to occur (Los et al., 1995). On the other hand, cells that undergo apoptosis due to neglect or damage depend on mitochondrial changes but caspase activation is not required for death. The activation of caspases is common and responsible for the morphology associated with apoptosis, but blocking caspase activity does not block cell death. Neglect by serum deprivation or DNA damage are two situations whereby blockage of caspase activity does not inhibit cell death, and the death that occurs is more similar to necrosis in morphology (McCarthy et al., 1997; Mills et al., 1998).

Research on cell death in the nematode, *Caenorhabditis elegans*, has led to fundamental discoveries of the central molecular mechanisms of apoptosis. Genetic mutants with cell death defects were created and two proteins called CED-3 and CED-4 were found to be activators of apoptosis. CED-3 was later cloned, and shown to have homology to the mammalian cysteine protease, interleukin-1β converting enzyme (ICE) (Yuan et al., 1993). At the time, ICE was known to process IL-1β into an active form, but it had not been implicated in apoptosis. Further studies showed, indeed, that ICE overexpression could itself induce apoptosis (Miura et al., 1993). Since then several ICE-like proteases have been identified and were renamed caspases. To date 13 caspases have been identified. A unique characteristic of the caspase family is their specificity for cleavage after aspartate residues in proteins (Howard et al., 1991). There are many intracellular substrates for caspase cleavage that once cleaved can elicit a biological effect. The cleavage and activation of these proteins leads to the distinct morphology

seen in apoptosis. For example, caspase 3 has been shown to cleave and degrade proteins involved in the cytoskeleton, such as actin (Kayalar et al., 1996). In addition to generating morphological changes, the caspases can also act to amplify the death pathway by cleaving anti-apoptotic proteins such as Bcl-2, rendering them pro-apoptotic (Cheng et al., 1997). Therefore many of the cellular changes associated with apoptosis are due to caspase activity.

Along with action on cellular substrates caspases can cleave and activate themselves, thus rendering a cascade of activated caspases. The caspases exist in an inactive procaspase form. Both cytochrome c from the mitochondria and the newly identified CED-4 homologue, apoptotic protease activating factor (APAF-1), are necessary for caspase activation in apoptosis due to neglect or cell damage (Zou et al., 1997). Cell neglect, such as lack of growth factors or cell damage can result in the depolarization of the mitochondrial membrane and the release of cytochrome c (Vander Heiden et al., 1997). Cytochrome c is thought to bind APAF-1 and activate binding to procaspase 9 resulting in autocleavage of the procaspase and thus formation of an active caspase 9 (Srinivasula et al., 1998). It is then believed that caspase 9 activates caspase 3, ultimately resulting in a cascade of caspase activity (Hakem et al., 1998).

Caspase activation due to TNFR family mediated apoptosis (death in otherwise healthy cells) initiation proceeds by a different mechanism. TNFR family members that promote death have a homologous cytoplasmic region termed the death domain (DD) (Nagata, 1997; Tartaglia et al., 1993). The receptors include FAS (CD95), TNFR-I and the death receptors DR3, 4 and 5. The DD allows recruitment of adaptor molecules that can directly bind caspase 8 (Boldin et al., 1996; Muzio et al., 1996). Receptor

crosslinking via ligand binding is then thought to promote the cleavage of procaspase 8 into its active form, followed by activation of the caspase cascade. Mitochondrial membrane depolarization and the release of cytochrome c typically occurs in this death pathway, but it is not essential. Therefore it appears that there are at least two distinct mechanisms for initiating the caspase cascade, one that is dependent on the mitochondrial release of cytochrome c for caspase activation and another that can directly activate caspases via receptor ligation.

Apoptosis Inhibition (Bcl-2 Family of Proteins)

Before the discovery of caspases the homologue for the *C. elegans* cell survival protein CED-9 was discovered. As previously stated, a gene translocation in B cell follicular lymphoma resulted in the discovery of Bcl-2. It was later discovered that CED-9 and Bcl-2 shared sequence and functional homology (Hengartner and Horvitz, 1994). Prior to this observation it was discovered that Bcl-2 could functionally replace CED-9 as a cell survival factor in *C. elegans* (Vaux et al., 1992). Bcl-2 is part of a large family of proteins. The proteins are defined as having at least one of four Bcl-2 homology domains (BH1, 2, 3 or 4). Interestingly, several members of the family have anti-apoptotic potential, whereas others can induce apoptosis. Family members that promote survival (e.g. Bcl-2 and Bcl-X long isoform; Bcl-X_L) contain each of the BH domains. Death promoting members (e.g. Bax, Bad and Bcl-X short isoform; Bcl-X_S) vary in the number of BH domains. Rather than functioning independently, it is commonly accepted that the ratio of pro-death versus anti-death factors determines the activity. It is thought that heterodimerization, between pro-apoptotic and anti-apoptotic proteins, disrupts activity

by disturbing homodimerization. Therefore, the protein in excess will form functional homodimers. It is also believed that the majority of Bcl-2 family members are targeted to membranes via a transmembrane tail (Nguyen et al., 1993). In fact early observations suggested that Bcl-2 elicited its effects by maintaining the mitochondrial membrane potential.

The exact function of the Bcl-2 family members has not been fully elucidated, but in recent years progress has been made. In 1996 the structure of Bcl-X_L was determined using x-ray crystallography and nuclear magnetic resonance (Muchmore et al., 1996). This led to speculation that Bcl-X_L could potentially control mitochondrial membrane homeostasis via pore formation due to structure similarities to bacterial toxins that function by forming membrane pores. Since then Bcl-X_L, as well as Bcl-2 and pro-apoptotic Bax, have been shown to form ion channels in synthetic lipid bilayers (Minn et al., 1997). Functionally, Bcl-2 has been shown to maintain mitochondrial polarization and block the release of cytochrome c into the cytosol (Marchetti et al., 1996). Bax, on the other hand, promotes mitochondrial membrane depolarization and cytochrome c release from the inner mitochondrial membrane space (Jurgensmeier et al., 1998). Observations that Bax-regulated release of cytochrome c could occur prior to membrane depolarization had also been made. This suggested that perhaps Bax might function as a channel for cytochrome c release, but this has not been proven and remains a subject of debate. Although the mechanism of action of these proteins have not been completely defined, it has become widely accepted that the Bcl-2 family members do play an important role in regulating mitochondrial activity in apoptosis.

Recent studies on the regulation of Bcl-2 family members, have provided evidence that their function can be modulated by intracellular signaling cascades. It was commonly believed that the major regulatory mechanism was via expression levels of the anti and pro-apoptotic molecules. Studies on the pro-apoptotic member, Bad, have indicated phosphorylation as another regulatory mechanism (Zha et al., 1996). Bad was discovered in 1995 (Yang et al., 1995). It varies from other pro-apoptotic family member in that it contains only the BH3 domain and does not have a membrane binding motif. Bad has been shown to be phosphorylated on the BH3 domain. This results in association with the cytosolic protein 14-3-3. Further investigation showed that in an IL-3 dependent cell line, IL-3 addition resulted in activation of the protein kinase AKT (or protein kinase B) (Datta et al., 1997). AKT activation led to the phosphorylation and cytosolic sequestration of Bad by 14-3-3. Removal of IL-3 resulted in the dephosphorylation of Bad and subsequent heterodimerization with Bcl-X_L. This resulted in inactivation of Bcl-X_L function and allowed for Bax homodimerization and pro-apoptotic function. Interestingly, the cytokine IL-4 has also been shown to activate AKT and promote thymocyte survival (Cerezo et al., 1998). A proposed model of Bad function is presented in Figure 1.7. These fairly recent findings stimulate the hypothesis that perhaps cytokine induced survival may at least in part proceed by the inactivation of Bad activity. Cytokine induced survival will be discussed further later in this chapter.

In addition to phosphoryl-regulation through Bad, direct phosphorylation of Bcl-2 may be another regulatory mechanism. The Raf-1 serine/threonine kinase has been shown to associate with Bcl-2 at the mitochondrial membrane (Wang et al., 1994). It can phosphorylate Bcl-2 leading to its inactivation. This presents an interesting paradox,

since the Raf-1 activating MEK pathway promotes cell survival, rather than cell death. The SAPK pathway has also been indicated in Bcl-2 phosphorylation (Maundrell et al., 1997). Considering the death promoting activity of the SAPK pathway, inactivation of Bcl-2 is a logical progression. It is clear that the regulation of Bcl-2 family members via cell signaling cascades has not been fully elucidated and will remain an active area of research.

Bcl-2 Family Members in B Cell Lymphopoiesis

The role of Bcl-2 family members in the development of mammals has been addressed by creating gene knockout mice. Both Bcl-2 -/- and Bcl-X_L -/- mice result in lethality (Kamada et al., 1995; Motoyama et al., 1995; Veis et al., 1993). Bcl-2 -/- mice die around three weeks after birth as a result of renal failure. In contrast, Bcl-X_L knockout mice die prior to birth at approximately embryonic day 13. Lethality appears to be due to faulty development of the brain. The immune system in both models also exhibits dysregulation. In Bcl-X_L -/- mice, lymphocyte development in the embryonic liver is blocked. To determine the effects of Bcl-X_L on lymphopoiesis, Bcl-X_L deficient embryonic stem cells were used to generate chimeric mice (Ma et al., 1995). In this case, survival of developing thymocytes and B lymphocytes was dramatically reduced. The Bcl-2 -/- mice initially generated mature B and T lymphocytes, but by three weeks of age both B and T cells were significantly reduced. These results suggested that both Bcl-X_L and Bcl-2 were independently involved in generating functional lymphocytes. Bcl-X_L appears to play a significant role in early generation of lymphocytes, whereas Bcl-2 functions in survival after the initial generation of the lymphoid compartment.

The expression of Bcl-2 in murine B lymphocytes was shown to correlate with developmental populations that were shown to be resistant to apoptosis *in vitro*. High levels of Bcl-2 were shown to be present in pre-pro and mature B lymphocytes from the BM; early-pro B cells showed lesser, yet significant expression levels (Li et al., 1993; Merino et al., 1994). These and other experiments have indicated that normal *in vitro* apoptosis and dex-induced apoptosis is greatest in pre B cells (Garvy et al., 1993; Nunez et al., 1990). In transgenic mice and B cell lines with Bcl-2 expression induced throughout the developmental stages, the pre B cells were resistant to apoptosis. This indicated that Bcl-2 was likely important to normal B cell development and that its varied expression might be important in selection processes. Although some *in vivo* studies by our lab had looked at B lymphocyte sensitivity in IgM^- verses IgM^+ B cells (as described earlier), the more defined B lymphocyte populations had not been studied (Garvy et al., 1993). Therefore this thesis addresses the sensitivity the various specific B lymphocyte developmental substages to Cs elevation *in vivo*, to determine if cell populations that express Bcl-2 are more resistant to losses.

Cytokine-Withdrawal Induced Apoptosis

In multicellular organisms each cell depends on signals from its environment to survive. The context in which a cell is maintained provides for the homeostasis and identity of the cell. When a cell is removed from its environment, and functioning properly, it undergoes apoptosis. It has been proposed that each cell contains the necessary components to undergo apoptosis and that extracellular signals are critical for maintaining survival signals that repress the death process (Raff, 1992). This theory was

developed by observations that removing cells from their environment or by blocking gene transcription results in apoptosis. A classic model for this has been observed in immune cells, by withdrawal of cytokines that are necessary for survival (Boise et al., 1993; Rebollo et al., 1995; Sohur et al., 2000). This is a commonly observed form of apoptosis termed factor-withdrawal cell death. The recent discovery of IL-4 induced suppression of Bad activity via the PI3 Kinase/AKT kinase pathway, indicated that cytokine receptors may function as survival signals by a common mechanism. Recent studies had also shown that both IL-3 and IL-7 could induce translocation of the pro-apoptotic Bax protein from the cytosol to the mitochondrial membrane (Khaled et al., 1999). It has been proposed that the removal of these cytokines in relevant cell types resulted in an intracellular increase in pH that caused a conformational change in Bax, allowing for its membrane binding and ultimately apoptosis. In addition to pro-apoptotic actions following withdrawal, the presence of several cytokines (including IL-7) had also been indicated in the upregulation of the anti-apoptotic Bcl-2 protein (Dancescu et al., 1992; Karawajew et al., 2000). These observations suggested that cytokine signals can result in direct activation of proteins involved in the control of apoptosis, yet the ability of cytokines to protect cells from apoptosis induction from other sources had not been studied in depth. This thesis will explore the potential of IL-7 and SCF to protect normal BM B lymphocytes from factor-withdrawal induced apoptosis and Cs-induced apoptosis.

Another molecule that appeared to have significant anti-apoptotic function via cytokine signaling was STAT5 (signal transducers and activators of transcription) (Nosaka et al., 1999; Rosa Santos et al., 2000). In studies using STAT5 deficient B and T cell lines, the readdition of the molecule resulted in the upregulation of c-myc, Bcl-2 and

Bcl-x (Lord et al., 2000). STAT5 activation is promoted by signaling through the janus kinase 3 (Jak3) phosphorylation pathway. The IL-2 common γ receptor had been shown to recruit and activate Jak3 upon ligand binding, suggesting that the cytokines that share this domain (IL-2, IL-4, IL-7, IL-9 and IL-15) may send this common signal, promoting cell survival. This was also suggested by the lymphoid deficiencies observed in Jak3 knockout mice, since these mice are deficient in B cell lymphopoiesis. (Thomis et al., 1997). It therefore appeared that certain cytokines, especially those sharing the common γ chain receptor, could potentially promote survival by inhibiting apoptotic factors and promoting anti-apoptotic factors.

Glucocorticoid induced Apoptosis

Although Gc-induced apoptosis has been studied for decades, the exact mechanism by which these steroids induce death in cells of the immune system has not been elucidated. This may be because it affects several cell survival and death processes within the cell. The biological effect of Gc on immune cells was studied long before apoptosis became an active area of interest. It was observed that Gc downregulated metabolism in lymphoid cells. Cells treated with Gc showed decreased glucose uptake, decreased intracellular ATP levels and down regulation in protein and nucleotide synthesis was observed (Montague and Cidlowski, 1995). Although a general decrease in transcription and translation was observed, Gc was also known to enhance the transcription of many genes (reviewed in (Burnstein and Cidlowski, 1989). Experiments that blocked Gc-induced transcription by coaddition of actinomycin D or protein synthesis with cycloheximide, eliminated Gc-induced death in thymocytes (McConkey et

al., 1989). This led to the hypothesis that Gc-induced apoptosis caused, at least in part, by inducing what were termed "death genes". Many efforts were put into identifying and defining genes that were upregulated or downregulated in Gc-induced apoptosis. Further investigation was not able to indicate any one of these genes as sufficient or central to cell death.

More recent efforts have been applied to determine how Gc may induce apoptosis by affecting specific signaling pathways. Using mouse thymocytes, studies have indicated that ceramide release, by sphingomyelinase (Smase) activity, may be one way that Gc effects death (Cifone et al., 1999). One group used several indirect methods to inhibit Smase activity, thus inhibiting ceramide production and apoptosis. In addition, they showed that blocking phosphatidylinositol phospholipase C (PI-PLC) activity blocked the activation of Smase and ceramide production and apoptosis. These events were shown to be upstream of GcR mediated transcription. This data contrasts other observations indicating that Gc-induced apoptosis is dependent on GcR mediated transcription (McConkey et al., 1989). One transcriptionally regulated pathway that has been suggested in the mediation of Gc induced apoptotic effects is the NFκB pathway. It has been shown that downregulation of NFκB activity and one of its gene targets, c-myc, by GcR mediated upregulation of IκB may be another mechanism by which Gc induces apoptosis. Overexpression of NFκB or c-myc in dex treated thymocytes resulted in a decrease in Gc-mediated apoptosis (Wang et al., 1999). Clearly, more research is needed to fully elucidate the mechanism of Gc-induced apoptosis, but it is likely that the mechanism will involve multiple intracellular processes.

Summary

Over the past two decades the study of apoptosis has shown that cell death is critical in the homeostasis of multicellular organisms. Intensive research efforts were applied to discovering the mechanism of this process. In the currently proposed models, both caspases and mitochondrial dysregulation are the effectors of cell death. Mitochondrial release of cytochrome c can induce the activation of caspases due to death initiated by damage or neglect. Caspase activation is not required for death in this case, but normally occurs and provides the apoptotic phenotype. Death via death receptor ligation can directly initiate the caspase cascade and death, but mitochondrial release of cytochrome c enhances this process. There is also a family of intracellular proteins (the Bcl-2 family) that have either pro-apoptotic or anti-apoptotic function. It is believed that they function as regulators of the mitochondrial membrane. Less is known about how cell death is initiated. Factor withdrawal induced death is one form of apoptosis commonly observed. Certain cytokines (such as IL-4) can promote survival by inhibiting the expression and activity of pro-apoptotic proteins. Their withdrawal then allows the activation of pro-apoptotic proteins and results in the downregulation of anti-apoptotic proteins. Glucocorticoid induced apoptosis induces death as seen in neglected or damaged cells. Developing B and T lymphocytes are two cell types that undergo massive apoptosis due to Gc exposure. It is thought that this may play a role in regulating negative selection of these cell types during lymphopoiesis. Current evidence suggests that Gc-induced apoptosis is a complex process that likely occurs via mediating transcription of genes directly involved in the death process, by downregulating survival pathways and by inducing other signaling pathways to induce death. Here Cs-induced

apoptosis in B lymphocytes will be thoroughly studied *in vitro*. Additionally, the effect of other factors, such as cytokine signaling by IL-7 and SCF or the effect of stromal cells as a whole, will be investigated for the ability to modify Cs action on the developing B cells. This is a whole new approach to studying Cs-induced death in normal B cells that explores how survival-inducing and death-inducing pathways affect B lymphocyte responses when they are co-initiated.

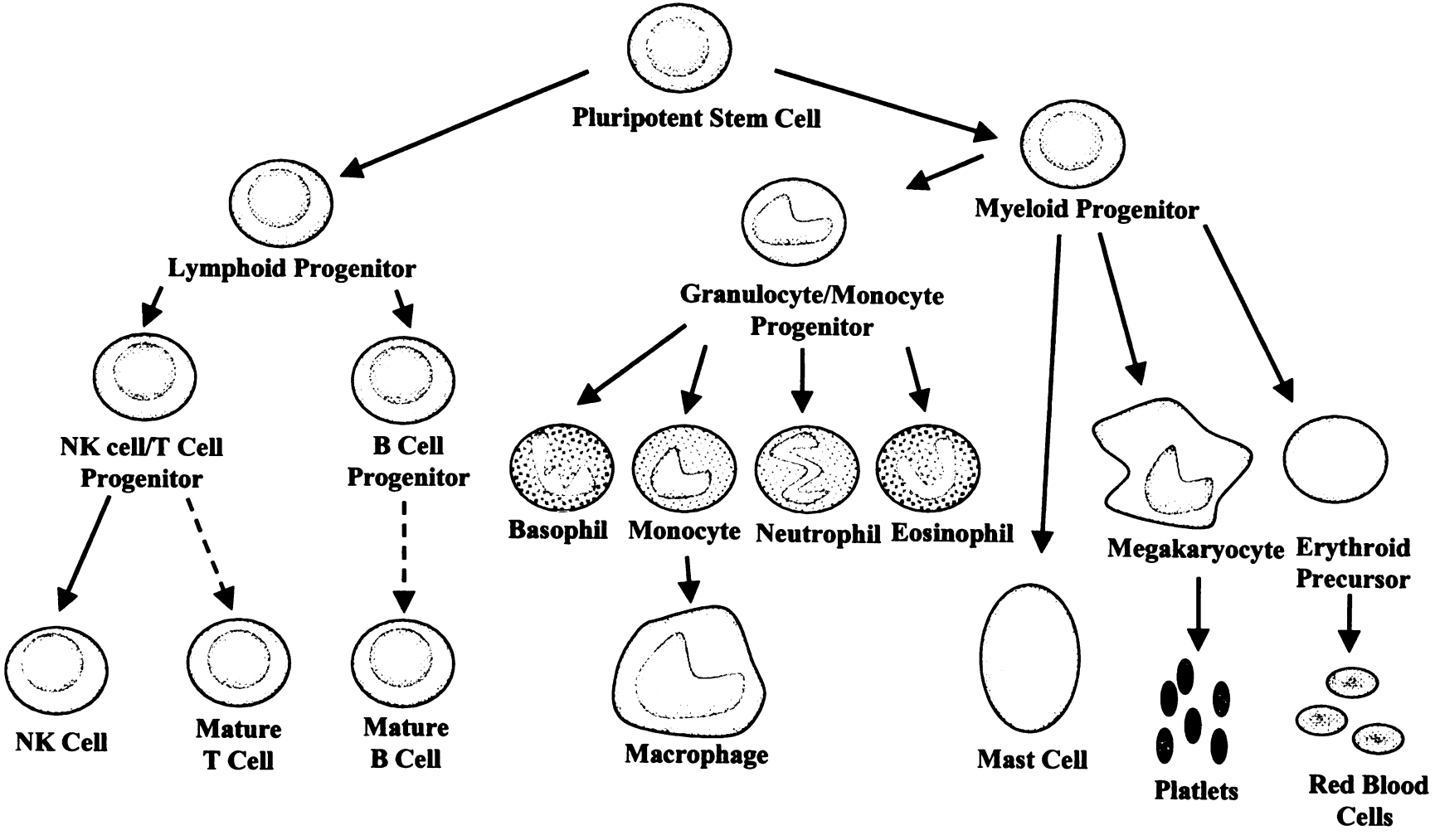


Figure 1.1 Differentiation of various blood cell lineages from a pluripotent stem cell

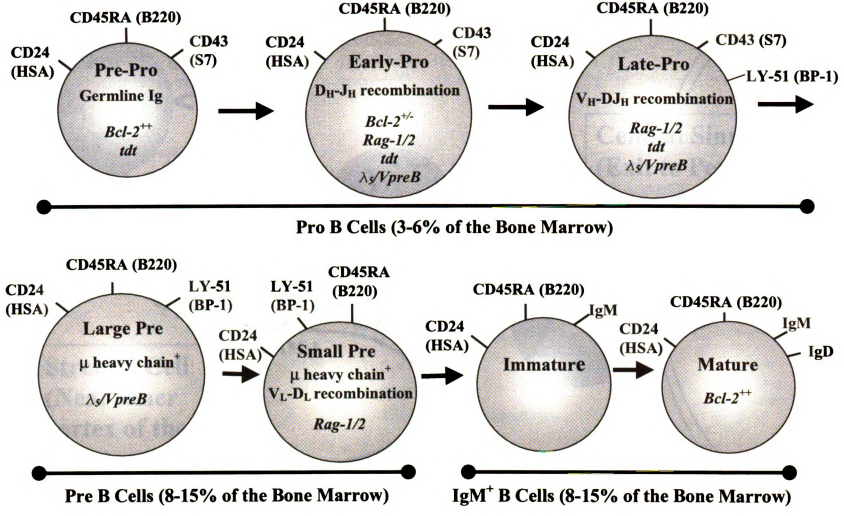


Figure 1.2 Bone marrow B lymphoycte development scheme

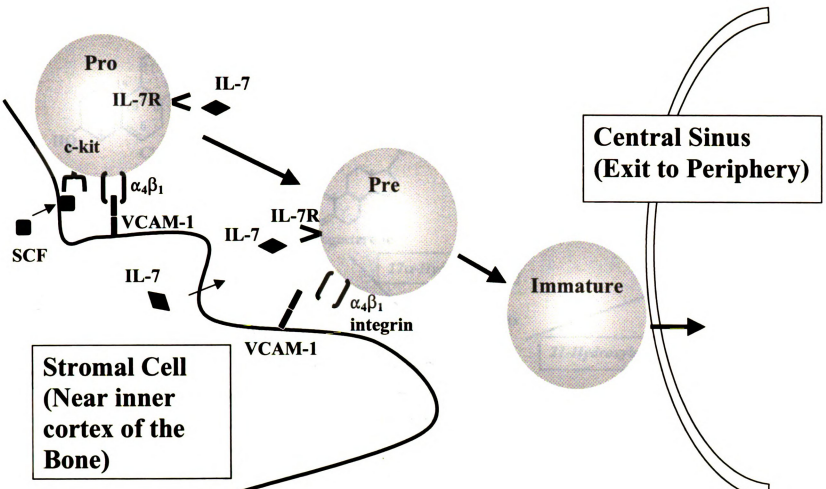


Figure 1.3 Developing B lymphocyte association with stromal cells in the bone marrow

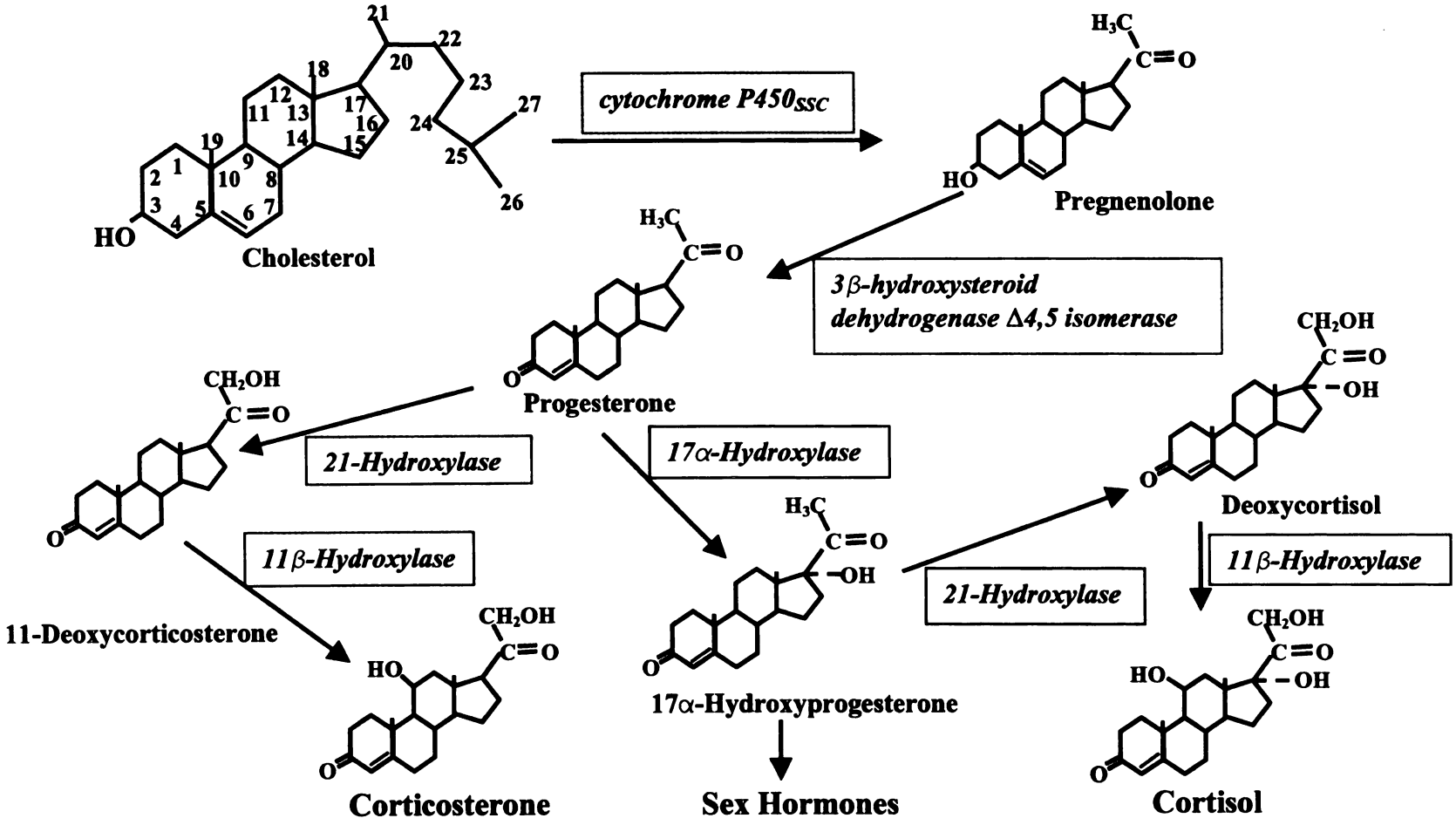
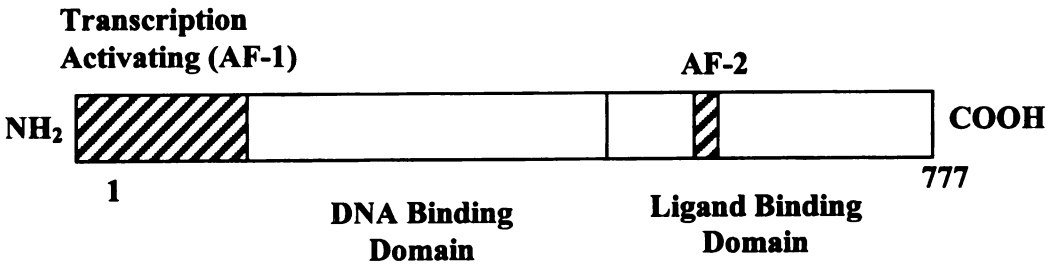


Figure 1.4 Corticosteroid biosynthesis

A) Receptor Structure



B) Receptor Activation

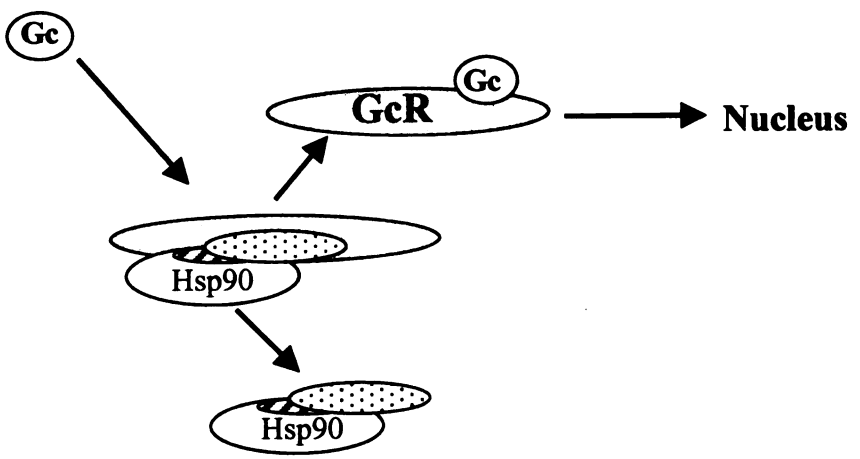


Figure 1.5 The glucocorticoid receptor

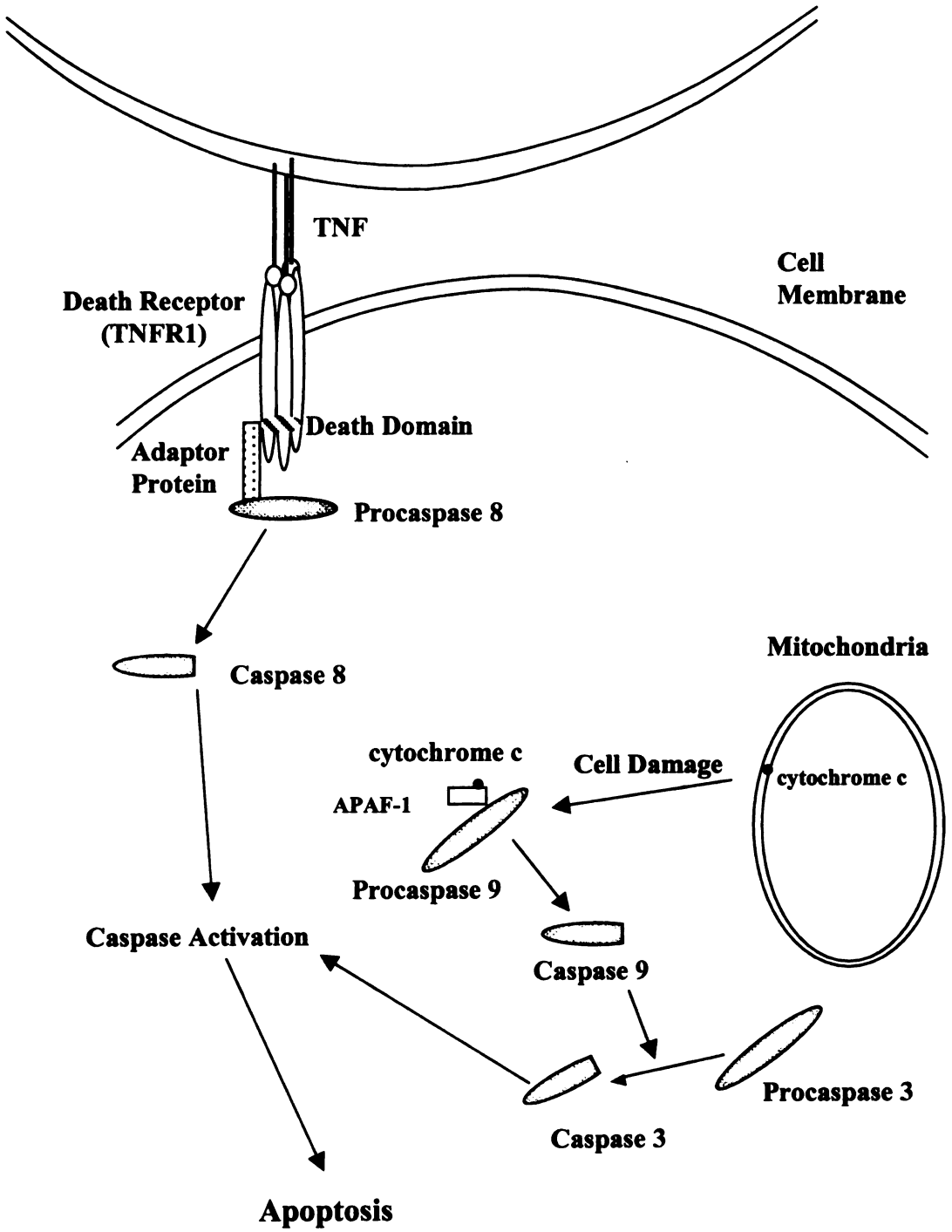
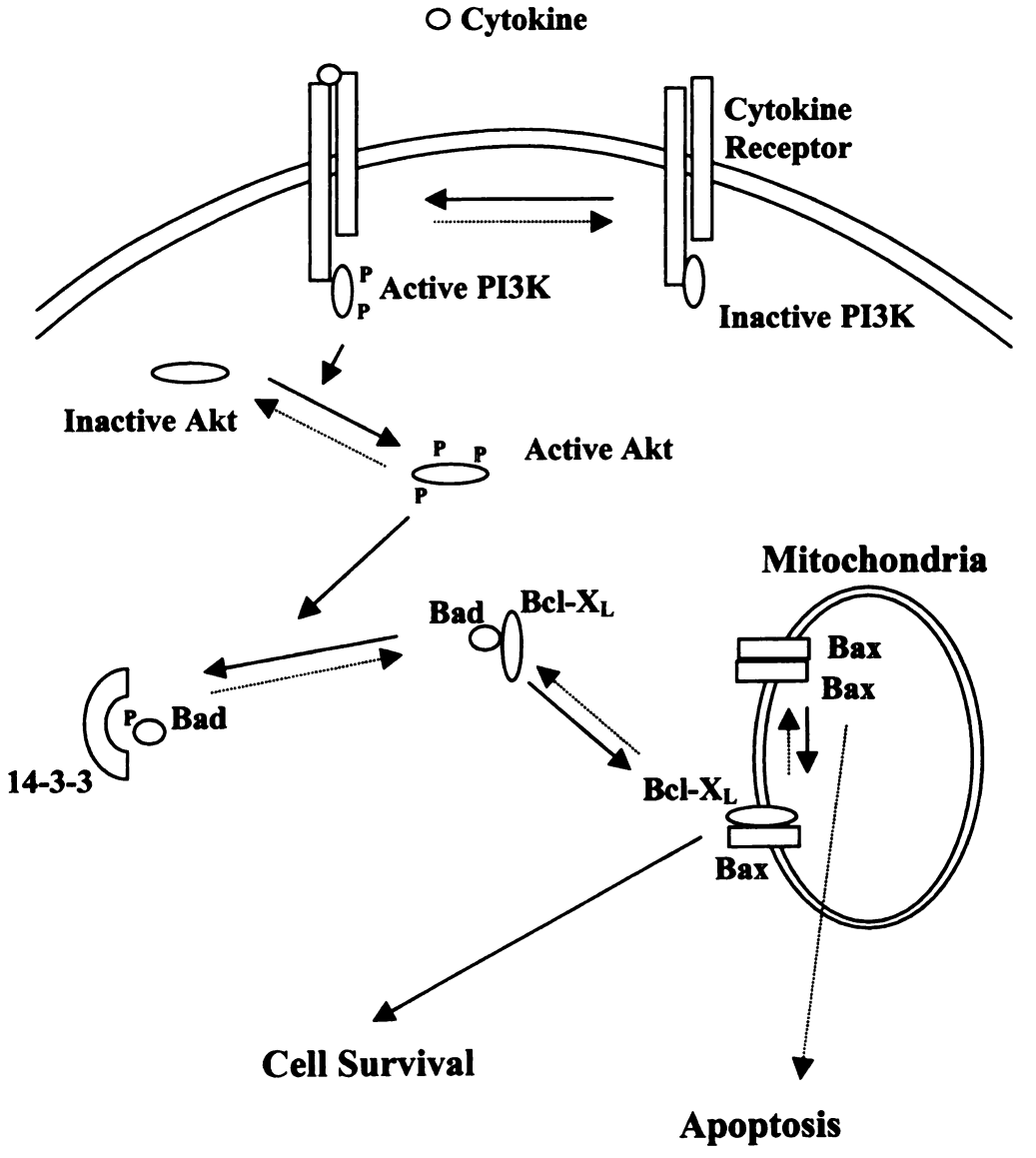


Figure 1.6 Apoptotic pathways



..... = No Survival Promoting Cytokine

——— = Survival Promoting Cytokine

Figure 1.7 Cytokine survival via Bad inactivation

CHAPTER 2: THE *IN VIVO* EFFECT OF CORTICOSTERONE ON DEVELOPING B LYMPHOCYTES: FROM COMMITTED PROGENITOR TO MATURITY

ABSTRACT

Chronic increases in circulating glucocorticoids (Gc), resulting from activation of the stress axis, can cause decreased levels of circulating B lymphocytes and ultimately compromise host defense. Reduced B cell development in the bone marrow (BM) has been indicated as a mechanism contributing to Gc-induced lymphopenia, yet the affect of Gc on each B cell developmental substage was not known. Utilizing multiparameter flow cytometry, the effect of the natural Gc, corticosterone (Cs), on B lymphocyte development, from progenitor cells to maturity, was investigated. The results showed that a modest elevation in plasma Cs concentrations, analogous to concentrations seen during physiological stress, caused a significant reduction in the BM B cell population as early as 12 hours; by 24 and 36 hours nearly 50% of the developing B lymphocytes were lost. Thirty-six hours after increased exposure to Cs, 80% of the surviving cells were $IgM^{+}IgD^{+}$ mature B cells. The populations exhibiting the greatest losses were those that had been reported to be undergoing active Ig gene rearrangement; the early-pro through the pre B cell stages of development. The earliest committed progenitors, the pre-pro B cells, with Ig genes in the germline configuration, were somewhat resistant to losses, decreasing by only 23% after 24 hours and 34% after 36 hours. Immature B cells did not show significant losses after 24 hours, but were nearly eliminated by 36 hours. The remaining pro and pre B cells, normally cycling populations, decreased in cells in the $S/G_2/M$ phases of the cell cycle by 74% and 85%, respectively. Therefore, Cs appeared to negatively affect developing BM B lymphocytes by severely depleting all but the earliest progenitors of developing B cells and by preventing their expansion by inhibiting

cell cycling. Additionally, analysis of major hematopoietic lineages of the bone marrow indicated that whereas Cs had a negative effect on BM B lymphocytes, it appeared to promote myelopoiesis by expanding the granulocyte compartment. Therefore modest increases in Cs, comparable to concentrations seen during the induction of the neuroendocrine stress axis, can result in the selective reduction of developing B lymphocytes in as little as 12 hours of exposure. Interestingly, after 36 hours the other developing blood cell lineages in the BM were either not affected or actually expanded in response to increased Cs. Thus, it appears that during stress the energy-expensive process of B cell development is downregulated, but perhaps compensated for by an upregulation in the production of cells that provide the first line of immune defense.

INTRODUCTION

There are important physiological and pharmacological reasons for interest in the effect of glucocorticoids (Gc) on the immune system. The natural steroid hormones, like corticosterone (Cs) or cortisol, are produced and released from the adrenal gland at basal levels and can become elevated in response to stress (Selye, 1947). Zinc deficiency, trauma, burns and neuroendocrine diseases are examples of chronic stress that can cause enhanced production of Gc (DePasquale-Jardieu and Fraker, 1980; Raber, 1998). Conversely, synthetic Gc are commonly used at pharmacological concentrations as anti-inflammatory drugs for diseases such as arthritis, asthma and autoimmune diseases. Natural or pharmacological increases in Gc concentrations can cause a decline in the number of peripheral B and T cells. This lab has shown that a major cause of this decrease could be the downregulation of B and T cell development in the primary immune tissue (Garvy et al., 1993). Using Cs implants, Garvy et al, produced concentrations of circulating Cs in mice analogous to those seen during stress, potentially resulting in a compromised host defense. This resulted in a dramatic decrease in thymus weight and a decrease in developing B lymphocytes in the bone marrow. It was also noted that early B lymphocytes, not expressing IgM, were very sensitive to Cs induced losses. However little was known about how Cs affected the various subpopulations of B lymphocytes as they matured from progenitor to maturity. Therefore it was important to determine whether the losses were specific to certain developmental stages or if Cs negatively affected all developing B cells to the same extent.

Such studies have been limited due to the complexity associated with the identification of subsets of cells within the B cell lineage, especially since they reside in a heterogeneous tissue, the bone marrow (BM). All cells of the hematopoietic lineage originate and most mature in the BM of adult mammals. One exception are the T lymphocytes, which are generated in the BM, then migrate to the thymus to mature. B lymphocytes develop entirely in the marrow in adults and comprise approximately 30% of murine BM cells. Over the last decade fluorochrome conjugated antibodies against B cell surface proteins has been utilized for the flow cytometric identification of distinct stages in B lymphocyte development. The B lymphocyte lineage is identified by an antibody that recognizes CD45RA (B220) (Coffman and Weissman, 1981). Using the phenotypic scheme developed by Hardy et al. (Hardy et al., 1991) $B220^+$ B cells can be further subdivided, from the earliest progenitor to maturity, as follows: pre-pro ($S7^+HSA^-BP1^-$), early-pro ($S7^+HSA^+BP1^-$), late-pro ($S7^+HSA^+BP1^+$), pre ($S7^-IgM^-$), immature (IgM^+IgD^-) and mature (IgM^+IgD^+). The status of Ig gene rearrangement has been determined for each of these stages of development (Faust et al., 1993; Li et al., 1993). Pre-pro B cells have Ig genes in a germline configuration; recombination of the heavy chain begins in early-pro and is completed in late-pro B cells. Pre B cells contain a completed heavy chain and undergo light chain gene rearrangements. At the immature stage of development IgM rearrangement is complete and it is expressed on the surface of the cell. This well developed phenotypic system provides the tools needed to better determine the effect of Gc on cells of the B lineage.

During the Ig rearrangement process it is estimated that nearly 80% of developing B cells are lost due to faulty or anti-self recombination events (Osmond, 1986). A

downregulation of the anti-apoptotic protein, Bcl-2, has been observed in cells undergoing Ig recombination, suggesting that induction of apoptosis may be a major mechanism of precursor B cell deletion of unwanted cells in the bone marrow (Li et al., 1993; Merino et al., 1994; Nunez et al., 1990). Only the pre-pro and mature B cells express substantial amounts of Bcl-2, therefore, it was of interest to determine if increased endogenous production of Cs might adversely affect those stages in B cell development that do not express Bcl-2. This would suggest that apoptosis might play a role in the Gc mediated downregulation of B cell lymphopoiesis seen both *in vivo* and *in vitro* (Garvy et al., 1993; Garvy et al., 1993; Merino et al., 1994).

Subdermal implantation of a Cs containing pellet can cause increased concentrations of circulating Cs being analogous to concentrations produced during physiological stress. Previous studies from this lab focussed on the effect of Cs on B lymphocyte after days or weeks of exposure. Beyond two days maximum depletion of B lymphocytes was apparent and remained virtually unchanged for two weeks. Thus the studies here were performed at 12, 24 and 36 hours after Cs implantation, to determine the initial effects of increased concentrations of circulating Cs on developing B lymphocytes and the thymus. A comprehensive investigation of the onset of Cs-induced suppression of B cell lymphopoiesis has not been investigated. These experiments demonstrate that the stages during B lymphocyte development where Ig gene rearrangement is occurring, undergo dramatic cell losses after just a few hours of exposure to steroid. Indeed losses began as early as 12 hours in early-pro and pre B cell populations and maximum effects were seen in these and late-pro and immature populations by 36 hours. In contrast, the populations that had been shown to contain

significant levels of Bcl-2 (pre-pro and mature B cells) were more resistant to losses due to chronic exposure to Cs. Therefore in the time period of 12 to 36 hours the negative effect of Cs on B cell lymphopoiesis in mice was dramatic.

Previous *in vitro* studies have shown that Gc causes lymphoid cells to undergo cell cycle arrest in G_0/G_1 (Andreau et al., 1998; King and Cidlowski, 1998). Pro and pre B cells normally undergo expansion, whereas the immature and mature B cells are quiescent in the bone marrow. Although a decrease in cycling IgM^- precursors after prolonged Cs exposure was previously noted, the initial affect on the individual pro and pre B cells was not investigated. These experiments show a rapid and similar decrease in cycling of surviving pro and pre B cells. Therefore in addition to inducing B cell losses, Cs also rapidly reduced cell proliferation.

Interestingly the BM cellularity did not change subsequent to exposure to Cs, yet there was a significant decrease in B lymphocytes. It seemed probable that other cell lineages were expanding during chronic exposure to Cs. Using a recently defined phenotypic labeling system, the cellular composition of key populations of cells of the marrow was determined (de Bruijn et al., 1994). Differential expression of the cell surface proteins ERMP12 (CD31) and ERMP20 (CD59), allows for the delineation of granulocytes, monocytes, hematopoietic progenitors, erythroid progenitors and lymphocytic cells. Interestingly, mice with Cs implants had a significant increase in the percent of granulocytic cells concomitant with a decrease in lymphocytic cells whereas the other lineages appeared unaffected. These observations suggested that Cs, while downregulating B cell development, might promote the generation of granulocytic cells. Therefore a reduction in B cell development can occur within 12 hours of modest

physiological increases in Cs. After 24-36 hours B cell development is nearly completely eliminated except for the earliest progenitors, yet the development of the other blood cell lineages is not downregulated and in some cases their development is upregulated. Thus it appears that very early after the induction of the stress axis the development of blood cells shifts from lymphopoiesis towards myelopoiesis.

MATERIALS AND METHODS

Mice and Cs Implantation

Young adult male Balbc/J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and were used from 8 to 12 weeks of age. Mice were housed in the laboratory animal facility in the Biochemistry department and all protocols were approved by the University Animal Use Committee at Michigan State University, East Lansing, MI. The facility was maintained at 25°C with 12-hour light and dark cycles.

Methoxyflurane inhalation was used to anesthetize the mice and tablets containing 20 mg corticosterone (Sigma, St. Louis, MO) and 20 mg cholesterol were implanted subcutaneously. Sham controls received tablets containing 40 mg of cholesterol. Post-surgery the mice were housed in the animal facility on sterile bedding and fed commercial rodent chow (Purina, St. Louis, MO) and acidified water.

Tissue Harvest and Processing

At 12, 24 or 36 hours after tablet implantation, mice were bled under anesthesia and sacrificed by cervical dislocation. The plasma was separated from the blood and Cs concentrations were determined as previously described (DePasquale-Jardieu and Fraker, 1980). Thymuses were removed to determine the amount of thymic atrophy by weight. Bone marrow was flushed from femurs with approximately 1 ml harvest buffer (Hanks' balanced salts, 1 mM HEPES pH 7.2 and 4% FBS) using a syringe and 22 gauge needle. The marrow was processed into a single cell suspension and red blood cells were removed by lysis. The cells were then washed in harvest buffer, resuspended in 0.5 ml

label buffer (Hanks' balanced salts, 1 mM HEPES pH 7.2, 0.1% sodium azide and 2% FBS) and placed on ice. Cell counts were performed and trypan blue exclusion was used to determine bone marrow cell number and viability. Following cell counts, two million cells were aliquoted into 5 ml polystyrene tubes (Becton Dickinson, Franklin Lakes, NJ) for phenotypic analysis.

Immunophenotyping and DNA Staining

Three separate phenotypic protocols were used to determine the various stages in B lymphocyte development. All antibodies were used at a dilution predetermined to provide optimum labeling. To identify pro, pre and IgM^+ cells the following antibodies were used: phycoerythrin (PE) conjugated rat anti-mouse CD45RA (B220), fluorescein (FITC) conjugated rat anti-mouse CD43 (S7) and biotinylated (biotin) goat anti-mouse IgM $F(ab')_2$ (IgM) were added simultaneously to cell aliquots. Antibodies against B220 and S7 were purchased from Pharmingen (San Diego, CA) and anti-IgM was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Cells were incubated for thirty minutes, then washed two times with label buffer. The fluorochrome red 670 (R670) conjugated to streptavidin (Av) (Gibco, Grand Island, NY) was added to cells for conjugation to biotin-anti-IgM. Cells were incubated for 20 minutes, washed and fixed with 1 ml of 1.25% paraformaldehyde for 40 minutes at room temperature. For DNA staining cells were washed with label buffer two times and resuspended in 0.5 ml 1μg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) and incubated for at least one hour at room temperature. For the identification of immature and mature B cells PE conjugated anti-B220, biotin conjugated anti-IgM and FITC conjugated rat anti-mouse

IgD (Pharmingen, San Diego, CA) were added simultaneously. Samples were incubated for 25 minutes, washed and incubation with Av-R670 for 20 minutes. Following phenotyping cells were fixed with paraformaldehyde and stained with DAPI, as described above.

To identify the pro B cell subsets (pre-pro, early-pro and late-pro) four-color phenotypic analysis was used. The antibodies (all purchased from Pharmingen) were as follows: PE conjugated anti-B220, FITC conjugated anti-S7, biotin rat-anti-mouse CD24 (HSA) and purified rat anti-mouse LY-51 (BP-1, 6C3 clone). Cells were first incubated with anti-BP-1 for 30 minutes and washed. An aminomethylcoumarin (AMCA) conjugated goat anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was added for identification of the BP-1 primary antibody. Cells were washed two times and 10 μg of Rat Ig was added for 10 minutes to block any unbound anti-IgG antibody. The antibodies against B220, S7 and HSA were then added at their appropriate dilutions and the cells were incubated for 25 minutes and washed. Av-R670 was then added for conjugation to HSA-biotin. Following labeling and washing, the cells were fixed with 0.5 ml of 1.25% paraformaldehyde and stored at 5°C until flow cytometric analysis.

To determine the composition of the major cell lineages within the BM, additional antibodies were used to determine the percent of granulocytes, monocytes, lymphocytes (as a whole), hematopoietic progenitors and erythroid progenitors. For this purpose biotinylated rat anti-mouse CD31 (ERMP12) and FITC conjugated rat anti-mouse CD59 (ERMP20), (Bachem, King of Prussia, PA) were utilized. Differential surface expression of CD31 and CD59 on the various cell lineages allowed for their identification as

follows: granulocytes (ERMP12$^-$ERMP20^{moderate}), monocytes (ERMP12^{moderate}ERMP20^{bright}), lymphocytes (ERMP12^{moderate}ERMP20$^-$), hematopoietic progenitors (ERMP12^{bright}ERMP20^{moderate}) and erythroid progenitors (ERMP12$^-$ERMP20$^-$). To label BM cells, ERMP12 and ERMP20 were added simultaneously to 2 X 10^6 cells on ice at a predetermined dilution. Samples were incubated for 30 minutes then washed with label buffer. Following phenotyping, cells were fixed by the addition of 0.5 ml 1.25% paraformaldehyde and stored at 5°C until FACS analysis

Flow Cytometry and Data Analysis

Samples were analyzed on a Becton Dickinson FACS Vantage flow cytometer. FITC, PE and R670 fluorochromes were excited at 488 nm, and emission was detected at 530, 575 and 670, respectively. DAPI and AMCA were excited at 365 nm and emission was detected at 470 nm and 450 nm, respectively. To reduce the spectral overlap of fluorochromes voltage compensation was performed by subtracting non-specific fluorescence from each wavelength analyzed.

For the 3-color phenotypic and DNA analysis, debris and cellular aggregates were excluded from analysis by gating using size and DNA content. Cell size was determined by forward and side light scatter and DNA content was determined by a DAPI pulse processed width versus area signal; cells with hypodiploid DNA were considered apoptotic, as previously described (Telford et al., 1991). A region was drawn around $B220^+$ cells to identify the B lymphocytes of the marrow and depending on the antibody combination used, the $B220^+$ subpopulations were determined as follows: pro B

($S7^+IgM^-$), pre B ($S7^-IgM^-$), immature (IgM^+IgD^-) and mature (IgM^+IgD^+) B cells. Isotype-matched samples were used as negative controls.

To determine the subpopulations of pro B cells, the 4-color phenotypic samples were analyzed by flow cytometry. Cells were gated based on size to exclude debris and aggregates. To define pro B cells a region was drawn around $B220^+S7^+$ cells and then analyzed as follows: pre-pro (HSA^-BP1^-), early-pro (HSA^+BP1^-) and late-pro (HSA^+BP1^+). The percent of each population was determined and isotype-matched control samples were used to determine negative fluorescence.

Most samples were run in duplicate and averaged. Data was processed and analyzed using PC-LYSIS (Becton Dickinson) and WinList (Verity Software House, Inc., Topsham, ME) software. Microsoft Excel was used for statistical analysis and graphing.

Statistics

The Student's t-test was used to determine significant differences ($p < 0.05$) between experimental and sham mice where mean data ± the standard deviations were reported.

RESULTS

Plasma Cs Concentrations, Thymic Weight and BM Cellularity

Under conditions of chronic stress in mice the plasma Cs increases 2 to 10-fold above concentrations seen in non-stressed animals. Therefore the concentration of plasma Cs was determined for both sham-control mice and mice receiving a Cs-implant (Figure 2.1). Mice containing the Cs tablet showed increased concentrations of plasma Cs at 12 hours of approximately 95 μg/dl. At 24 and 36 hours serum Cs plateaued between 60 and 70 μg/dl. These concentrations of Cs are consistent with those seen under conditions of stress (Osati-Ashtiani et al., 1998). The slightly higher concentration of Cs, apparent in sham mice at 12 hours, is likely due to some contribution of steroid due to surgical stress.

Thymic atrophy is a common observation under conditions of chronic increases in circulating Cs (Garvy et al., 1993). The thymuses of mice that underwent a sham surgery with a cholesterol tablet implant or mice that had a Cs-containing tablet implant were weighed to determine the affects on thymic integrity. Much of the thymus is composed of developing T cells and this lab has shown that a decrease in weight is often correlated with a decrease in thymic cellularity (personal communication, King and Fraker). Figure 2.2 shows the thymic weights for each mouse. As early as 12 hours after surgery, the thymus of mice containing the Cs tablet implant had decreased by 25%. After 24 and 36 hours thymic weight had decreased 54% and 69%, respectively. This is of interest since BM, in contrast, did not decrease in total cell numbers. Table 2.1 shows the total cellularity of mouse femurs 12 and 36 hours after sham or Cs implantation. No

significant change in BM cellularity was seen and the absolute cell numbers did not change in the BM of mice exposed to Cs. Since the BM is composed of many different cell lineages, this suggested that the reduction in a population such as B lymphocytes might have been compensated for by an expansion in another cell lineages.

Significant Changes in B-Lineage Cells of the Bone Marrow Induced by Cs Exposure

Although distinct stages in B lymphocyte development have been defined based on surface expression of proteins, Ig gene rearrangement status and the expression of proteins relevant to B cell lymphopoiesis, the *in vivo* effect of Cs on the various stages from committed progenitor to maturity had not been assessed. Figure 2.3 and Figure 2.4 show the dramatic changes in the composition of cells of the B lineage subsequent to only a few hours of exposure to Cs. Figure 2.3 shows actual flow cytometric profiles of the various B lymphocyte developmental populations 24 hours following implantation of a sham or Cs tablet. Chronic Cs exposure resulted in a decrease of almost 60% in pre B cells and a decrease of about 55% in pro B cells. Further analysis of the pro B cell substages showed that pro B cell response to Cs varied between the populations. Pre-pro B cells were relatively resistant to losses, decreasing by only 17%. Early-pro B cells, in contrast, showed extensive losses, decreasing 70% and late-pro B cells decreased 33%. Analysis of the immature and mature B cell populations indicated that, while immature cells were not substantially different from the negative control, mature cells actually increased to 164% of the proportion noted in sham mice. These profiles are from representative mice and indicate that the pre B cells (where Ig light chain rearrangement

is initiated) and early-pro B cells (where heavy chain rearrangement is initiated) undergo dramatic losses in less than 24 hours of exposure to somewhat modest increases in Cs that were analogous to those produced by the stress axis.

The effect of chronic exposure to Cs on the composition of the B lymphocyte developmental substages over the course of 36 hours is shown in Figure 2.4. The earliest committed B cell progenitors, the pre-pro B cells, were not significantly affected by enhanced exposure to Cs in the first 12 hours. Relative to the other precursor populations the decrease in these cells after 24 or 36 hours of 23% and 34%, respectively, were modest. Early-pro B cells, in contrast, were dramatically decreased by 60% after only 12 hours of heightened Cs exposure. These cells were nearly eliminated, at 24 and 36 hours, decreasing by approximately 80%. The next developmental stage, the late-pro B cells, did not show significant decreases until 24 hours post tablet implant. At 24 a decrease of 62% was observed and they continued to decrease by 72% through 36 hours. The pre B cells, like the early-pro B cells, were significantly decreased after only 12 hours of Cs elevation. They continued to decrease by 70% and 94% after 24 and 36 hours, respectively. Immature B cells were somewhat resistant to losses during early exposure, not showing significant decreases until 36 hours post-implantation. At 36 hours, these cells were nearly eliminated, they decreased from approximately 7% of the BM population to less than 1% of the BM. Interestingly, mature B cells were not adversely affected by Cs and as early as 12 hours after heightened exposure to Cs these cells actually increased in the BM. They continued to increase through 36 hours, where experimental mice displayed nearly 3-fold more mature B cells in their BM compared to sham controls. Mature B cells in sham mice made up around 4% of the BM, whereas

they made up 12% of the marrow in Cs-treated mice. Clearly, Cs severely affected the development of B lymphocytes in the BM. Early-pro, late-pro, pre and immature B lymphocytes were so dramatically decreased that together they made up less than 3% of the BM as compared to 24% of the BM in sham mice. In contrast the pre-pro B cells were somewhat resistant to losses and mature B cells actually increased dramatically in the BM. Since the total cellularity of the bone marrow of sham and experimental mice did not change these increases represent actual increases in the overall cell numbers for mature B cells in the bone marrow. Therefore Cs exposure can begin to negatively affect B cell lymphopoiesis as early as 12 hours and maximum losses were noted by 36 hours.

Cs Induced Changes in Cell Cycle Status of Pro and Pre B Lymphocytes

The reduction in the B cell compartment of the marrow was largely due to losses in numbers of developing cells, but a decrease in proliferation of the remaining cells might have also played a role. To investigate this, the cycling status of pro and pre B cells (IgM^+ B cells have very few cells in a cycling state) after Cs exposure was determined. For this purpose the BM was phenotyped with DAPI to determine the DNA content. A dramatic decrease in the percent of pro and pre B cells in the $S/G_2/M$ phases of the cycle was observed 24 hours after Cs implantation. Figure 2.5 shows both the change in the cell cycle distribution of these cells as compared to sham mice as well as the overall percentage of cells in the $S/G_2/M$ phases for both treatment groups. The average percent of pro B cells in the $S/G_2/M$ phases of the cell cycle in sham controls was $25.5 \pm 2.8\%$ whereas mice exposed to increased Cs had only $6.2 \pm 0.2\%$ cells in the cycling phases (a 75% decrease). The remaining pre B cells displayed a dramatic 84%

decrease in cells in the $S/G_2/M$ phases of the cell cycle 24 hours after Cs exposure. Therefore chronic Cs exposure, analogous to that produced by the stress axis, resulted in dramatic decreases in cell cycling in the remaining pro and pre B cells in the BM. This suggested that at 24 hours the remaining cells were not proliferating and could not reconstitute the B cell compartment. Taken together this data shows that chronically elevated Cs can not only cause dramatic decreases in pro and pre B cells, but also substantially reduced the proliferative capacity of the surviving cells during exposure.

The Effect of Cs on Major Hematopoietic Cell Lineages of the BM

Considering that approximately half of the B lymphocytes in the BM are eliminated due to Cs exposure, an analysis of the composition of the key hematopoietic cell lineages of the BM was performed to determine why the overall BM cellularity was unchanged. Using the ERMP12 and ERMP20 labeling system granulocytes, monocytes, lymphocytes, hematopoietic progenitors and erythroid progenitors could be differentiated (de Bruijn et al., 1994). The composition of the BM of sham mice and mice containing a Cs tablet insert after 36 hours is shown in Figure 2.6. The lymphocyte compartment that represented 32% of the cells of the marrow of sham mice had been reduced by 40%, to 19% of the BM, in mice with heightened exposure to Cs. Interestingly, the granulocyte population increased by 31%, comprising 42% of the marrow in sham mice and 55% of the marrow in mice with a Cs tablet implant. These results strongly suggested that the overall BM cellularity did not change due to Cs, because the granulocyte population expanded almost proportionately to the decrease in lymphocytes. Thus Cs can have two different effects on hematopoiesis by negatively affecting the generation of B

lymphocytes while positively affecting the generation of granulocytes, thereby potentially skewing the immune system towards myeloid defense as lymphoid mediated immune responses are decreased.

DISCUSSION

The experiments herein showed that increased concentrations of circulating Cs caused substantial losses of developing B lymphocytes and reduced proliferation among surviving cells. The early-pro through pre B cell stages of development, where Ig gene rearrangement has been shown to occur, were the most sensitive to Cs-induced losses. These populations are likely intrinsically susceptible to apoptosis, due to the natural elimination of up to 80% of developing B cells that occurs during selection for correct Ig gene rearrangement and against faulty rearrangements. These are also the developmental populations where the expression of the anti-apoptotic protein, Bcl-2, had been shown to be downregulated (Merino et al., 1994). This suggests that Cs might, at least in part, elicit its negative effect on developing B cells by inducing apoptosis in cells that are sensitive to cell death, due to low levels of Bcl-2. In fact Chapters three and four of this thesis will show *in vitro* studies where Cs directly induced apoptosis in bone marrow B lymphocytes with pre B cells exhibiting the greatest susceptibility to cell death.

The loss of B lymphocyte cellularity also appears to be due to a reduction in cell cycling. These data showed that the remaining pro and pre B cells, normally cycling populations, displayed dramatically reduced cycling after 24 hours of Cs exposure. Considering that newly generated cells would likely be eliminated by apoptosis due to heightened Cs, proliferation of these cells would be a futile process. The data presented later in this thesis will also demonstrate that, *in vitro,* Cs caused reduced cell cycling in the non-apoptotic pro and pre B cells. It is not known whether the decrease in proliferating cells *in vivo* was due to a selective elimination of cycling cells and/or as a

result of a direct reduction in cycling by Cs. The latter scenario would be congruent with the literature that reports a direct link between Cs and cell cycle downregulation (King and Cidlowski, 1998).

The negative effect of Cs on the BM, appeared to be specific for the B lymphocyte lineage. Results here suggest that increased Cs exposure might actually have a positive effect on the generation of other cell lineages, specifically granulocytes. This generates an intriguing hypothesis on how the immune system might respond to stress. Heightened Cs might selectively reduce the highly error-prone B cell development process while promoting granulopoiesis to maintain and enhance a first line of defense to try to compensate for a decreased second line of defense. Clearly, this area of research needs to be studied further to determine the effect of Cs on granulopoiesis for longer time periods and to determine the mechanism whereby Cs enhances the granulocyte population. This might simply be due to a non-responsiveness of granulocytes to Cs, resulting in expansion since loss of B lymphocytes would allow for more physical space in the BM. Expansion may also result if Cs has a proliferative and/or cell survival effect on BM granulocytes. Relatively recently studies have shown that Gc, in contrast to its negative effect on lymphocytes, promotes the survival of neutrophils (Liles et al., 1995). Therefore further investigation of Gc effects on the myeloid lineage is clearly warranted.

The experimental system used here has proven to be a reliable and reproducible model for inducing physiological-like increases in serum Cs. Surprisingly little was known of the effects that the induction of the stress axis could have on developing cells of the immune system. Most studies had focussed on the effects of Gc on peripheral, mature immune cells. Here it was shown that the development of B cells is reduced, thus

likely contributing to the lymphopenia often seen during chronic stress. Considering the early onset of BM B cell downregulation and the modest increases in Cs, it appears that lymphocyte development is one of the initial immune functions to be negatively affected by the steroid. It therefore seems likely that somehow protecting these cells from heightened Cs exposure might provide immune resilience to stress.

With the advent of B lymphopoiesis supporting long term bone marrow cultures and more advanced technology for studying cells of the marrow, it has become clear that stromal cells can play a major role in both lymphopoiesis and myelopoiesis by the production of critical cytokines (Whitlock and Witte, 1982). It is also clear that the addition of Gc to long term cultures, where stromal cells are present, selectively promotes the development of the myelopoietic lineage. Considering the results presented herein, it might be that Cs modifies the production of key molecules produced by stromal cells to support a more myelopoietic-like environment. It may also be that the exogenous addition of lymphopoiesis supporting factors could override Cs negative effects on B cell development.

Interleukin-7 is one cytokine produced by stromal cells that can act as a critical proliferative, survival and differentiation signal in early stages of B cell development (Namen et al., 1988). It might be that this cytokine could potentially act as an immunotheraputic agent to protect B cell development against increases in Gc. Therefore studies investigating the effect of IL-7 and other factors, critical to lymphopoiesis, on Cs-induced downregulation of B cells could have important implications in the treatment of stress-related immunodeficiencies. Later chapters in this thesis investigate the potential of stromal cells and/or exogenous IL-7 to modulate the negative effects of Cs on B

lymphocytes and also investigate the direct effect of Cs on the production of two key cytokines involved in blood cell development, IL-7 and SCF.

Further understanding of the effect of Gc on immune cell development could provide important advances in health studies, since both physiological stress and pharmacological administration of Gc is widespread. *In vivo* studies to determine the effect of Cs on the development of the myeloid lineage is warranted, as are studies investigating possible immunotheraputic agents on B lymphocyte development. Additionally, *in vitro* experiments could provide insight into the mechanism whereby Gc downregulates B cell development and how various factors might modulate that mechanism. Clearly the work presented here has provided some key insight into the effect of Gc on blood cell development and likely could provide a foundation for further investigations.

Figure 2.1 The plasma Cs levels for mice receiving a 40 mg cholesterol implant (sham) (solid bars) or a 20 mg Cs plus 20 mg cholesterol implant (dotted bars) at 12, 24 and 36 hours are shown. The data are expressed as μg/dl and standard deviations are shown (n=4). Significant differences between control and experimental mice were established using the Student's t-test ($p < 0.05$) and are indicated (*).

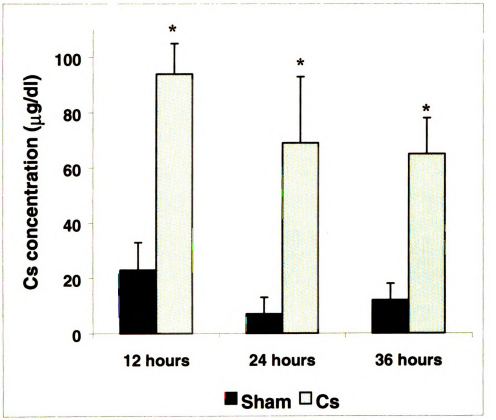


Figure 2.1 Plasma corticosterone concentrations

Figure 2.2 The individual thymic weights for sham control mice (X) and mice receiving a Cs implants (O) for 12, 24 and 36 hours are shown. Six mice were analyzed per group where some individual data points are hard to distinguish due to overlap. The mean of each group is indicated and an asterisk indicates significant differences between sham control and experimental mice as determined by the Student's t-test ($p < 0.05$).

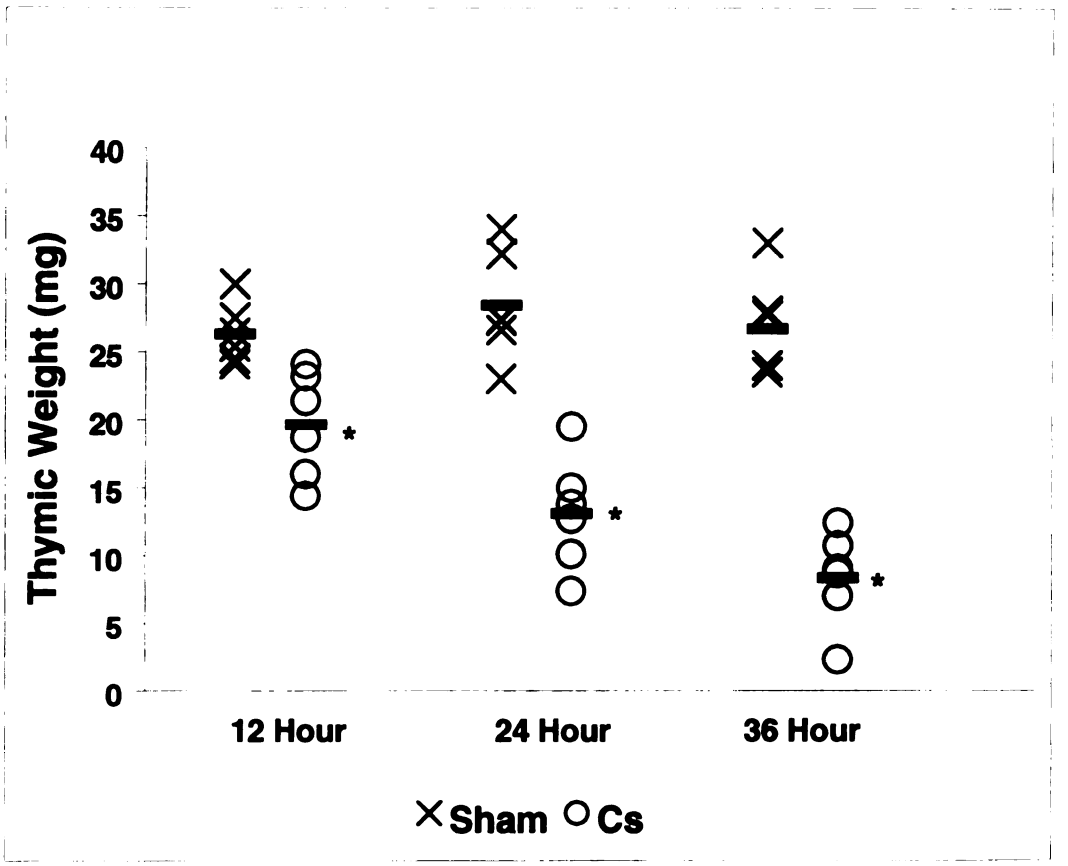


Figure 2.2 The effect of Cs or cholesterol implants on thymic weights

Table 2.1 Bone Marrow cellularity of Cs or sham implanted mice

Implant Time/Type		Total Cell Number [a] (Femurs)
12 Hour	Sham	$2.58 \pm 0.19 \times 10^7$
12 Hour	Cs	$2.75 \pm 0.46 \times 10^7$
36 Hour	Sham	$2.20 \pm 0.29 \times 10^7$
36 Hour	Cs	$2.32 \pm 0.46 \times 10^7$

[a] There was no significant difference between sham or experimental mice and data shown are mean ± standard deviation where n = 6 mice per treatment group.

Figure 2.3 Flow cytometric data for pre-pro through mature B lymphocytes where representative results from a sham control mouse and a Cs exposed mouse 24 hours post-implantation are shown. Panel (A) shows the $B220^+$ gated cells and gives the percent of pro ($B220^+S7^-IgM^-$), pre ($B220^+S7^-IgM^-$) and IgM^+ ($B220^+S7^-IgM^+$) cells in the BM of sham controls and Cs-treated mice. Regions were drawn around each population and the cell type and percent are shown. Panel (B) shows the $B220^+$ gated cells and the percentage of immature ($B220^+IgM^+IgD^-$) and mature ($B220^+IgM^+IgD^+$) B cells in the BM. Panel (C) shows $B220^+S7^+$ cells and the percent of pre-pro ($B220^+S7^+HSA^-BP^-1^-$), early-pro ($B220^+S7^+HSA^+BP\text{-}1^-$) and late-pro ($B220^+S7^+HSA^+BP\text{-}1^+$) B cells. Percentages were based on total nucleated BM cells. Data is representative of at least six mice per treatment group.

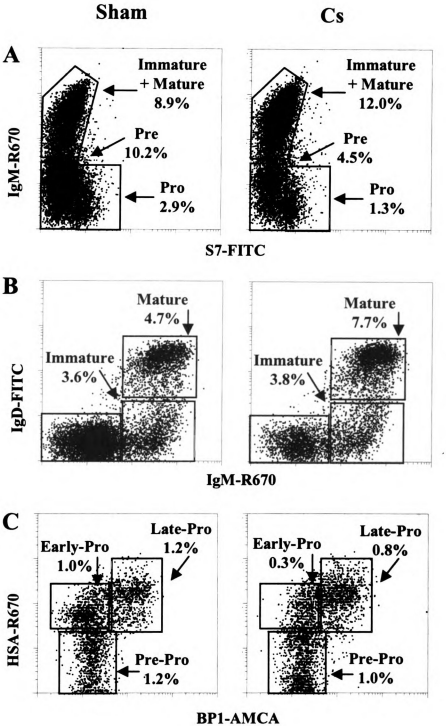


Figure 2.3 Flow cytometric profile of developing bone marrow B lymphocytes

Figure 2.4 The percentage of pre-pro, early-pro, late-pro, pre, immature and mature B cells in the BM are shown for sham (solid lines) and Cs-implanted mice (dashed line) at 12, 24 and 36 hours after implantation. Six mice were averaged per treatment group and the mean ± standard deviations are shown.

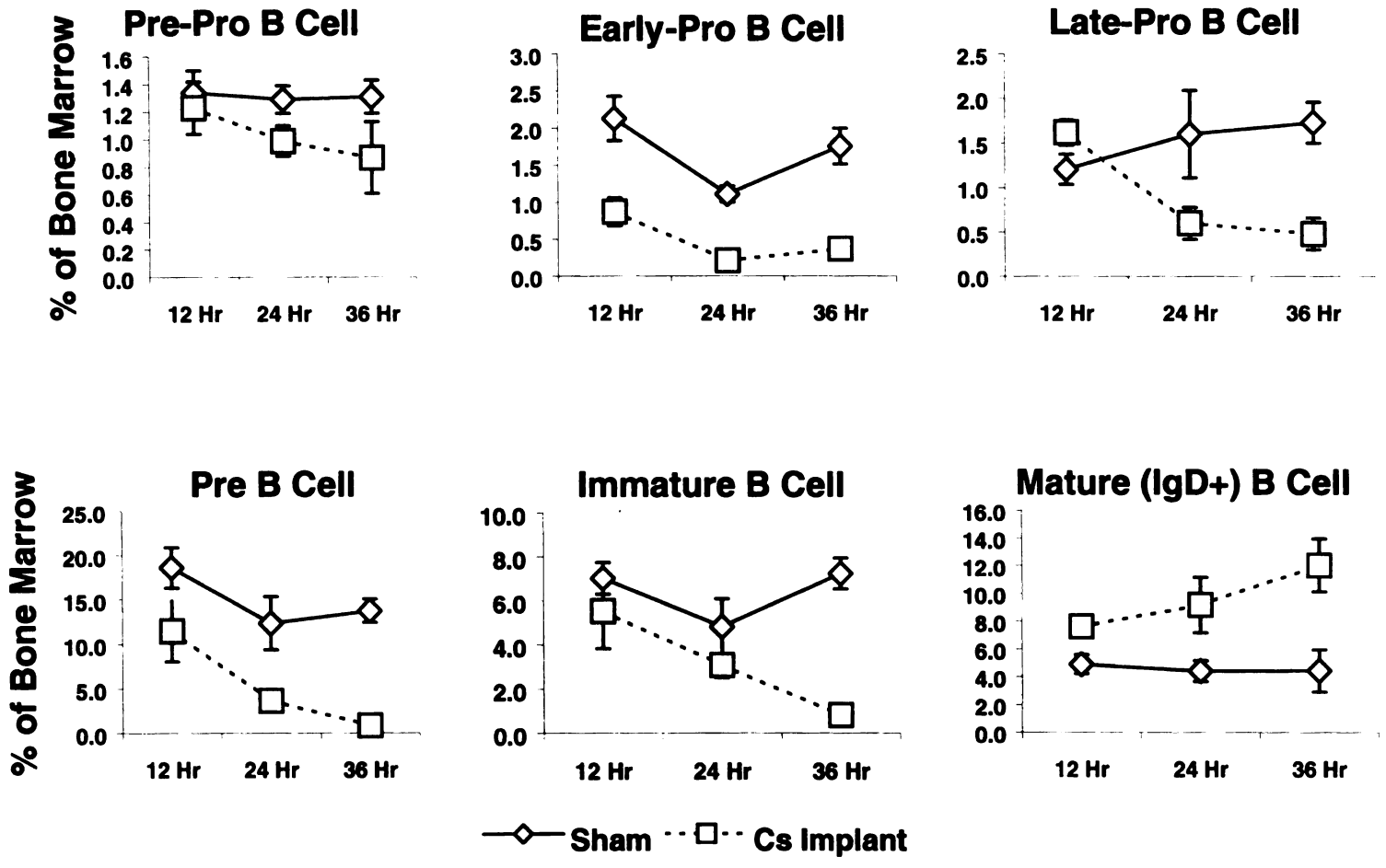


Figure 2.4 Cs-induced changes in bone marrow B lymphocyte populations over time

Figure 2.5 The cell cycle distribution for pro and pre B cells of sham and Cs-treated mice at 24 hours was determined. The main panel shows the change in the percentage of cells found in the $S/G_2/M$ phases of the cell cycle for pro and pre B cells. The actual percentages of cells in $S/G_2/M$ are shown in the panel insert. The data are for six mice from both sham and Cs-implanted mice where the mean ± standard deviations are shown and significance is indicated with an asterisk.

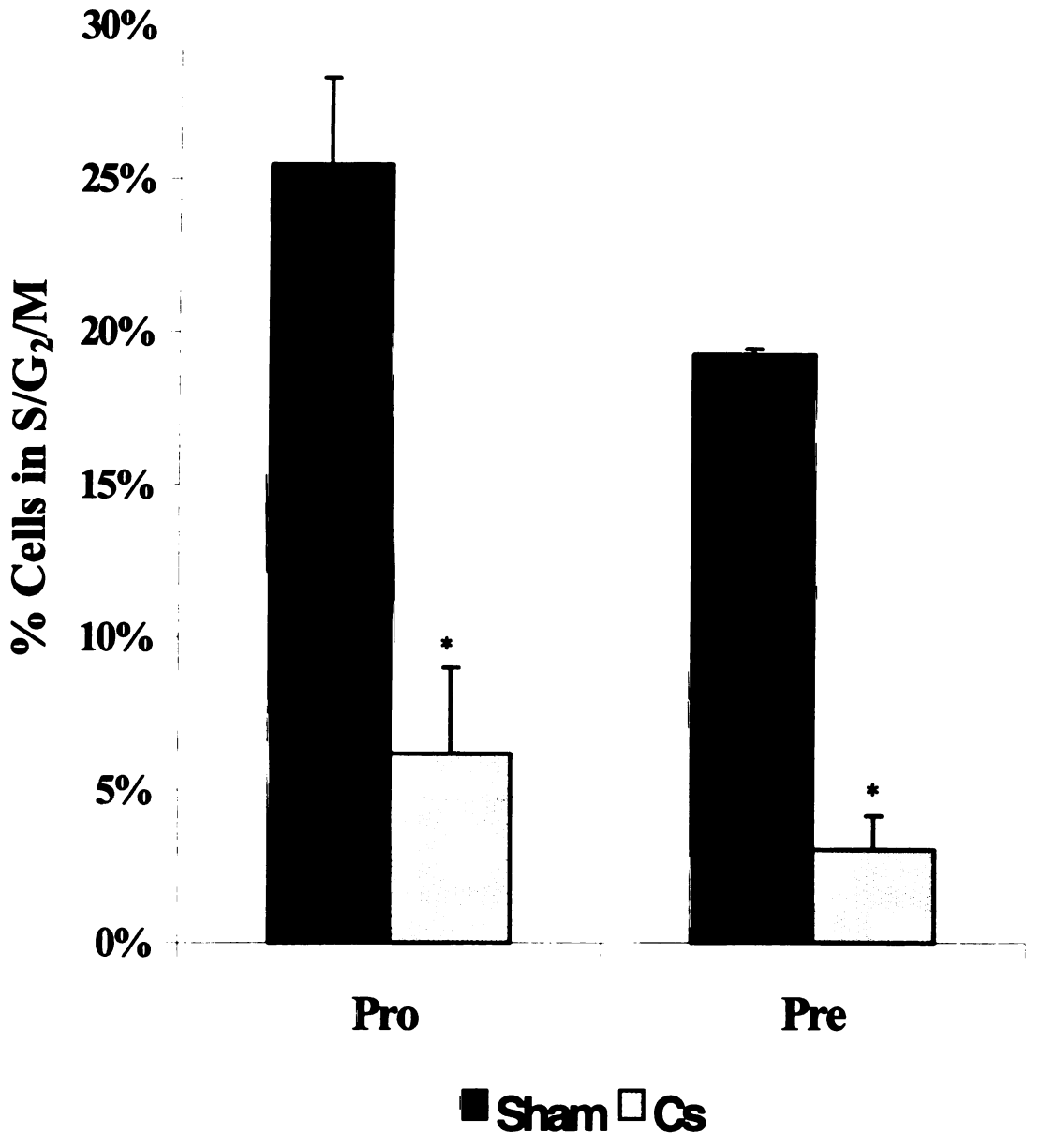


Figure 2.5 Cs-induced cell cycle changes in pro and pre B cells

Figure 2.6 The cellular composition of the BM from sham and Cs-treated mice after 36 hours is shown. Percentages were determined by flow cytometric analysis of the phenotypic distribution of granulocyte, lymphocyte, hematopoietic progenitors, monocyte and erythroid progenitors. These results are based on the expression of the ERMP12 and ERMP20 cell surface antigens. The means for sham control mice (n=5) and Cs-implanted mice (n=3) are shown.

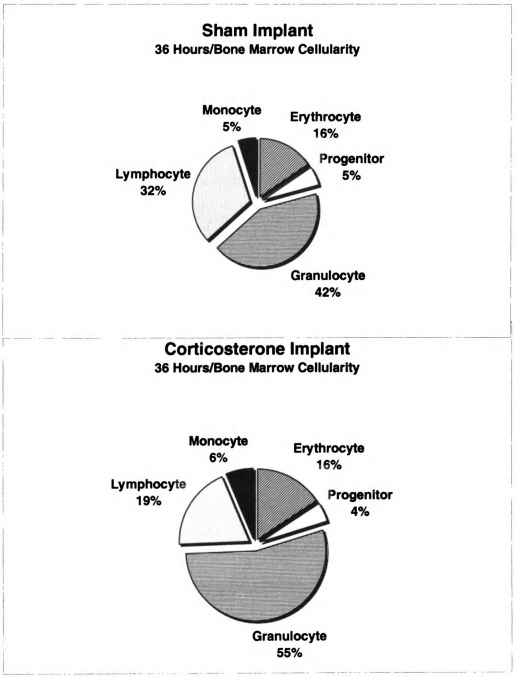


Figure 2.6 Cs effect on major hematopoietic lineages in the bone marrow

CHAPTER 3: STROMAL CELL PROTECTION AGAINST CORTICOSTERONE-INDUCED APOPTOSIS IN BONE MARROW B LYMPHOCYTES

ABSTRACT

Glucocorticoids (Gc) can induce apoptosis and cell cycle arrest in developing bone marrow (BM) B cells both *in vitro* and *in vivo*. Stromal cells are critical to B lymphocyte development in the BM and in long-term BM cultures. They can provide support to the developing B cells through direct cell-cell interactions and by the production of lymphopoietic cytokines. Here it is shown that soluble factors produced by and direct interactions with stromal cells can protect BM B lymphocytes from short-term exposure to a natural Gc, corticosterone (Cs). Indeed, stromal cells could reduce the ability of the steroid to initiate apoptosis by up to 30-50% among pro, pre and IgM^+ B cells. However, neither soluble factors nor direct interactions with the stromal cells restored cell cycling in developing B cells exposed to Cs. However, the exogenous addition of the lymphopoiesis promoting cytokine, interleukin-7 (IL-7), augmented the stromal cell protection against Cs-induced apoptosis in pro and pre B cells and also restored cycling to normal levels among pro B cells. In addition the potential of Gc to directly modulate the expression of genes important in hematopoiesis was investigated. Stromal cell expression of IL-7 and stem cell factor (SCF), a cytokine that supports early blood cell progenitors from many lineages, was therefore determined. After one day, Cs caused SCF mRNA to increase 2-3 fold, but it did not have an affect on IL-7 expression. Therefore the direct affect of Cs on BM B lymphocytes can be influenced by other cells normally found in the BM microenvironment, namely stromal cells. Additionally Cs appeared to also directly effect stromal cells by causing the upregulation of a general hematopoiesis supporting cytokine, SCF. Taken together these experiments demonstrate

the complex nature of studying BM B cells, since cells and factors that would normally be present *in vivo* can modulate the responses of developing cells presented with a death inducing molecule such as Cs. Thus, some of the negative effects of Gc on B cell development during physiological stress could potentially be alleviated by factors produced by stromal cells and/or exogenous IL-7.

INTRODUCTION

The successful generation of mature B lymphocytes is a complex multi-stage process consisting of differentiation and proliferation. It is accepted that during B cell development apoptosis plays an important role by eliminating the high population of precursor cells with faulty immunoglobulin (Ig) gene rearrangements. Detection of apoptosis *in vivo* has been very difficult due to the rapid phagocytosis of dying cells and the difficulties associated with studying subsets of cells in heterogeneous tissues like the bone marrow. Short-term *in vitro* culture systems and flow cytometry have allowed for the phenotypic analysis of the various stages in B cell development to include DNA analysis for cell cycle status and apoptosis (Garvy et al., 1993; Hardy et al., 1991; Telford et al., 1994). Our lab and others have shown that, *in vitro,* precursor B cells normally undergo low but significant levels of apoptosis in short-term cultures (less than 24 hours) (Garvy et al., 1993; Merino et al., 1994). Additionally, glucocorticoids (Gc), such as dexamethasone or corticosterone (Cs), have been shown to cause a dramatic increase in apoptosis among these cells. Therefore *in vitro* models have proven valuable in studies that otherwise could not be performed *in vivo*.

A major concern when studying B lymphocyte apoptosis and cell cycle *in vitro* is that *in vivo* B lymphocytes normally develop in close association with fibroblast-like cells, termed stromal cells, that support their development (Osmond, 1990). In the mid-1980's Whittlock and Whitte developed a long-term B lymphopoiesis supporting culture system (Whitlock and Witte, 1982). Stromal cells were key to the successful long-term maintenance of lymphopoiesis in this culture system. Later a variety of stromal cell lines

were generated that could help support B cell lymphopoiesis for long periods of time (Collins and Dorshkind, 1987; Dorshkind et al., 1986). Without stromal cells, lymphocytes do not survive beyond a few days (Borghesi et al., 1997). Interestingly, with the modification of culture conditions B lymphopoietic long-term bone marrow cultures and stromal cell lines could be converted from the promotion of B cell lymphopoiesis to the production of granulocytes (Dorshkind et al., 1986). Myelopoiesis supporting cultures were first described by Dexter (Dexter et al., 1977) and vary from lymphopoietic cultures in that exogenous Gc is added to the media. This suggested that the presence of Gc aided in changing the environment from the support of lymphocytes to the support of granulocytes in long-term cultures. Therefore Cs, in a period of weeks, could dramatically modify the culture environment. However, few studies have investigated the more immediate effect of stromal cells on lymphoid cells treated with an apoptosis inducing factor such as Cs. One study by Borghesi, et al showed that a stromal cell line, BMS2, could protect precursor B lymphocytes (IgM^-) from dexamethasone induced apoptosis, but effects on the specific pro, pre and IgM^+ populations was not investigated.

In addition to directly inducing apoptosis in developing B cells, it may be that Cs directly modulates stromal cell gene transcription. Many effects of Gc on their target cells are mediated by transcription modulation of certain genes via Gc receptor binding to glucocorticoid response elements. Under B lymphopoiesis supporting conditions, stromal cells produce certain cytokines, such as interleukin 7 (IL-7) and stem cell factor (SCF) that promote B cell development. Exogenous IL-7 can promote the proliferation and survival of pro and pre B cells without stromal cell support (Namen et al., 1988;

Namen et al., 1988). Elimination of IL-7 in culture resulted in the loss of B cells. Moreover it was determined that IL-7 deficient transgenic mice do not produce B cells beyond the pro-B cell stage (von Freeden-Jeffry et al., 1995). SCF, in combination with IL-7, promoted commitment to the B lineage and enhanced proliferation of the pro B cell population (McNiece et al., 1991). Whereas IL-7 was shown to be specific and critical for lymphocyte development, SCF has been shown to promote the development of progenitors of several cell lineages (Ashman, 1999). Therefore it might be that IL-7 plays an important role in Whitlock-type cultures whereas SCF supports both B lymphopoietic and myelopoietic Dexter-type cultures. Since the presence of Gc is the main difference between these culture systems it is possible that the steroid can modulate these cytokines that are involved blood cell development.

The stromal cell lines S10 and S17 and stromal-like cells sorted from long term bone marrow cultures were used to determine the effect of stromal cells on Cs-induced apoptosis and cell cycle status in pro, pre and IgM^+ B cells. Additionally, the effect of S10 and S17 stromal cell derived soluble factors, alone, on Cs-induced apoptosis was determined by culturing the BM B cells suspended over stroma with a membrane insert. These soluble factors modestly protected each B lymphocyte population from Cs-induced apoptosis while direct interactions with the stromal cells afforded slightly more protection. Although stromal cells demonstrated some protection against Cs-induced apoptosis they did not protect against the Cs-induced cell cycle arrest. The exogenous addition of IL-7 augmented stromal cell protection against Cs-induced apoptosis in pro and pre B cells and restored cycling among pro B cells. Additionally, the direct effect of Cs on the expression of SCF and IL-7 mRNA was determined. In the time frame studied

here, Cs caused a significant increase in SCF mRNA levels; the cytokine that promotes both lymphoid and myeloid development. Interestingly, the cytokine that is specific for lymphoid development, IL-7, did not increase. Therefore Cs might promote myelopoietic cultures by upregulating cytokines involved in this process.

MATERIALS AND METHODS

Harvesting of Bone Marrow from Mice

Young adult male Balb c/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the animal facility in the Biochemistry department at Michigan State University, East Lansing, MI; protocols were approved by the All-University Committee on Animal Use and Care.

Mice were used from 6 to 14 weeks of age and marrow was flushed from femurs with approximately 1 ml harvest buffer per bone (Hanks' balanced salts, 1 mM HEPES pH 7.2 and 4% FBS) using a 22 gauge needle. The marrow was processed into a single cell suspension and red blood cells were removed by lysis.

Cell Culture/Cell Lines

Bone marrow derived murine stromal cell lines S10 and S17 were generous gifts from the laboratory of Dr. Kenneth Dorshkind. These lines were grown at 37°C with 7.5% CO_2 and maintained in RPMI 1640 containing 5 x 10^{-5} M 2-mercaptoethanol, 1 mM HEPES pH 7.2, 1000 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 5% fetal bovine serum (FBS). FBS was purchased from HyClone (Logan, UT) and was tested for optimal performance; the same lot was used throughout. S10, S17 or sorted stromal cells were plated at 1x10^5 cells/ml in 24-well (1ml/well) or 12-well (2ml/well) Corning Costar (Corning, NY) tissue culture plates and were incubated overnight to insure complete adherence. To obtain stromal cells sorted from long-term bone marrow cultures, cultures were initiated and maintained as previously described (Whitlock and

Witte, 1982) and VCAM-1$^+$ (Southern Biotechnology Associates, Inc., Birmingham, AL) cells were sorted via fluorescence activated cell sorting (FACS). FACS sorted cells were maintained under the conditions used for the stromal cell lines; the sorted VCAM-1$^+$ cells had typical stromal cell morphology and were MAC-1$^-$ (Pharmingen, San Diego, CA).

Bone marrow was plated at 1-2 x 10^6 cells/well either in 24-well plates in media only, in 24 well plates directly onto confluent stroma or onto 0.4 μM transwell inserts suspended over stroma in 12-well plates. Corticosterone (Cs), purchased from Sigma (St. Louis, MO), and/or recombinant murine interleukin 7 (IL-7), purchased from R&D Systems (Minneapolis, MN), were added to cultures at 0.1 μM Cs and at 0.1 ng/ml IL-7. BM cells were dislodged from culture by the addition of 0.02% EDTA.

Immunophenotyping/DNA staining

Antibodies to B cell surface antigens were added to samples at a predetermined dilution to phenotypically label different stages involved in B cell lymphopoiesis. phycoerythrin (PE) conjugated anti-CD45R (B220), fluorescein (FITC) conjugated anti-CD43 (S7) and biotinylated anti-IgM F(ab')2 (IgM) were added to samples simultaneously and incubated for 25 minutes. Antibodies against S7 and B220 were purchased from Pharmingen (San Diego, CA) and anti-IgM was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Following primary staining, streptavidin-red670 (R670), purchased from Gibco (Grand Island, NY), was added at a predetermined dilution for conjugation to biotinylated anti-IgM. Samples were incubated with R670 for 20 minutes and then washed two times with label buffer. Following phenotyping, cells were suspended in 50% FBS and fixed with the slow addition 1.2 ml

of ice cold 70% ethanol. To determine DNA content for cell cycle and apoptosis, samples were stained with 0.5 to 1.0 ml of 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) for at least one hour prior to flow cytometric analysis.

Flow Cytometry

Samples were analyzed on a Becton Dickinson FACS Vantage flow cytometer. FITC, PE and R670 fluorochromes were excited at 488 nm, and emission was detected at 530, 575 and 670, respectively. DAPI was excited at 365 nm and emission was detected at 470 nm.

Debris and cellular aggregates were excluded from analysis by gating based on size and DNA content. Cell size was determined by forward and side light scatter. DNA content was determined by a DAPI fluorescence and cells with hypodiploid DNA were considered apoptotic, as previously described (Telford et al., 1994). $B220^+$ B lymphocyte subsets were defined as follows: pro B cells were $S7^+IgM^-$, pre B cells were $S7^-IgM^-$ and IgM^+ B cell were $S7^-$. Flow cytometric data was processed using either PC-LYSIS (Becton Dickinson) or WinList (Verity Software House, Inc.) software

Ribonuclease protection assay

RNA was isolated from S10, S17 or sorted stromal cells using Pharmingen's Total RNA Isolation Kit and protocol (San Diego, CA). In general 5×10^6 to 1×10^7 cells were used per isolation yielding approximately 80-150 μg total RNA. RNA was resuspended in 0.6 ml RNase-free water and stored at -80°C until analysis.

The IL-7 and SCF probe templates were purchased from Pharmingen. Probe synthesis was performed as described in Pharmingen's Riboquant Multi-Probe RNase Protection Assay (RPA) System. Briefly, the probe template was transcribed with ^{32}P labeled uridine triphosphate (New England Biolabs). The radiolabeled probe was then added to 50 μg stromal cell RNA and hybridized overnight. For positive control samples 10 μg of RNA from IL-7 overexpressing cell lines Psi 5-20 and N59 and the SCF overexpressing BHK-MK1 cell line were used. Control lines were very generous gifts from the laboratory of Dr. Richard Schwartz. Negative control samples contained 2 μg of tRNA. RNA samples and radiolabeled probe were hybridized overnight at 56°C. Following hybridization, ribonuclease treatment was performed as described by Pharmingen. Samples were electrophoresed on a 5% denaturing polyacrylamide gel and visualized by phosphorimaging. To obtain a standard curve for fragment mobility, the probe was run at 2000 counts per minute per lane of the gel. The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and L32 were used to normalize variances in the amount of sample applied to the gels. Phosphorimaging was used for quantitation and Excel was used to analyze the data.

Data Analysis and Statistics

Data analysis was performed using Microsoft Excel software and mean ± standard errors are reported. Statistical significance was determined using the Student's t-test ($p < 0.05$).

RESULTS

Pro, Pre and IgM$^+$ B Lymphocytes Undergo Spontaneous and Cs-Induced Apoptosis

To determine the effect of stromal cells on spontaneous verses Cs-induced apoptosis it was important to assess the degree of apoptosis in each cell type. To choose an appropriate culture time frame where significant levels of apoptosis were induced but where necrosis or cell losses were minimal, the degree of spontaneous and Cs-induced apoptosis at 15, 24, and 48 hours was determined. Figure 3.1 shows a time course of apoptotic pro, pre and IgM$^+$ B lymphocytes incubated in media alone (Panel A) or with 0.1 μM Cs (Panel B). The data show that over time, the percent of spontaneous apoptosis increased in each developmental population from 8%, 14% and 9% in pro, pre and IgM$^+$ cells at 15 hours to 29%, 36% and 23% in the pro, pre and IgM$^+$ populations at 48 hours, respectively. Freshly isolated marrow contained fewer than 2% apoptotic B lymphocytes (data not shown). The addition of Cs to culture caused a significant increase in apoptosis. At 15 hours Cs caused a modest induction of apoptosis in pro, pre and IgM$^+$ of 24%, 44% and 36%. Greater levels of death were apparent at 24 hours; 32%, 64% and 48% in the pro, pre and IgM$^+$ cells, respectively. After 48 hours nearly all of the pre and IgM$^+$ cells were apoptotic. Therefore a 15 hour culture period resulted in a significant induction of spontaneous and Cs-induced apoptosis, yet these levels were more modest compared to later time points, thus making the modulation of B cell death by stromal cells more apparent.

Protective Effect of Stromal Cell Lines on Spontaneous and Cs-Induced Apoptosis in B Lymphocytes

Pro, pre and IgM^+ B lymphocytes underwent low levels of spontaneous apoptosis at 15 hours and the addition of Cs caused increased apoptosis, as shown above. Therefore, whether stromal cells and/or the soluble factors they produce could modulate this apoptosis in the B cell populations was determined. BM cells were cultured as a single cell suspension either in media alone, directly on confluent stromal cells or suspended above the stromal cells using a 0.4 μM permeable membrane insert. Samples were incubated for 15-16 hours after which the composition of cells of the B lineage and degree of apoptosis were determined by flow cytometry. Figure 3.2 (A) shows the effect of either soluble factors from and/or direct interactions of BM B lymphocytes with the S17 stromal cell line on spontaneous apoptosis. Direct incubation with S17 resulted in a significant decrease in spontaneous apoptosis of 40-50% in each pro, pre and IgM^+ B cells. However soluble factors, alone, did not reduce spontaneous apoptosis in any stage of development. The S10 stromal cell line also had similar effects on the BM B cells (data not shown). Therefore, direct interaction with the stromal cell lines, but not soluble support alone, could reduce spontaneous apoptosis in pro, pre and IgM^+ B cells.

Figure 3.2 (B) shows the effect of soluble factors from and/or direct interactions with S17 on Cs-induced apoptosis in pro, pre and IgM^+ B cells. Direct interaction with S17 resulted in the greatest protection against Cs-induced apoptosis in pro B cells (44% reduction). IgM^+ cells were also significantly protected with a 39% decrease and pre B cells showed a more modest 29% decrease. Soluble factors produced by S17 also protected B lymphocytes from Cs-induced apoptosis. A 31%, 20% and 28% reduction in

apoptosis by soluble factors alone was observed in pro, pre and IgM^+ cells, respectively. Although this trend suggested lesser protection by soluble factors, the reduction was not statistically different from those obtained from direct stromal contact. S10 stromal cells had similar protective effects on the B lymphocytes (data will be presented below). The data suggests that soluble factors produced by these stromal cell lines provided significant protection to each stage of B lymphocyte development from Cs-induced cell death in short-term cultures. Interestingly, soluble factors from the stromal cell lines did not appear to protect B lymphocytes from spontaneous apoptosis, suggesting that either one or more soluble molecules produced by stromal cells could specifically protect against Cs-induced apoptosis without affecting basal levels of apoptosis.

Stromal Cell Effect on Cs-Induced Cell Cycle Arrest in Pro and Pre B Lymphocytes

Data here and in Chapter two showed that along with causing apoptosis in B lymphocytes, Cs also caused a reduction in cycling pro and pre B cells. The extent to which these two Gc-induced phenomenon are interrelated is not fully understood. Here experiments were performed to determine the effect of S17 on Cs-induced cell cycle arrest. BM B lymphocytes were incubated with or without S17 as previously described and in media alone. Table 3.1 shows the percent of pro, pre or IgM^+ cells in the $S/G_2/M$ phases of the cell cycle. Samples incubated without Cs showed that neither soluble nor direct interaction with S17 changed the proportion of cycling cells in control cultures. Addition of 0.1 μM Cs, on the other hand, resulted in a significant decrease in the percent of cycling pro and pre B cells similar to the decreases seen in *in vivo* studies. Cells in $S/G_2/M$ decreased by 54% in pro B and 71% in pre B cells when cultured with Cs.

Neither soluble factors nor direct interaction with S17 were able to restore the cycling population in the pro or pre B cells. S10 similarly did not effect the cell cycle status and these results will be shown below. Therefore, although stromal cells could protect B lymphocytes from Cs-induced apoptosis they were not able to protect the developing pro and pre cells from the Cs-induced cycling decrease.

The Effect of Exogenous IL-7 on BM B Lymphocytes Cultured with Stromal Cells

Chapter 4 in this thesis shows the effect of exogenous addition of IL-7 alone on B lymphocytes exposed to Cs. The cytokine protected pro B cells from Cs-induced apoptosis and cell cycle arrest and also modestly protected pre B cells from the Cs-induced apoptosis, but not the cell cycle arrest. It was therefore important to determine if the exogenous addition of IL-7 to cells incubated with either S10 or S17 could reproduce the effect on B lymphocytes seen with IL-7 alone. Figure 3.3 shows actual flow cytometric data for apoptosis and $S/G_2/M$ in pro, pre and IgM^+ cells cultured with no treatment, 0.1 μM Cs, Cs and S10 or Cs, S10 and IL-7 for 16 hours. As compared to background levels, Cs clearly induced substantial induction of apoptosis in pro, pre and IgM^+ cells. Additionally significant reductions in pro and pre B cell cycling were apparent. Direct incubation with S10 resulted in a 50%, 51% and 55% decrease in Cs-induced apoptosis in pro, pre and IgM^+ cells, respectively. The exogenous addition of IL-7 further reduced the percent of apoptotic pro B cells by an additional 62%, bringing the percent apoptosis to below background levels. IL-7 caused a more modest decrease in apoptosis in pre B cells of 43% as compared to pre B cells cultured with S10 alone. The cytokine did not cause a further reduction in apoptosis in IgM^+ cells. These data are

consistent with the independent effect of IL-7 on these cell types. IL-7 addition to B lymphocytes cultured with S17 resulted in similar responses to those seen with S10 (data not shown).

As seen with the S17 data above, S10 alone did not affect the decrease in pro and pre B cells in $S/G_2/M$ induced by treatment with Cs. The addition of IL-7 to cells incubated with S10, on the other hand, resulted in a restoration in pro B cell cycling to that seen in background cultures. Again the effect of exogenous IL-7 addition to these cultures reproduced the effect of IL-7 alone on pro B cell cycling. Therefore recombinant IL-7 has effects on Cs-induced cycle arrest that S10 alone could not provide and S10 alone appears to protect IgM^+ cells from Cs-induced apoptosis whereas IL-7 did not augment this response.

The Effect of Cs on Stromal Cell Expression of SCF and IL-7

The major mechanism whereby Cs elicits its effects in cells is by transcription regulation via the Gc receptor. Although the protective effect of S10 and S17 on B lymphocytes could be attributable to soluble factors other than IL-7, it was important to determine whether this cytokine was expressed in each stromal cell type and whether that expression was modified by Cs-treatment. Additionally the cytokine SCF could be a target for regulation by Cs, since it generally promotes hematopoietic progenitors. In contrast to IL-7, SCF likely could play a role in long term myelopoietic cultures, where Cs is part of the culture medium. Figure 3.4 shows the relative RNA expression of IL-7 and SCF in S17, S10, sorted stroma and from positive control cell lines. Clearly message for both cytokines was present in each stromal cell type, with sorted stromal cells

expressing higher levels of IL-7. Relative to IL-7, SCF was expressed at dramatically higher levels.

Figure 3.5 (A) shows the change in SCF mRNA levels in S10, S17 and sorted stromal cells treated with 0.1 μM or 10 μM Cs for 20-24 hours. Cs treatment (10 μM) resulted in a 2-3 fold elevation in SCF mRNA expression in all stromal cell types tested. Although the increase caused by 0.1 μM Cs was less overall it was not statistically different from the increases induced by 10 μM treatment. These data show that Cs caused an upregulation of SCF as early as one day after treatment.

IL-7 mRNA was detectable in S17, S10 and sorted stroma. S17 and S10 showed very low but similar expression levels. Sorted stroma, in contrast, had approximately 5-10 fold higher production of IL-7 mRNA (as shown in Figure 3.4). Figure 3.5 (B) shows that there was no significant change in IL-7 mRNA expression with Cs treatment. Therefore, Cs does not appear to regulate IL-7 expression after 20-24 hours exposure to Cs, suggesting that the modest protection provided by stromal cells was not due to upregulated IL-7 expression.

DISCUSSION

The experiments presented here have shown that in short-term primary bone marrow cultures significant but low levels of spontaneous apoptosis occurs in pro, pre and IgM^+ B cells. The addition of Cs at concentrations analogous to physiological rather than pharmacological concentrations resulted in substantial increases in apoptosis, with pre B cells being the most susceptible to death. Additionally the steroid dramatically reduced cycling pro and pre B cell numbers. Soluble factors produced by, or direct interactions with, the S10 or S17 stromal cell lines caused a modest reduction in apoptosis in each B cell population, but in contrast the stromal cells did not affect the Cs-induced reduction in cell cycling. This suggests that factors produced by stromal cells could potentially protect developing B cells from Cs-induced apoptosis over a short period of time.

At least one soluble factor produced by the stroma appeared to be responsible for the decrease in Cs-induced apoptosis. IL-7 would be surmised to be one likely candidate for this protective function, since it is critical and specific for lymphopoiesis rather than myelopoiesis in the bone marrow (Namen et al., 1988; von Freeden-Jeffry et al., 1995). The S17 cell line had been reported to not produce biologically active levels of this cytokine but here these cells protected the B lymphocytes to the same extent as S10, which has been reported to produce biologically active IL-7 (Henderson et al., 1990). However, S17 was also reported to not produce IL-7 mRNA by RT-PCR but the experiments herein show that S17 in fact displayed low levels of IL-7 gene expression being comparable to that seen in S10 (Faust et al., 1993). Therefore these S17 stromal

cells did express IL-7 mRNA in contrast to previous publications. The difference could potentially arise from a reversion of the cell line to an IL-7 producing stromal cell. Both S10 and S17 were derived from multiple passages of long term bone marrow cultures to obtain a self-renewing clonal cell type, therefore there is no enforced selection of cells to maintain characteristics such as the lack of expression of certain cytokines.

The independent protective effect of exogenous IL-7 on B lymphocytes is thoroughly presented in Chapter 4 of this dissertation. The cytokine clearly protected pro and pre B cells from Cs-induced apoptosis and eliminated the cell cycle arrest in pro B cells. Those experiments suggested that IL-7 could potentially have immunotheraputic value in lymphocyte development during stress. Here the exogenous co-addition of this cytokine to stromal cell supported cultures, reproduced the protective effects seen by the cytokine alone in addition to stromal cell protection. Therefore the immunotheraputic potential of IL-7 was further supported by these experiments.

Regardless of the potential effects that low levels of IL-7 produced by stromal cells might have, it seems likely that other soluble factors produced by the stromal cells have a protective effect on B cells exposed to Cs. Evidence for this comes from the protection from Cs-induced apoptosis observed in IgM^+ cells. IL-7 alone or in combination with stromal cells did not protect this more mature cell population and previous studies have indicated that IgM^+ cells in the marrow are mostly unresponsive to IL-7 (Sudo et al., 1993). One stromal cell derived cytokine that could potentially elicit this effect is thymic stromal lymphopoietin (TSLP). Recent studies on the positive effect of TSLP on developing B lymphocytes indicated that this cytokine could potentially support B cells from Cs-induced apoptosis, because it supports B cell

development throughout IgM^+ stages (Levin et al., 1999). Further studies to identify active factors that can protect all stages of B lymphocyte development from Cs-induced apoptosis would therefore be of value.

The studies on the expression of SCF message levels have shown that Cs can directly affect bone marrow stromal cells and potentially can modulate the hematopoietic environment by transcriptionally regulating cytokines important to blood cell development. Cs treatment for one day significantly upregulated SCF gene expression. Since SCF is a cytokine that positively affects a variety of progenitor blood cell types and that Cs promotes myelopoiesis, it may be that the upregulation of SCF acts to promote the initiation of a myelopoietic-like environment. IL-7 on the other hand, which does not promote myelopoiesis, was not upregulated in response to Cs. Since long-term exposure to Cs causes the stromal cell environment to promote myelopoiesis it is logical that the steroid would cause the upregulation of a general cytokine involved in blood cell development, but would not upregulate a cytokine specific for lymphopoiesis which the environment does not support. It could be that after extended culture with Cs, stromal cells might actually downregulate IL-7 expression. Advances in technology have created the potential to screen the expression of a variety of cytokine genes simultaneously and Cs treatment of stromal cells could be a valuable area of investigation.

It is clear that the study of B lymphocyte responses to physiological factors is a very complex area of study with several variables to take into account when studying cells *in vitro*. Nevertheless, the complete understanding of B cell development and how that development can be modified remains a very important area in health and disease.

Therefore it is important to study these cells and attempt to understand the potential roles of factors that would normally be found in the BM on them.

Figure 3.1 The percent of apoptotic cells in pro, pre and IgM^+ B lymphocytes from BM are shown. Apoptosis was determined after culturing for 15, 24 or 48 hours, by DNA staining and analysis for hypodiploid DNA. Panel A shows apoptosis among cells cultured in media only and Panel B shows the percent apoptosis in the B cell populations cultured with 0.1 μM Cs. Data are the average of duplicate samples ± the standard deviations and is representative of several experiments. Error bars not seen are smaller than the data symbols.

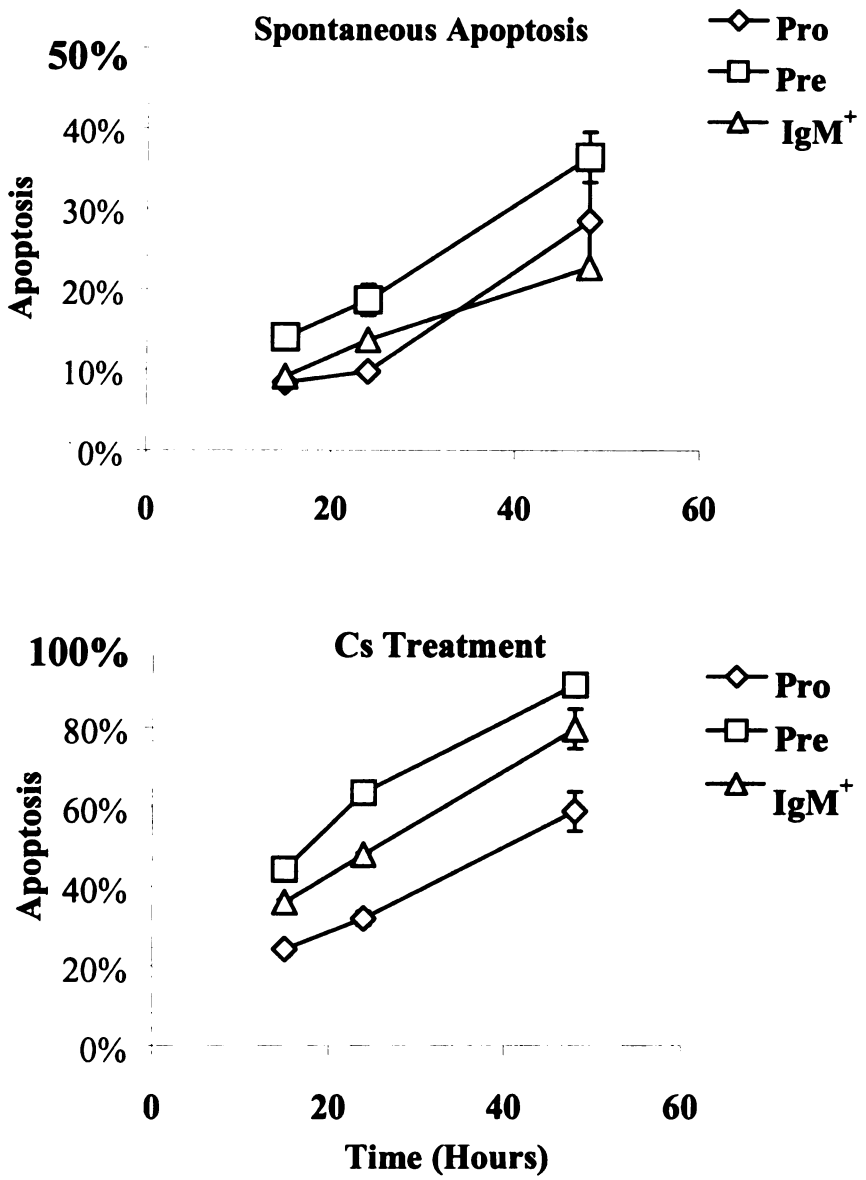


Figure 3.1 Spontaneous and Cs-induced apoptosis over time in pro, pre and IgM^+ B cells *in vitro*.

Figure 3.2 Apoptosis was measured among pro, pre and IgM+ B cells with and without Cs to determine potential protective effects of stromal cells (direct contact) or factors produced by stromal cells (membrane insert). BM was processed into a single cell suspension and plated in media alone (solid bars), on a 0.4 μM transwell insert suspended over S17 stromal cells (dots) or directly onto a confluent layer of S17 stromal cells (stripes). Following 15-16 hours in culture the BM cells were harvested and phenotyped for pro, pre and IgM^+ B cells, fixed and stained with DAPI, a DNA dye, for analysis of apoptosis. Panel (A) shows the change in apoptosis in cells cultured alone or with or without direct stromal cell contact in media and (B) shows the change in apoptosis among cells exposed to 0.1 μM Cs alone or when in direct or indirect contact with stromal cells. All data were normalized to the percent of apoptosis among cells cultured without S17 and are the averages of at least three experiments. The actual percent apoptosis for pro, pre and IgM^+ cells in control cultures without Cs or stromal cells were 7%, 14% and 12%, respectively. Control samples treated with Cs were 21%, 51% and 41% apoptotic in pro, pre and IgM^+ cells, respectively. Standard error bars are shown and significant differences from control samples was determined using the Student's t-test ($p < 0.05$) and indicated by an asterisk.

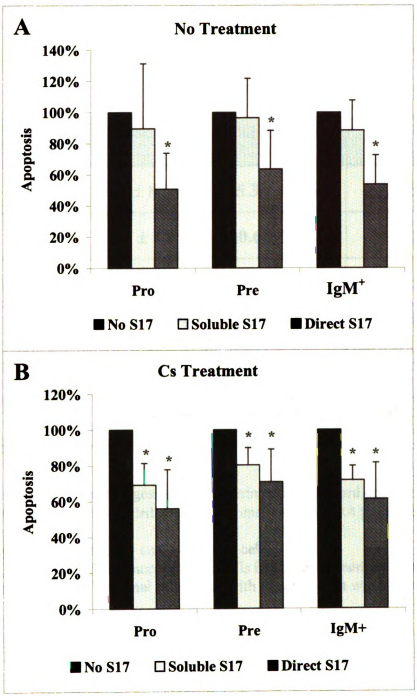


Figure 3.2 Stromal cell modulation of spontaneous and Cs-induced apoptosis

Table 3.1 Bone marrow B cells in $S/G_2/M$ phases of the cell cycle

	Cell Type	No Stroma	Soluble Factors[a]	Direct Contact[b]
No Cs	Pro	16.6 ± 8.5%*	15.3 ± 6.8%[c]	13.1 ± 4.6%[c]
	Pre	12.6 ± 1.2%	10.6 ± 1.9%	11.9 ± 1.8%
	IgM^+	4.8 ± 2.0%	4.8 ± 0.8%	4.2 ± 1.7%
0.1 μM Cs	Pro	7.7 ± 0.5%	8.0 ± 1.2%	6.25 ± 2.1%
	Pre	3.6 ± 2.0%	3.8 ± 1.5%	3.4 ± 1.0%
	IgM^+	3.6 ± 1.4%	3.6 ± 0.3%	3.3 ± 1.1%

* Data are presented as the averages of three experiments ± standard deviations

[a] B lymphocytes cultured suspended over S17 stromal cells by a 0.4 μM membrane insert

[b] B lymphocytes cultured directly on S17 stromal cells

[c] There were no statistical differences between cells incubated in media alone, with soluble factors from the S17 stromal cell line or with direct contact with the stromal cells

Figure 3.3 Flow cytometric DNA histograms show the Cs-induced change in apoptosis and cell cycle status for pro, pre and IgM^+ B cells and modulation of these Cs-induced changes by stromal cells or a combination of stromal cells and IL-7. BM was processed into a single cell suspension and incubated for 16 hours in culture media only (A), with 0.1 μM Cs (B), directly on a confluent S10 stromal cell layer and with 0.1 μM Cs (C) or with S10, Cs and 0.1 ng/ml IL-7 (D). Following culture BM cells were harvested and phenotyped for pro, pre and IgM^+ cell types, the samples were fixed with ethanol and the DNA stained with DAPI. Cells were analyzed by flow cytometry as follows: pro B ($B220^+S7^+IgM^-$), pre B ($B220^+S7^-IgM^-$) and IgM^+ cells ($B220^+S7^-IgM^+$). Debris and cell aggregates were excluded from analysis by gating based on cell size and each population was analyzed for apoptosis (A_0) and cell cycling ($S/G_2/M$) as indicated. These data are representative of several experiments.

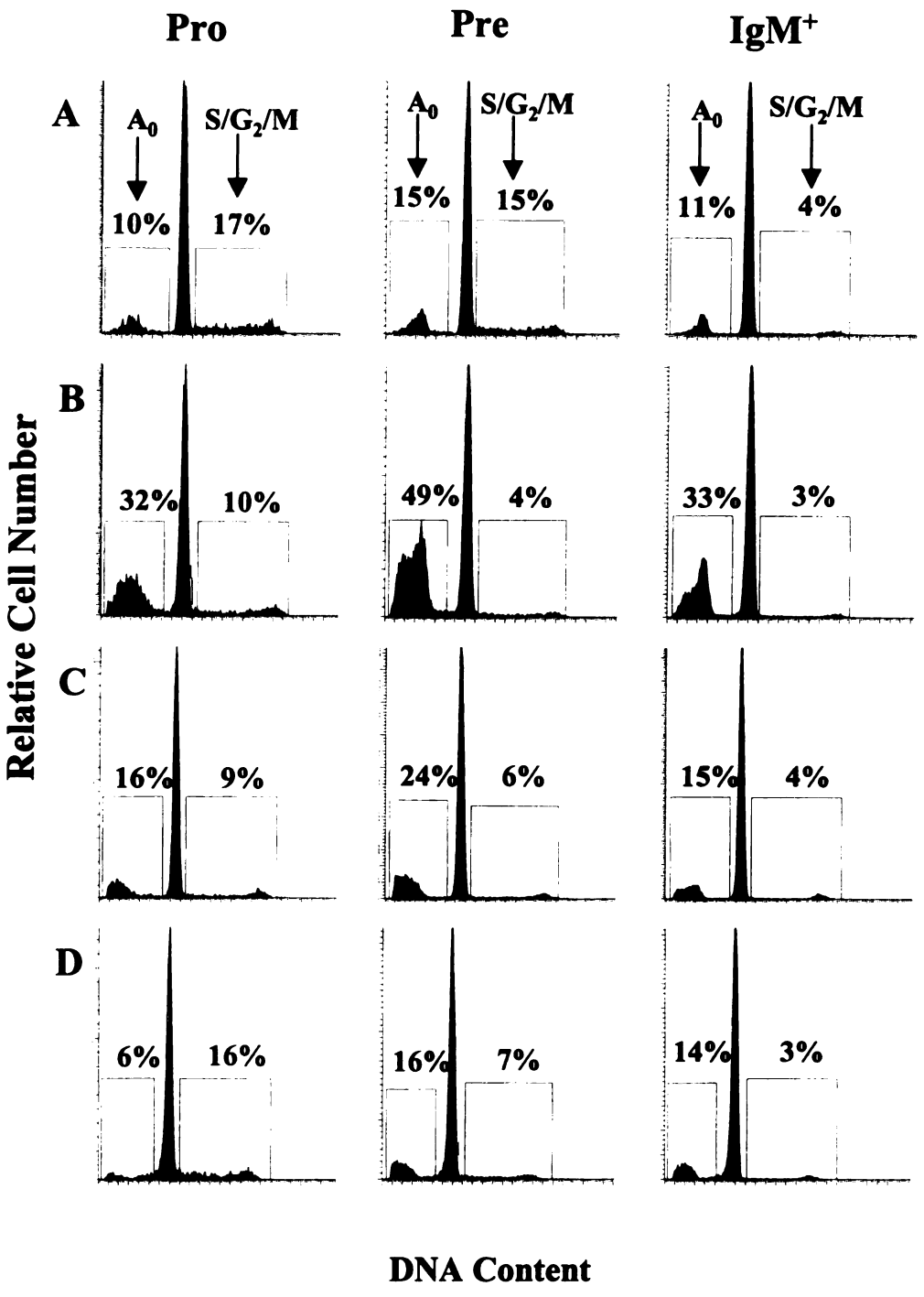


Figure 3.3 Flow cytometry of S10 and/or IL-7 effects on apoptosis and cycling of pro B cells

Figure 3.4 The relative RNA expression between stromal cell types is shown as the actual phosphoimage obtained from the ribonuclease protection assay. RNA was extracted and the ribonuclease protection assay were performed as described by Pharmingen using 50 μg stromal cell RNA and 10 μg positive control cell line RNA. The samples are shown as follows: Lane (A) unprotected probe, (B) S17 stromal cells, (C) S10 stromal cells, (D) sorted stromal cells, (E) IL-7 positive control cell line N59, (F) SCF positive control cell line BHK-MK1. The approximate electrophoretic mobility for each is indicated on the left hand side of the figure.

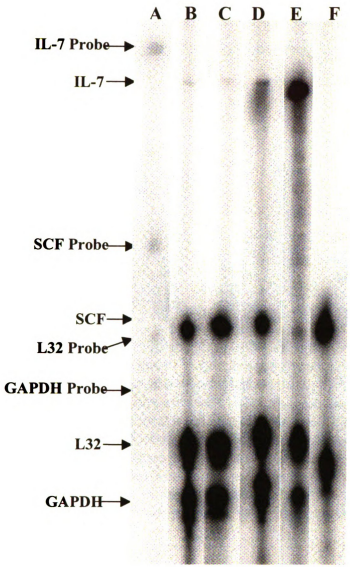


Figure 3.4 Relative expression of IL-7 and SCF message

Figure 3.5 The relative change in messenger RNA expression levels of SCF (A) and IL-7 (B) for stromal cells treated with 0.1 μM or 10 μM Cs for 20-24 hours. Stromal cells tested were the S10 and S17 cell lines and stromal-like cells sorted from long term bone marrow cultures. Cells were sorted based on large size and VCAM-1 surface expression. These cells had fibroblast like morphology typical of stromal cells and did not express MAC-1 on their surface. The stromal cells were grown just to confluence and spent media was replaced with fresh media for approximately one day before Cs addition. Following culture, whole cell RNA was extracted from approximately 5 X 10^6 - 1 X 10^7 stromal cells using Pharmingen's Total Cell RNA Isolation Kit. RNA analysis was performed using the ribonuclease protection assay and quantified using phosphorimaging. The results shown are the average changes relative to the corresponding non-treated cells for at least two experiments and standard deviation bars are shown. Asterisks represent significant differences as determined by the Student's t-test ($p < 0.05$) from untreated samples.

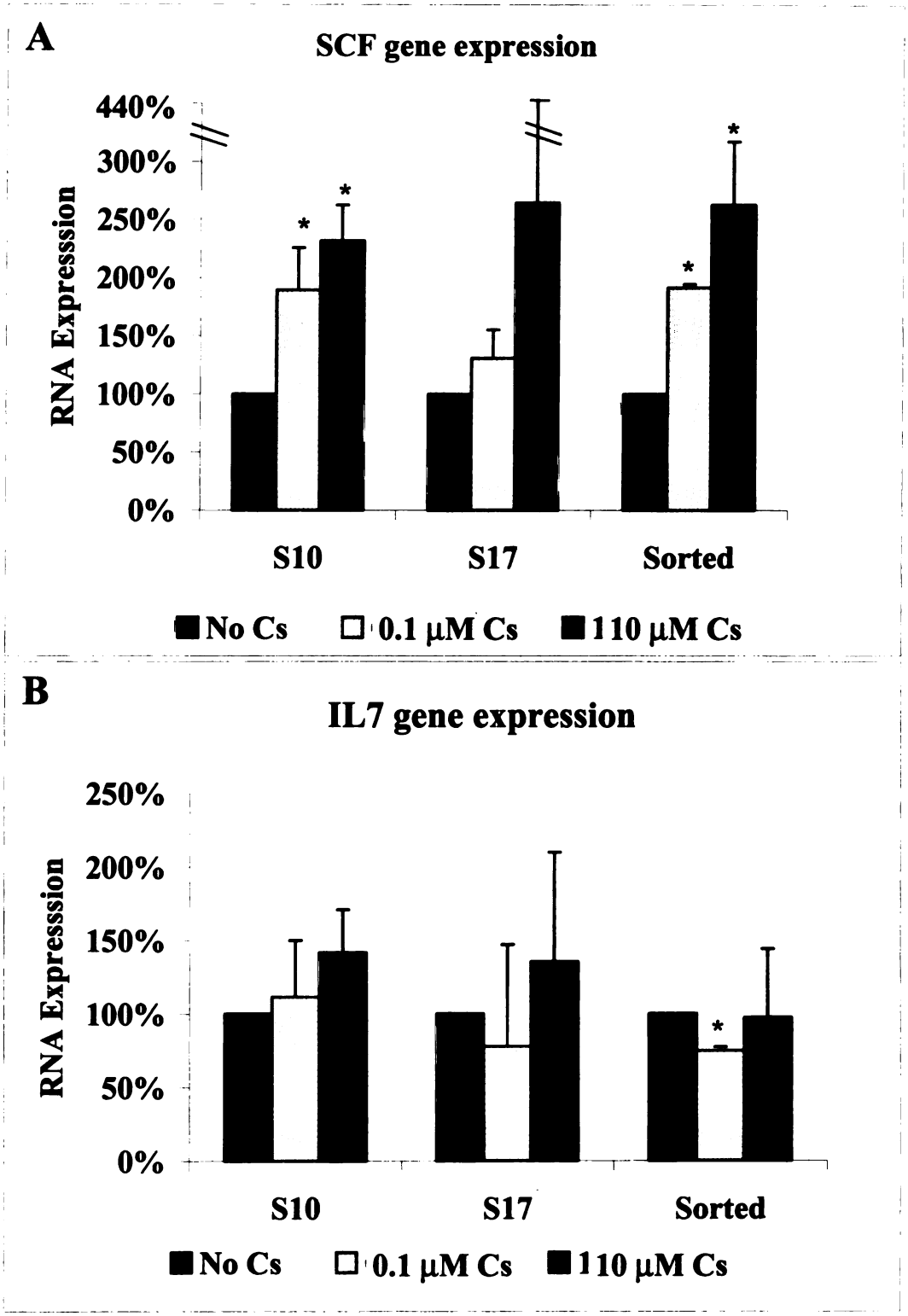


Figure 3.5 Cs effect on stromal cell expression of SCF and IL-7 RNA

CHAPTER 4: INTERLEUKIN-7 PROTECTS EARLY STAGES IN B LYMPHOYCTE DEVELOPMENT FROM CORTICOSTERONE-INDUCED APOPTOSIS AND CELL CYCLE ARREST

ABSTRACT

Independent and combined effects of the cytokines interleukin-7 (IL-7) and stem cell factor (SCF) on spontaneous verses glucocorticoid (Gc) induced apoptosis in developing B lymphocytes was investigated. These cytokines could potentially protect developing B cells from the negative effects of culturing and Gc exposure, since they act as positive factors during the early stages of normal B cell lymphopoiesis. Murine bone marrow was cultured as a single cell suspension, without stromal cell support, and the various B lymphocyte developmental stages, levels of apoptosis and cell cycle status were determined by flow cytometry. Exogenous addition of IL-7 reduced spontaneous apoptosis in the pro B cell population by nearly half, but had little effect on the survival of the more mature pre and IgM^+ B cells. This cytokine also dramatically protected the pro B cell population from apoptosis induced by the natural murine Gc, corticosterone (Cs), decreasing levels of apoptosis by 60%. IL-7 also afforded modest protection to pre B cells from Cs-induced apoptosis but only minimal protection was provided to IgM bearing cells. Cs exposure resulted in a profound decrease in cycling among pro and pre B cells but once again IL-7 protected pro B cells by eliminating this cell cycle arrest while providing little cell cycle protection to pre B cells. SCF, which can augment lymphopoietic development, similarly caused a 40% decrease in background apoptosis in pro B cells when added alone to short-term cultures. However, in contrast to IL-7, it did not appear to have a significant affect on apoptosis induced by Cs. When SCF was used in combination with IL-7, it did not augment the protection against Cs-induced apoptosis in pro B cells. Therefore although both cytokines could protect pro B lymphocytes from

spontaneous apoptosis, only IL-7 protected early B lymphocytes from Cs-induced apoptosis. Additionally, IL-7 also provided substantial protection of pro B cells from Cs-induced cell cycle arrest. Clearly this cytokine has the potential to dramatically protect early stages in B cell development from agents that alter proliferation or induce apoptosis. It reinforces the importance of IL-7 to the early phases of lymphopoiesis and indicates it has immunotheraputic potential.

INTRODUCTION

Chronic increases in circulating levels of glucocorticoids (Gc) are evident during physiological stresses such as in burn victims, trauma patients, the aged and the malnourished and can cause impaired immune function and lymphopenia (Schleimer et al., 1989). *In vitro*, dexamethasone (a synthetic Gc) has been shown to induce apoptosis and decrease cycling in developing lymphocytes in the marrow, with precursor cells (IgM^-) showing greater sensitivity to these negative effects (Borghesi et al., 1997; Garvy et al., 1993; Merino et al., 1994). Our lab has also demonstrated, *in vivo*, that chronically elevating corticosterone (Cs), resulted in a selective reduction in early B lymphocytes that had not begun to express surface immunoglobulin (Garvy et al., 1993). Therefore Cs might elicit its lymphopenic effect *in vivo*, at least in part, by inducing apoptosis in the early stages of B cell development. Recent data also showed that Cs could dramatically reduce cycling among both pro and pre B cells, suggesting this as another mechanism whereby the steroid might downregulate B cell development (Laakko and Fraker, unpublished observation; King and Cidlowski, 1998).

Blocking Gc-induced cell death and cell cycle reduction in B lymphocytes could potentially help protect against the lymphopenia that can occur under conditions of stress. Cytokines that are critical to B lymphocyte development might be of benefit in protecting these cells from Gc induced apoptosis. IL-7 and SCF are cytokines that could potentially protect early stages in B lymphocyte development from apoptosis. IL-7 was originally identified as a factor produced by stromal cells in long term bone marrow cultures that, independently, promoted proliferation of precursor B cells *in vitro* (Namen et al., 1988).

When added to B lymphocyte cultures, with no other apparent cytokine or stromal cell support, IL-7 promoted the survival of pro B cells that would otherwise rapidly undergo apoptosis in culture (Gibson et al., 1996; Lee et al., 1989). In addition, IL-7 caused dramatic increases in cycling in early B cell progenitors, not expressing surface IgM. Recent evidence suggested that IL-7 might promote survival via phosphatidyl inositol 3 kinase (PI3K) activation of the AKT pathway and/or by upregulation of the anti-apoptotic Bcl-2 protein (Lavagna-Sevenier et al., 1998; Lu et al., 1999). Another stromal cell derived cytokine, SCF, also can promote B cell lymphopoiesis. In combination with IL-7 it promoted the commitment of progenitor cells to the B lineage and it increased IL-7 induced proliferation among pro B cells (McNiece et al., 1991). In addition to amplifying lymphopoiesis, SCF had also been shown to positively affect many progenitors from various lineages when in combination with growth factors specific for those lineages (Ashman, 1999). PI3K mediated activation of the AKT cell survival pathway has also been indicated in SCF signaling via its receptor c-kit (Blume-Jensen et al., 1998). Although both IL-7 and SCF have shown positive effects on precursor B cell development and survival, what effects these cytokines might have on apoptosis among early B cells has not been determined. Moreover the effect of these cytokines on developing B lymphocytes exposed to a potent death-inducing factor, such as Cs, are not known.

The potential of IL-7 or SCF to suppress spontaneous or Cs-induced apoptosis in a short-term (16 hour) culture system was determined here. Murine bone marrow was used as the source for developing B lymphocytes and the degree of apoptosis and cell cycle status was determined using flow cytometry. An established phenotypic scheme

using fluorochrome conjugated monoclonal antibodies to cell surface proteins, developed by Hardy et al (Hardy et al., 1991), was used to identify three stages in B lymphocyte development. The stages are listed in order of least to most mature as follows: pro B cells, pre B cells and IgM^+ cells consisting of immature and mature B cells. The degree of apoptosis and cell cycle status was also determined for each population by measuring the DNA content. The results show that IL-7 and SCF individually provided substantial protection against background apoptosis among pro B cells prior to the induction of cell proliferation, but there was no appreciable protection provided by either cytokine to pre or IgM^+ cells undergoing spontaneous death. Interestingly, IL-7 and SCF did not have the same effect on Cs-induced apoptosis. IL-7 protected pro B cells, and to a lesser extent pre B cells, from Cs-induced apoptosis. In the pro B cell population IL-7 also inhibited the cell cycle arrest induced by Cs, but this was not the case for the pre B cell population. SCF did not appear to have a significant affect on Cs-induced apoptosis either alone or in combination with IL-7. Therefore, both of these cytokines appear to promote survival of cells early in B lymphocyte development but only IL-7 can protect early B cells from Cs-induced apoptosis. This is the first direct evidence that a cytokine may be able to help protect early stages in B cell lymphopoiesis from Cs exposure.

MATERIALS AND METHODS

Bone Marrow Preparation and Cell Culture

Young adult male Balbc/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a temperature and light controlled facility. All protocols used herein were approved by the All-University Committee on Animal Use and Care at Michigan State University.

Bone marrow was flushed from femurs with approximately 1 ml of harvest buffer (Hanks' balanced salts, 1 mM HEPES pH 7.2 and 4% FBS) per bone using a 22 gauge needle. The marrow was processed into a single cell suspension and red blood cells were removed by lysis. Bone marrow cells (1-2 x 10^6 cells/ml) were cultured at 37°C in 7.5% CO_2 using RPMI 1640 containing 5 x 10^{-5} M 2-mercaptoethanol, 1 mM HEPES pH 7.2, 1000 units/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 5% fetal bovine serum (FBS). FBS was purchased from HyClone (Logan, UT) and the same lot of serum was used throughout.

Corticosterone was purchased from Sigma (St. Louis, MO) and both recombinant murine IL-7 (rmIL-7) and recombinant murine SCF (rmSCF) were purchased from R&D Systems (Minneapolis, MN). Cs was used at 0.1 μM, unless otherwise stated. Both SCF and IL-7 were titered for maximum activity and used at 100 ng/ml and 0.1 ng/ml, respectively, unless otherwise stated. The standard culture time was 15 to 16 hours.

Immunophenotyping/DNA staining

Antibodies to B cell surface antigens were added to samples at optimum concentrations and samples were maintained at approximately 5°C throughout

phenotyping. Cells were incubated for 25 minutes, simultaneously, with phycoerythrin (PE) conjugated anti-CD45R (B220), fluorescein (FITC) conjugated anti-CD43 (S7) and biotinylated anti-IgM F(ab')2 (IgM) in label buffer (HBSS containing 2% FBS, 1mM HEPES and 0.1% sodium azide). Antibodies against S7 and B220 were purchased from Pharmingen (San Diego, CA) and anti-IgM was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Following primary staining, Streptavidin-Red670 (R670), purchased from Gibco (Grand Island, NY), was added for conjugation to biotinylated anti-IgM for 20 minutes and then washed two times with label buffer.

Following phenotyping, cell pellets were resuspended in 50% FBS and fixed by slow addition of 1.2 ml of ice cold 70% ethanol with gentle mixing. Cells were left in the fixative for at least one hour and for as long as three days. At least one hour before FACS analysis, fixed samples were incubated at room temperature in 0.5-1.0 ml of DAPI staining solution (1μg/ml DAPI and 0.01 mM EDTA in phosphate buffered saline) for DNA analysis of apoptosis and cell cycle.

Flow Cytometry

Samples were analyzed on a Becton Dickinson FACS Vantage flow cytometer. FITC, PE and R670 fluorochromes were excited at 488 nm, and emission was detected at 530, 575 and 670 nm, respectively. DAPI was excited at 365 nm and emission was detected at 470 nm.

Debris and cellular aggregates were excluded from analysis by gating based on size and DNA content and fluorochrome conjugated isotype antibodies were used as

negative controls. Cell size was determined by forward and sideward light scatter and DNA content was determined by DAPI width versus area. According to the phenotypic scheme developed by Hardy (Hardy et al., 1991), B lymphocyte subsets were defined as follows: pro B cells were $B220^{+}S7^{+}IgM^{-}$, pre B cells were $B220^{+}S7^{-}IgM^{-}$ and immature and mature cells were defined as $B220^{+}IgM^{+}$. Each population was gated and analyzed for apoptosis (hypodiploid DNA) and for cell cycle status. PClysis (Becton Dickinson) or WinList (Verity Software House, Inc., Topsham, ME) software were used to process flow cytometric data.

Statistics

Microsoft Excel software was used to analyze data and perform statistics. Data are presented as the mean ± the standard deviation and significance was established using the Student's t-test ($p < 0.05$).

RESULTS

Analysis of the Effect of IL-7 and Cs on Short-Term Marrow Cultures

Cytokine signals are often required for the survival of cells of the immune system. Early stages in B cell lymphopoiesis depend on IL-7 for proliferation, differentiation and long-term survival (Lee et al., 1989; Mertsching et al., 1996; Sudo et al., 1989; Valenzona et al., 1998). Here a short-term culture system where stromal cells and their effects were negligible was used to directly analyze the effect of IL-7 on developing B cells. For this type of culture system it was also important to use a rather short time period where necrosis and cell losses due to culturing and Cs addition were minimal. This was to ensure accurate comparisons between treatment groups and to determine IL-7 effects independent of its proliferative potential. Thus the majority of studies were performed in 16 hours.

Table 4.1 shows the recovery and the viability of nucleated cells from bone marrow after 16 hours in culture with media alone, 0.1 ng/ml IL-7, 0.1 μM Cs or IL-7 in combination with Cs. Microscopic cell counts were performed to determine recovery and viability by trypan blue exclusion; apoptotic cells exclude trypan blue and were considered viable in cell counts. The viability at the initiation of cultures was approximately 90% (data not shown). At the end of a typical 16 hour period there was excellent viability in control cultures (93%) with some expansion of cells (126% recovery). Cells incubated with Cs alone had only a slight reduction in viability, decreasing from 90% viable at culture initiation to 82% post-culture. Therefore under these culture conditions there was a modest expansion in B cells and the viability did not

differ by more than 10% of the viability of freshly plated cells. The effect of IL-7, alone, on recovery and viability was analogous to untreated cultures, however it provided good protection to Cs-treated cells keeping proliferation and survival to levels analogous to media alone.

Protective Effects of IL-7 on Spontaneous and Cs-Induced Apoptosis Among Pro B Cells

To determine the specific effect of IL-7 on the survival of cells of the B lineage, the marrow was phenotyped and DNA analysis of the pro, pre and IgM^+ cells was assessed by flow cytometry. Figure 4.1 shows that the addition of IL-7 resulted in a 40% decrease in spontaneous apoptosis among pro B cells (Panel A). However, this protection from apoptosis was not evident in either the pre B cell or IgM^+ cell populations. Panel B of Figure 4.1 shows IL-7 also provided dramatic protection from Cs-induced apoptosis causing a 60% decrease in apoptosis among pro B lymphocytes. The IL-7 reduction in Cs-induced apoptosis in pre and IgM^+ B lymphocytes was much less than that seen in pro B cells; there was a 30% reduction in pre B cell apoptosis and only a 15% decrease in IgM^+ cell apoptosis. Therefore, IL-7 protected pro B lymphocytes from both spontaneous apoptosis and Cs-induced apoptosis during a 16 hour culture period. Interestingly, although IL-7 had been shown to act as a growth factor for pre B cells (Mertsching et al., 1996; Sudo et al., 1993), it did not appear to inhibit the background apoptosis that occurred when these cells were incubated in culture media alone, but did modestly protect the pre B cells from Cs-induced apoptosis. Therefore the mechanism of IL-7 protection varies between protection from spontaneous apoptosis

verses protection against Cs-induced apoptosis, since the cytokine reduced Cs-induced apoptosis without affecting spontaneous apoptosis.

The Ability of IL-7 to Maintain Cell Cycling of B cells in the Presence of Cs

While IL-7 can enhance proliferation in early B lymphocytes, Cs can induce cell cycle arrest in G_0/G_1 in some cell types, especially early B cells (King and Cidlowski, 1998). Since the above data showed that IL-7 dramatically protected pro B cells, and to a lesser extent pre B cells, from Cs-induced apoptosis it was important to determine if it could protect against changes in cell cycle induced by Cs. Data in Figure 4.2 shows the change in cycling status of pro and pre B cells when IL-7 was added alone, Cs was added alone or Cs and IL-7 were added in combination to short-term BM B lymphocyte cultures. Data was normalized to control levels of pro and pre B cells in the $S/G_2/M$ phases of the cell cycle cultured in media alone. During 16 hours of culture, the addition of IL-7 did not enhance the number of pro or pre B cells in the $S/G_2/M$ phases of the cell cycle. A significant increase in IL-7 induced cycling in pro and pre B cells was not observed until 24 hours after culture; by 48 hours there was nearly a 4-fold increase in pro B cells in $S/G_2/M$ and a 2.7-fold increase in pre B cells in $S/G_2/M$ in cells treated with IL-7 (data not shown). The latter showed that the IL-7 used was biologically active on precursor B cells. Addition of Cs to cultures resulted in a substantial decrease in pro and pre B cells in the $S/G_2/M$ phases of the cell cycle. Cells in a cycling phase were decreased by nearly 50% in pro B cells and by 57% in pre B cells. Interestingly, when IL-7 and Cs were added to cultures simultaneously, IL-7 was able to completely override the cell cycle arrest induced by Cs in pro B cells, maintaining the level of cycling to that

seen in media or IL-7 only treated cultures. By contrast, IL-7 had no effect on the Cs-induced decrease in cycling observed in pre B cells. These results show that IL-7 totally protected pro B cells from the Cs-induced cell cycle arrest. In contrast, IL-7 did not inhibit the cell cycle arrest induced by Cs in pre B cells, suggesting a variable effect by the cytokine on changes in cycling in pro and pre B cells caused by Cs.

DNA Histograms of IL-7 Protected Pro B cells

The above data indicate that IL-7 elicited its greatest effect on the earliest committed B cell progenitors, the pro B cell, both by protecting the cells from spontaneous and Cs-induced apoptosis and by eliminating the Cs-induced cell cycle arrest. Figure 4.3 shows the DNA distribution for representative pro B cells ($B220^+S7^+IgM^-$) as determined by flow cytometry. The percent of apoptotic cells (hypodiploid DNA content) and cells in the $S/G_2/M$ phases of the cell cycle (hyperdiploid DNA content) are indicated for each treatment group. Following 16 hours in culture in media alone, pro B lymphocytes underwent low levels of spontaneous apoptosis (less than 10% of pro B cells) and maintained a significant percentage of cells in the $S/G_2/M$ phases of the cell cycle (18.5% of pro B cells). The addition of 1 ng/ml IL-7 resulted in a reduction in background apoptosis of approximately 40%, but it did not dramatically affect the percent of pro B cells in $S/G_2/M$ phases. Therefore, IL-7 induced survival does not appear to be due to any enhancement of proliferation at 16 hours.

As discussed, the addition of Cs at 0.1 μM to short-term BM cultures resulted in an increase in apoptosis in pro B cells from 10% in background cultures to 25% apoptosis. Cs also dramatically reduced the percentage of pro B cells in the $S/G_2/M$

phases of the cell cycle by more than 50%. Whether Cs resulted in selective induction of apoptosis in cycling cells or induced an arrest of cycling cells in G_0/G_1 was not determined here, but previous studies have shown that Cs induces cell cycle arrest prior to induction of apoptosis. However as before, the addition of IL-7 to Cs-treated cultures resulted in a dramatic decrease in the percent of apoptotic pro B cells (Figure 4.3 D). In fact, the protection from Cs-induced apoptosis in this cell type appeared to be complete, since IL-7 reduced levels of apoptosis to less than levels seen in background cultures (Figure 4.3 A). Interestingly, the percent of cycling pro B cells was unchanged by IL-7 at the 16 hour time point but as before IL-7 offset the reduction in cycling created by Cs in pro B cells (Figure 4.3 D). Therefore, IL-7 appeared to inhibit both the cell cycle arrest and apoptosis induction by Cs in the pro B cell population after 16 hours in culture.

Continued Protection of Pro B cells by IL-7 at Higher Concentrations of Cs

The culture conditions presented in the above experiments were used to ascertain the potential of IL-7 to protect against the effects of Cs on developing B lymphocytes at concentrations analogous to that observed during physiological stress. In the next set of experiments higher concentrations of Cs, some that were analogous to pharmacological levels, were used to determine whether IL-7 continued to protect B cells against apoptosis while maintaining cell cycle status. Phenotypic distribution, degree of apoptosis, and cell cycle status were determined following 15 hours exposure to 0.1 μM, 1 μM or 10 μM Cs (Figure 4.4). At 1 μM Cs, apoptosis appeared to plateau and levels at 10 μM Cs were 32%, 73% and 46% apoptosis for pro, pre and IgM^+ B cells, respectively. IL-7 addition to cultures containing 0.1 μM or 1 μM Cs resulted in similar protection against apoptosis

among the B cell subsets. Apoptosis among pro B cells was decreased by approximately 60% with the addition of IL-7 at either 0.1 μM or 1 μM Cs. Pre and IgM^+ B cells treated with 0.1 μM or 1 μM Cs exhibited IL-7 induced decreases in apoptosis of 35% and 19%, respectively. The degree of protection afforded by IL-7 to cells treated with 10 μM Cs for pro and pre B-lymphocytes was not as extensive as for lower concentrations of steroid. IL-7 caused a 43% reduction in pro B cell apoptosis and a 27% decrease in pre B cell apoptosis of cell treated with 10 μM Cs. However 10 μM is a pharmacological level of this steroid. Consistent with the previous cell cycle data presented, IL-7 overrode the decrease in pro B cell cycling caused by Cs at each concentration. However pre B cycling, as before was substantially reduced by Cs and remained unprotected by the addition of IL-7 (data not shown). These results show that IL-7, even at high concentrations of Cs, maintained its ability to substantially protect pro B lymphocytes from apoptosis and decreases in cell cycling.

The Effect of SCF on Background and Cs-Induced Apoptosis in BM B Lymphocytes

Since SCF also has beneficial effects on early B cell development, its effects on background and Cs-induced apoptosis in B lymphocytes were also investigated. Figure 4.5 shows the effect of SCF on background and Cs-induced apoptosis in pro, pre and IgM^+ cells. SCF was added to cultures at predetermined concentration of 100 ng/ml and Cs was used at a concentration of 0.1 μM. The addition of SCF to cultures, resulted in a decrease in spontaneous pro B cell apoptosis of 40% but pre and IgM bearing cells were not significantly protected. In contrast to the dramatic effect of SCF on background apoptosis in pro B cells, only a small protective effect was seen on Cs-induced apoptosis

in these cells. In fact, the decrease of 17% in pro B cell apoptosis might be attributed to SCF protection from basal apoptosis as described above. Additionally, pretreatment of cells with SCF for 20 hours did not result in decreased apoptosis beyond that presented here (data not shown). Therefore, although SCF appeared to protect pro B cells from basal apoptosis in culture it did not necessarily protect these cells from Cs-induced apoptosis, in contrast to the effect of IL-7. This suggests that SCF might promote *in vitro* survival, similar to IL-7, but it does not protect these cells from the negative effect of Cs.

Since SCF acts synergistically with IL-7 to promote lymphopoiesis, it might synergize with IL-7 to protect pro B cells from Cs-induced apoptosis. To determine this, SCF together with IL-7 was added to cultures and their effects on apoptosis in pro B cells analyzed. Table 4.2 shows the percent of apoptosis in pro B cells cultured alone or with SCF, IL-7 and Cs in various combinations. IL-7 was also added at more dilute concentrations to obtain a lower level of protection from Cs-induced apoptosis, since at 0.1 ng/ml it reduced apoptosis to background levels in pro B cells therefore any effect by SCF may not have been apparent. The data indicated that SCF did not appear to synergize with IL-7 to protect pro B cells from Cs-induced apoptosis. Additionally the combination of SCF and IL-7 did not result in more protection from background apoptosis. Pretreatment with SCF for 20 hours also did not provide enhancement of the protective effect by IL-7 (data not shown). Clearly, although both SCF and IL-7 promote the survival of pro B cells in culture these cytokines do not synergize in the protection of these cells from Cs-induced apoptosis.

DISCUSSION

The survival of hematopoietic cells often depends on signals sent from cytokines. The limiting amounts of cytokines present in short-term marrow cultures can result in enhanced cell death. Here we have shown that both SCF and IL-7 appear to act as survival factors specifically for the pro B cell stage during B cell development by reducing the degree of background or spontaneous apoptosis in these cells after approximately 16 hours in culture. This reduction in spontaneous apoptosis preceded the enhancement of proliferation by either cytokine, which did not occur until 24 hours. This suggests separate mechanisms for cell cycle control and cell survival.

The promotion of pro B cell survival in culture did not necessarily mean that either IL-7 or SCF could protect these cells from the induction of apoptosis by Cs. Here it was shown that IL-7 dramatically reduced Cs-induced cell death in pro B lymphocytes and it also inhibited the cell cycle arrest caused by the steroid. Therefore IL-7 appears to block Cs signaling of apoptosis and/or to promote cell survival signals that overrides Cs death promoting and cycle blocking activity. Even though IL-7 prevented the cycle block it did not cause increased proliferation, above background levels, until after 24 hours in culture, suggesting that protection from apoptosis occurred earlier than the enhancement of cell cycling.

Gc-induced apoptosis has been studied for many years, yet its exact mechanism of eliciting death has not been established. It is becoming clear that Gc signaling through the GcR can affect many other intracellular signaling pathways. For example, Gc has been shown to negatively affect the nuclear factor kappa B (NFκB) and AP-1

transcription factors, both of which can promote survival and proliferation of lymphocytes (Auphan et al., 1995; Heck et al., 1994). Additionally, some evidence exists that Gc may promote apoptosis by the activation of phosphatidyl inositol dependent phospholipase C and subsequent activation of sphingomyelinase and ceramide release (Cifone et al., 1999). It is apparent that much more investigation will be needed to determine the exact mechanism of Gc-induced cell death and it is likely that different cell types may use different pathways to induce death.

The recently described AKT pathway, which promotes survival by causing the cytosolic sequestration of the pro-apoptotic protein Bad, may be a viable mechanism for the survival induced by both IL-7 and SCF. The receptors for both IL-7 (IL-7R) and SCF (c-kit) have been previously reported to have phosphatidyl inositol 3-kinase (PI3K) activity upon ligand binding (Blume-Jensen et al., 1998). AKT has been shown to be phosphorylated by the PI3K pathway, thus rendering it active to phosphorylate the cytosolic protein 14-3-3 that sequesters Bad (Datta et al., 1997).

Reports on the expression of the IL-7R on B lymphocytes have varied, but it now seems clear that the high affinity form of the receptor is present on IgM^- cells (pro and pre B cells) (Sudo et al., 1993). If receptor activity was the same in pro and pre B cells, the differences in IL-7 mediated protection from apoptosis and cell cycle arrest must be attributed to variations in intracellular conditions and signaling. Clearly, the use of phenotypic and apoptotic analysis provided great insight into the response of primary developing B lymphocytes from the BM to specific cytokines. The use of primary cells for these studies was very important in determining responses such as survival, since

normal cell survival, cell proliferation and cell differentiation are often compromised in cell lines.

Interestingly, IL-7 elicited a modest, yet significant protective effect against Cs-induced apoptosis in pre B lymphocytes even though it did not promote background survival in this cell type. This suggests that IL-7 may be utilizing different mechanisms to promote background survival (perhaps the AKT pathway) than that used in promoting protection from Cs-induced apoptosis. Since Bcl-2 upregulation by IL-7 has been previously reported, it could be that protection from Cs is mediated by increased expression of this anti-apoptotic factor (Hernandez-Caselles et al., 1995; Karawajew et al., 2000). Also of interest, although apoptosis was decreased, IL-7 did not inhibit the Cs-induced cycle decrease in pre B cells. Therefore it appears from the pre B cell results, that the protective effect of IL-7 on Cs-induced apoptosis can be separate from its ability to inhibit cell cycle arrest.

Considering the similar ability of IL-7 and SCF to provide protection of pro B cells from spontaneous cell death, it would be reasonable to assume similar effects on Cs-induced apoptosis. This was not the case. SCF provided no appreciable protection from Cs treatment, above that seen in background cultures, in any cell type. Also when used in combination with IL-7 it did not appear to augment the protective effects of IL-7. Poor activity of the recombinant SCF could be one explanation for the lack of apparent effects, but this is unlikely since titrations were performed to determine the maximum effect of SCF and SCF elicited protective effects on background apoptosis. Had there been no activity or low activity, it would be likely that SCF-induced protection from spontaneous apoptosis would not have been apparent.

By using methodology that allowed for the direct analysis of apoptosis in primary developing B lymphocytes it was determined that IL-7, in fact, could protect early B lymphocytes from Cs-induced apoptosis. Considering the devastating affect that Cs elevation can have on the immune system, IL-7 may warrant further investigation as an immunotheraputic agent. The mechanisms by which Cs elicits its effect or whereby IL-7 can inhibit those effects does not appear to be a simple process, the differential effects on apoptosis and cell cycle status between cell types suggests a complex interplay between these two signaling systems. It does appear that IL-7 mediated protection from background apoptosis and Cs-induced apoptosis utilizes at least two different pathways. Additionally, protection from apoptosis clearly occurs prior to induction of proliferation and the two processes appear to be regulated separately. SCF, while similar to IL-7 in protection from background apoptosis, does not appear to protect B lymphocytes from Cs-induced apoptosis. This suggests that either these cytokines initiate different modes of survival or that a different mechanism exists whereby IL-7 can also promote protection from Cs-induced death. A combinatorial approach between the analysis of biological responses and cell signaling analysis may aid in the elucidation of some of the interactions between Cs-mediated and IL-7-mediated responses.

Table 4.1 Recovery and viability of bone marrow cultured for 15 to 16 hours

	Media only	0.1 μM Cs	0.1 ng/ml IL-7	Cs and IL-7
Recovery[a]	**126 ± 9%[c]**	**106 ± 8%**	**127 ± 8%**	**123 ± 18%**
Viability*[b]	**93 ± 3%**	**82 ± 3%**	**90 ± 3%**	**86 ± 3%**

[a] Recovery was determined by dividing the viable and apoptotic cells obtained following culture by the number of viable cells added to the original culture
[b] Viability was determined by dividing the viable and apoptotic cells obtained following culture by the total of viable, apoptotic and trypan blue$^+$ cells (dead)
[c] Cells were cultured for 15 to 16 hours, the data are averaged from five separate experiments and standard deviations are shown
* Cell viability was approximately 90% at the initiation of cell cultures

Figure 4.1 The effect of IL-7 on spontaneous levels of apoptosis verses Cs-induced apoptosis in pro, pre and IgM^+ B lymphocytes. BM was cultured for 16 hours either with 0.1 ng/ml IL-7 only, 0.1 μM Cs only or with a combination of IL-7 and Cs. Phenotypic and DNA analysis were performed using flow cytometry. Panel (A) shows the change in spontaneous apoptosis elicited by the addition of IL-7. Data was normalized to the percent of apoptosis in cells cultured in media alone, with actual percent apoptosis of 7%, 16% and 11% for the pro, pre and IgM^+. Panel (B) shows the change in Cs-induced apoptosis caused by IL-7. Data was normalized to the percent apoptosis in cells incubated with Cs alone, with actual percentages of 23%, 53% and 39% for pro, pre and IgM^+ cells, respectively. IL-7 induced changes in apoptosis were determined for four separate experiments and were averaged with standard error bars shown. Significant differences were determined by the Student's t-test ($p < 0.05$) and indicated with an asterisk.

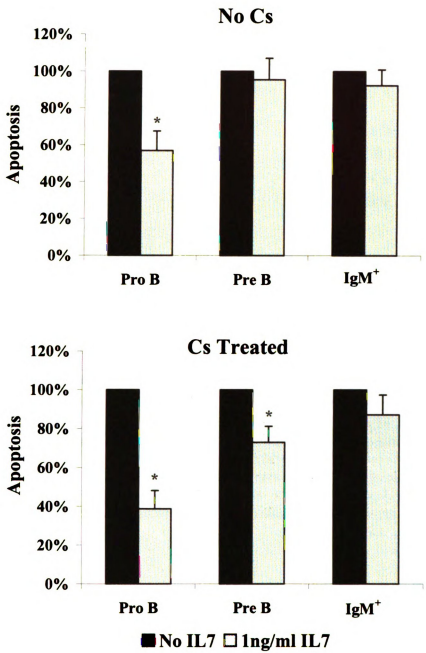


Figure 4.1 IL-7 modulation of spontaneous and Cs-induced apoptosis

Figure 4.2 Data show the effect of added IL-7 on the $S/G_2/M$ phases of the pro and pre B lymphocyte cell cycle of cells cultured in media alone or treated with Cs. BM was cultured for 16 hours in media alone (solid bars), with 0.1 ng/ml IL-7 alone (sparse dots), with 0.1 μM Cs alone (stripes) or with a combination of both IL-7 and Cs (dense dots). Data was normalized to 100% percent for pro or pre B cells in the $S/G_2/M$ phase of the cell cycle when cultured in media alone; actual percentages were 17% for pro B cells and 15% for pre B cells (controls). The percentage $S/G_2/M$ was determined via flow cytometric analysis of the DNA content and pro and pre B cells were identified by phenotyping. The data shown are the average of two separate experiments and standard error bars are shown. Significant differences were determined by the Student's t-test ($p < 0.05$) and indicated with an asterisk.

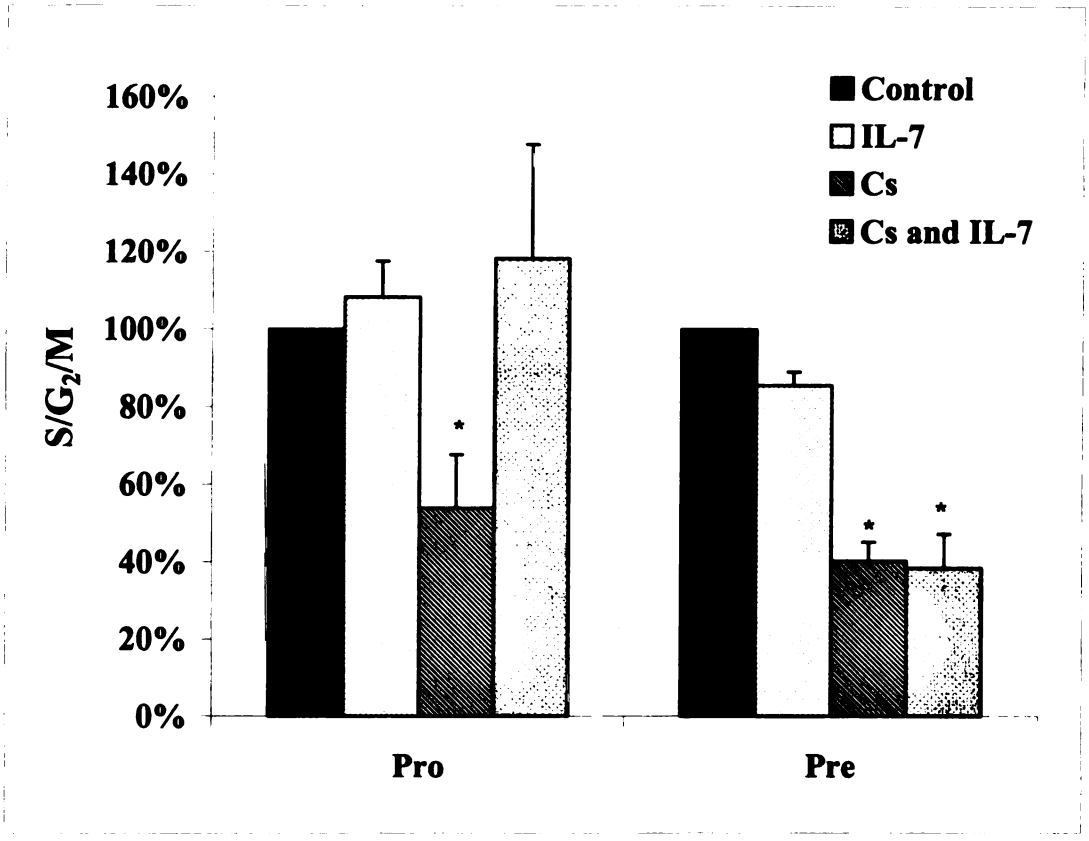


Figure 4.2 IL-7 and/or Cs effect on cell cycle distribution among pro and pre B cells

Figure 4.3 A representative DNA flow cytometric profile of pro B lymphocytes cultured for 16 hours was determined by DAPI staining of phenotyped BM B cells. The hypodiploid, or apoptotic, populations are indicated by "A_0". Panel (A) shows the DNA content of pro B cells incubated in media alone. Panel (B) shows the percent A_0 and $S/G_2/M$ for cells incubated with 0.1 ng/ml IL-7 alone and panel (C) shows pro B cells incubated with 0.1 μM Cs. Panel (D) is the DNA profile of pro B cells incubated with both IL-7 and Cs. These data are representative of several experiments.

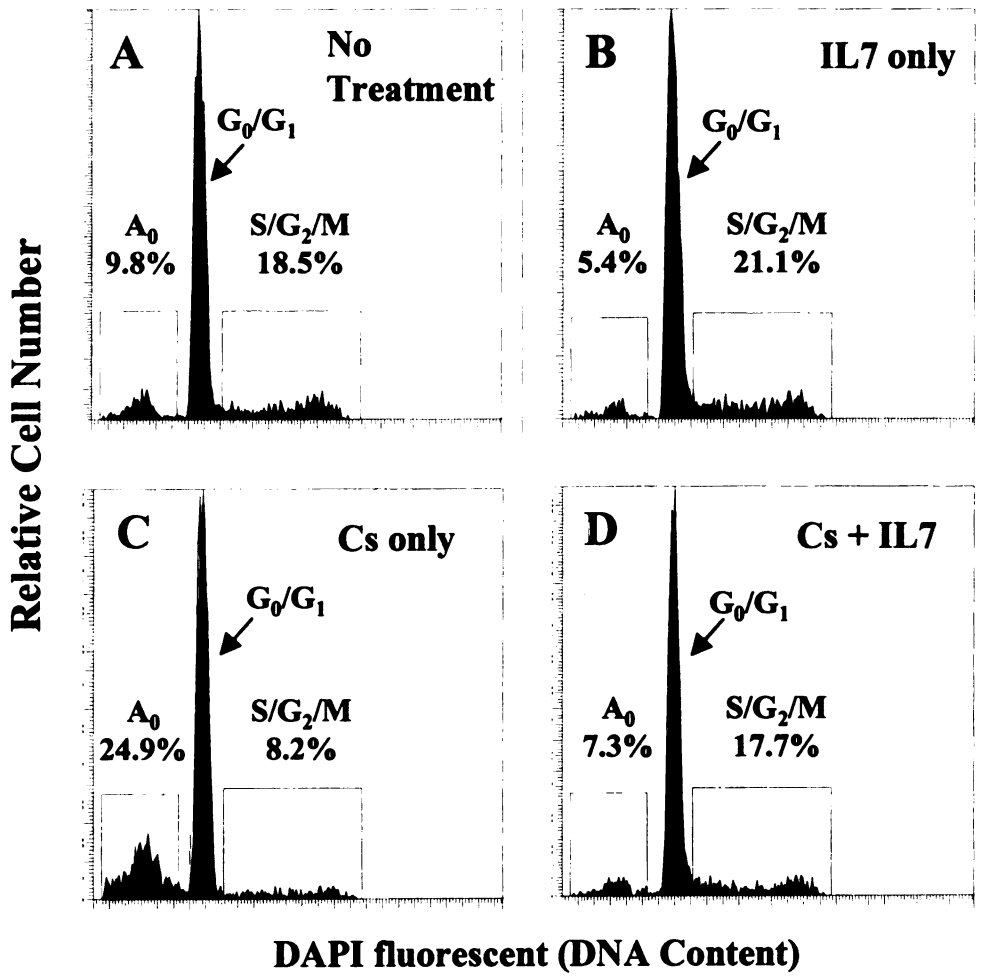


Figure 4.3 Flow cytometry of IL-7 and/or Cs effect on pro B cell apoptosis and cell cycle

Figure 4.4 Data show that IL7 protected B lymphocytes from Cs-induced apoptosis even at high concentrations of the steroid. The percent apoptosis, as determined flow cytometrically by DAPI stained DNA, is shown for **(A)** pro B **(B)** pre B and **(C)** IgM^+ B cells. BM was cultured for 16 hours either with no Cs, 0.1 μM, 1 μM or 10 μM Cs (solid line) and the percent apoptosis was plotted. The percent apoptosis for each population incubated with the above concentrations of Cs and with 0.1 ng/ml IL7 (broken line) is also shown. Standard error bars are plotted and the data shown are representative of at least two separate experiments.

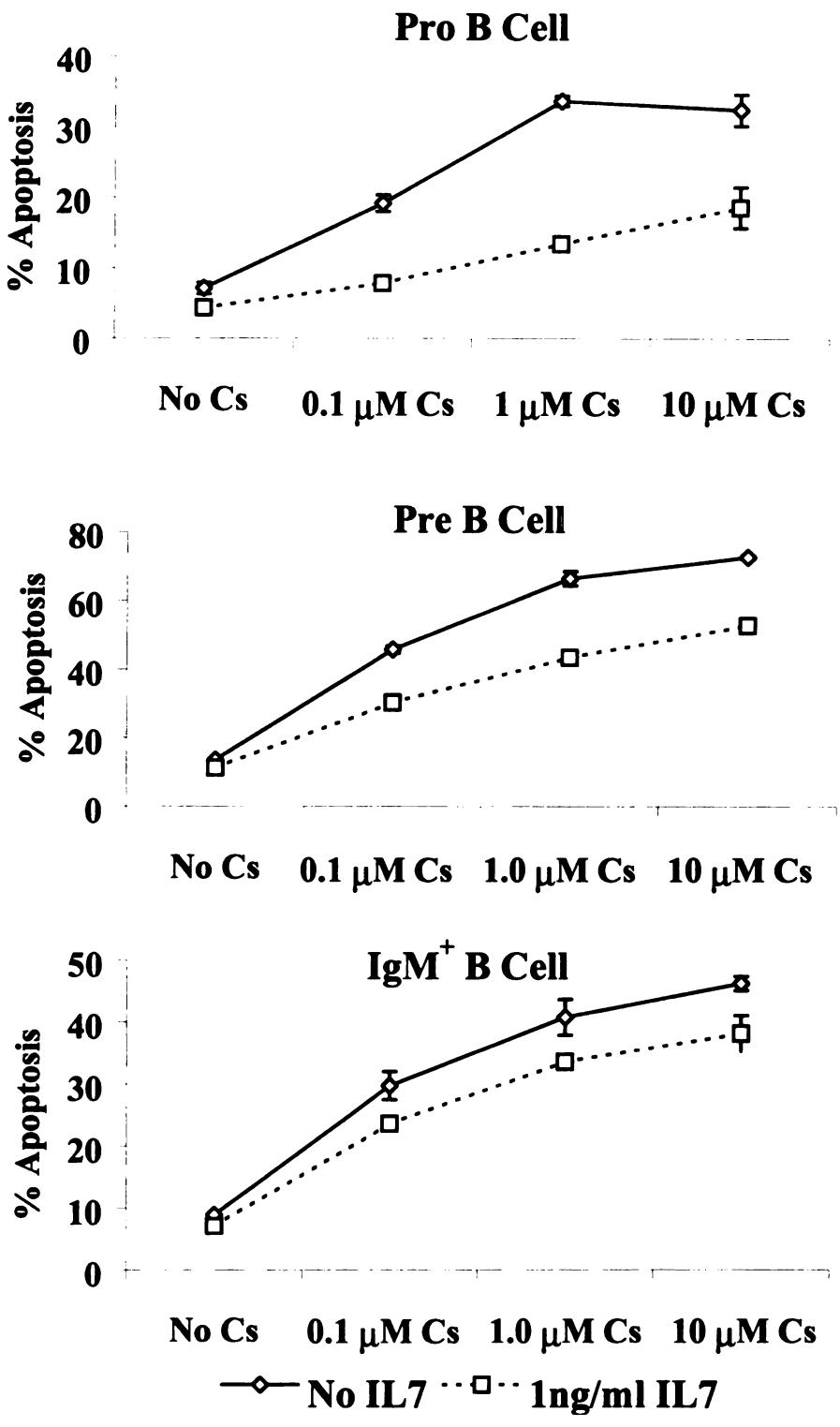


Figure 4.4 IL-7 effect on apoptosis induced by varying concentrations of Cs among B cells

Figure 4.5 The effect of SCF on spontaneous and Cs-induced apoptosis in bone marrow B cells. BM was cultured for 15 to 16 hours, cells were phenotyped for B cell subsets and DNA was stained with DAPI to determine the amount of apoptosis by flow cytometry. SCF was used at 0.1 μg/ml and Cs was used at 0.1 μM. Panel (A) shows the effect of SCF on spontaneous apoptosis in media alone. Data were normalized to the amount of apoptosis in cells cultured without SCF addition with actual percentages of 12%, 16% and 16% for pro, pre and IgM^+ cells, respectively. Panel (B) shows the effect of SCF on Cs-induced apoptosis. Data were normalized to the level of apoptosis in cells cultured with Cs only with actual control percentages of 22%, 49% and 40%, respectively. Standard error bars are shown for the average of three separate experiments where significant differences were determined by the Student's t-test ($p < 0.05$) and indicated by an asterisk.

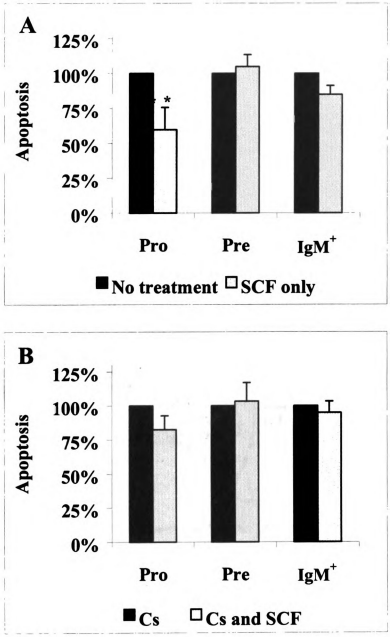


Figure 4.5 SCF effect on spontaneous and Cs-induced apoptosis

Table 4.2 **Effects of IL-7 and SCF on apoptosis in pro B cells**

	No Treatment	SCF[a]	Cs[b]	Cs and SCF
No IL-7	8.7 ± 0.5% [c]	4.3 ± 1.2%*	23.4 ± 4.1%	16.3 ± 1.9%**
0.1 ng/ml IL-7	3.6 ± 2.2%	3.6 ± 0.9%	7.3 ± 2.8%	7.1 ± 1.1%
0.01 ng/ml IL-7	N/D	N/D	14.1 ± 0.7%	13.8 ± 2.3%
0.001 ng/ml IL-7	N/D	N/D	18.1 ± 2.8%	19.2 ± 1.0%

[a] SCF was used at 100 ng/ml

[b] Cs was used at 0.1 μM

[c] Data are the averages of duplicate experiment ± the standard deviations and represent at least two experiments

* Significantly different from control sample ($p < 0.05$)

** Significantly different from Cs sample ($p < 0.1$)

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