ACTIVATION OF THYMIC T CELLS BY MHC ALLOANTIGEN CAN REQUIRE SYNGENEIC ACTIVATED CD4 T CELLS AND B CELLS AS APC

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ABSTRACT

An immunological mechanism to account for the regulation of peripheral self-reactive T cells, which escape central tolerance in the thymus, during the primary activation of naïve, foreign antigen-specific T cells remains to be established. Contemporary models of primary T cell activation that attempt to describe how this occurs differ significantly in the cellular interactions necessary for naïve CD4+ T helper cell activation. It is generally accepted that most CD8+ T cells are dependent upon CD4+ T helper cells for their activation.

The Infectious Non-Self and Danger Models of CD4+ T cell activation propose that interaction of a naïve T cell with an appropriately armed dendritic cell is sufficient, whereas the Two-step, Two-signal Model proposes additional cellular interactions are necessary. The major goal of this thesis was to establish and utilize an *in vitro* experimental system that would allow one to begin to delineate which model most validly describes the cellular interactions required for generation of primary immune responses from naïve T cells. Employing a population of naïve T cells uncontaminated with any partially or fully activated cells is essential for such a study.

The results presented in this thesis show, that when thymocytes are employed as a source of responding naïve T cells, cellular interactions, in addition to interaction with bone marrow derived dendritic cells, are required for the activation of naïve thymic T cells. The primary activation of thymic T cells to generate CD4+ IL-2 producing cells, and CD8+ IFN-γ producing cells and cytotoxic T cells upon stimulation with splenic allogeneic stimulator cells is critically dependent upon the

presence of a syngeneic population of radiation resistant, CD4+ T cells found in the spleen of normal mice. Additionally, when such cells are present as a source of "help" for thymocytes, allogeneic bone marrow derived dendritic cells fail to stimulate the generation of optimal cytotoxic and cytokine responses from naïve thymic T cells. However, they do stimulate thymocytes to cycle and up regulate the ligand for the costimulatory molecule CD40, CD40L.   The results presented within also show that the optimal activation of naïve thymic T cells to generate CD4+ IL-2 producing cells, and CD8+ IFN-γ producing cells and cytotoxic T cells, requires the presence of allo-MHC bearing Ig+ B220+ B cells. The removal of B220+ cells by magnetic cell sorting from the allogeneic spleen reveals that the generation of CD8+ cytotoxic T cells and IFN-γ producing cells from thymocytes is markedly reduced compared to unsorted allogeneic spleen cells. However, IL-2 and IL-4 cytokine producing cells are still detectable. Potential reasons for the generation of the latter cytokine producing cells are discussed. The results presented in this thesis have revealed insights into the cellular interactions involved in the activation of naïve thymic T cells.

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Ab	antibody
Adh	adherent cells
Ag	antigen
APC	antigen presenting cell
ATCC	American type culture collection
C'	complement
CD44	pgp-1
CD45RA	B220
CD62L	L-selectin
CD80	B7.1
CD86	B7.2
CD152	CTLA-4
CD154	CD40L
CD205	DEC-205
CTL	cytotoxic T lymphocytes
CCRx	chemokine receptor
CFSE	carboxyfluorescein diacetate succinimidyl ester
CPM	counts per minute
DTH	delayed type hypersensitivity
DCs	dendritic cells
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescence-activated cell sorter
FCS	fetal calf (bovine) serum
FITC	fluorescein isothicyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
HEL	hen egg lysozyme
ICAM	intracellular adhesion molecule
IFN-γ	interferon-gamma

Ig	immunoglobulin
IL	interleukin e.g. IL-2
L	ligand
LPS	lipopolysaccharide
LU	lytic unit
TLR	TOLL-like receptor
LT	lymphotoxin
MAb	monoclonal antibody
MHC	major histocompatability complex
MLR	mixed lymphocyte reaction
MS	maturation stimulus
OVA	ovalbumin
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline containing tween 20
PHS	pooled human serum
PRR	pattern recognition receptor
RAG	recombination activation gene
RBC	red blood cells
RPMI	Roswell Park Memorial Institute
Rx	treatment
SD	standard deviation
Spl	spleen
SRBC	sheep red blood cells
T	thymocyte
TCR	T cell receptor
Tg	transgenic
TGF	transforming growth factor
TNF	tumor necrosis factor e.g. TNF-α
T_{R-1}	T regulatory cell

1.0. INTRODUCTION

1.1. The Basic Foundations of Immunity

1.1.1. Brief Historical Background

The discipline of immunology, which is defined as the science that deals with the immune response and its humoral and cell-mediated aspects (Merriam-Webster, 2004), had its beginnings in the realization that exposure to an attenuated form of an infectious organism or to a related, non-pathogenic organism, could protect an individual from serious infection upon encounter with a more virulent strain of a pathogen (Clark, 1983; Silverstein, 1999). Since Jenner's demonstration in the 1700's of the generation of immunological protection against smallpox with pre-exposure or vaccination with cowpox virus, people have wanted to understand how vaccination results in the generation of protective immunity, and how this immunity is mediated. The field of immunology thus arose.

At the turn of the 20th century, attempts to delineate the protective immunity induced upon vaccination led to the early identification of two types of immune response. Metchnikoff attributed a major role for phagocytic cells in the defense against pathogens (Metchnikoff, 1968), while at the same time Ehrlich proposed antibody was more important (Ehrlich, 1900; Silverstein, 1999). The debate about

which arm of immunity was the most essential for providing immunological protection ensued for almost 50 years.

It was the realization that both humoral and cellular immune responses are essential parts of the body's specific immune defense against infectious organisms that sparked the beginnings of a modern era of cellular immunology (Clark, 1983; Silverstein, 1999). People wanted to understand what cells were responsible for mediating protection, where they originated, and more importantly how they recognized foreign and not self-antigens. In the 1940's and 50's, Burnet and Talmage formulated the Clonal Selection Theory to provide a rationale for how antibody is produced and how tolerance towards self is achieved (Baxter & Hodgkin, 2002; Burnet, 1959; Jerne, 1955; Silverstein, 2002; Talmage & Pearlman, 1963). Though originally describing the precursor of antibody-producing cells, the Clonal Selection Theory correctly predicted that antibody-producing cells express only a single type of specificity, and the population as a whole thus represents a large repertoire of potentially selectable entities envisaged free of virtually all self-reactive clones.

According to Burnet, antibody-producing cells capable of responding against self-tissues are deleted early in prenatal life (Burnet, 1957, 1959). This model of self non-self discrimination was supported by the experiments of Owen with non-identical dizotic twin cattle that were mutually tolerant of each other's blood cells (Owen, 1945), and the demonstration by Billingham, Brent, and Medawar that adult mice would accept foreign skin grafts if they had been injected in utero with cells from the donors (Billingham, Brent, & Medawar, 1953). According to Lederberg's

proposal, survival versus deletion of immune cells was temporal, and depended on the maturity of the cell and not the developmental state of the animal. Immature cells capable of responding against self-tissues become tolerant upon encounter with antigen and only mature cells are activatable (Lederberg, 1959, 1988). It became evident from Landsteiner's work with hapten-carrier conjugates that the nature of antigen was also important in determining the immunological outcome of administering an antigen (Landsteiner, 1945). Neither Burnet's nor Lederberg's model of self-non-self discrimination could explain how non-immunogenic haptens could be made immunogenic by conjugation to an immunogenic carrier. In order to accommodate all of these findings, Talmage and Pearlman proposed that antigens alone, in non-conjugated forms, result in the induction of tolerance, whereas, aggregates, that possibly associate with complement, deliver additional non-specific stimuli that result in clonal expansion and antibody production (Talmage & Pearlman, 1963).

The cells responsible for mediating immunological protection, and how they recognize antigen, self or foreign, were largely unknown when these models were formulated. Results indicating that circulating lymphocytes are the immunocompetent cells capable of mediating graft rejection and tuberculin immunity came from the experiments of Gowans and Medawar, and Chase, respectively (Billingham, Brent, & Medawar, 1954; Chase, 1945; Ellis, Gowans, & Howard, 1969). The demonstration that circulating lymphocytes are absent from mice thymectomized within a day of birth, and the subsequent susceptibility of these mice to infectious organisms and their inability to reject allogeneic tissue grafts

indicated that the thymus could be essential for immunological function (Claman & McDonald, 1964; Claman & Talmage, 1963; Miller, 1964; Miller, Mitchell, & Weiss, 1967; Mitchell & Miller, 1968b). From experiments originally designed to identify whether the origin of antibody producing cells was thymus or bone marrow dependent it became evident that different cells of the immune system interact with one another to result in the generation of immune responses (Claman, Chaperon, & Selner, 1968; Claman, Chaperon, & Triplett, 1966a, 1966b). The experiments of Miller with semiallogeneic mice confirmed these findings (Mitchell & Miller, 1968a), and the work of Rajewsky & Rottlander, and Mitchison with hapten carrier conjugates, demonstrated that cellular collaboration is involved in the generation of immune responses (Mitchison, 1966; Rajewsky & Rottlander, 1967; Rajewsky, Schirrmacher, Nase, & Jerne, 1969). The ability to break tolerance to self-antigens by conjugation of that antigen to immunogenic carriers (Weigle, 1964) or by challenge with cross-reactive antigens could not be easily explained by either Burnet's or Lederberg's models of self non-self discrimination.

It was at this time that Bretscher and Cohn put forth the two-signal model of lymphocyte activation (Bretscher & Cohn, 1970; Bretscher & Cohn, 1968). This model was originally put forth to describe the activation of antibody producing cells, but was generalized to also apply to cellular responses. Bretscher and Cohn postulated that a free antigenic determinant binding to an antigen-receptor would generate a paralytic signal within a cell, whereas, aggregation of two or more determinants by a "carrier antibody" would induce a second signal that would cause cellular activation (Bretscher & Cohn, 1968). In 1970, the cells capable of inducing

second signals for cell activation were predicted to be the thymus dependent lymphocytes (Bretscher & Cohn, 1970). The experiments of Lafferty focusing on transplant rejection indicated that the lymphocytes mediating rejection need to recognize a cosignal in addition to an antigenic difference (Lafferty, Walker, Scollary, & Killby, 1972). The cells involved in delivering the additional stimulus to lymphocytes were of heamatopoietic origin and needed to be metabolically active in order to deliver the "second" signal (Lafferty, Bootes, Dart, Radovich, & Talmage, 1976; Lafferty, Misko, & Cooley, 1974). Lafferty modified Bretscher and Cohn's two-signal model and suggested a stimulator cell that is bound to the responsive lymphocyte by means of an antigen-bridge provides signal two via a conformational change in an unidentified surface receptor-ligand combination; once activated, he postulated that the lymphocytes no longer require recognition of this second signal.

The discovery that the immune response genes which determine immune responsiveness encode the Major Histocompbatibity Complex molecules, and that T lymphocytes from different strains of mice recognize antigen in the context of these molecules (Doherty & Zinkernagel, 1975; McDevitt & Sela, 1965; Rosenthal & Shevach, 1973), opened up the possibility that reactivity against allografts and normal T cell behavior were similar. Lafferty and Cunningham subsequently suggested that T cells that saw antigen in the context of a self-MHC for autologous reactions, and allo-MHC for alloreactions, trigger the stimulatory cell to provide an additional stimulatory or costimulatory signal (Lafferty & Cunningham, 1975). According to their model, recognition of antigen by the T cell without recognition of the MHC would fail to result in the delivery of a second costimulatory signal.

Evidence in accord with the prediction that antigenic stimulation of mature lymphocytes in the absence of costimulatory signals induces a state of unresponsiveness and that the presence of additional costimulatory signals results in lymphocyte activation came from the work of Jenkins & Schwartz and Nossal (Jenkins & Schwartz, 1987; Nossal & Pike, 1980). The cell involved in delivering the "second signal" had since been identified as an antigen-presenting cell capable of digesting and displaying peptides derived from whole antigens in the context of MHC antigens on their surface (Harding, Leyva-Cobian, & Unanue, 1988; Unanue, 1984). Jenkins and Schwartz found that viable antigen presenting cells were required for the full activation of cloned lymphocytes, and that killed or fixed antigen-presenting cells failed to deliver the required costimulatory signals to T lymphocytes (Jenkins, Ashwell, & Schwartz, 1988). They proposed a new version of Bretscher and Cohn's two-signal model where antigen alone seemed to lead to an inert state, and antigen in the presence of costimulation from viable antigen-presenting cells leads to activation (Schwartz, Mueller, Jenkins, & Quill, 1989).

The observations of Jenkins and Schwartz were instrumental in the delineation of how the activation of lymphocytes occurs upon recognition of antigen (Jenkins, Pardoll, Mizuguchi, Quill, & Schwartz, 1987). In conjunction with the characterization of the T cell receptor (TCR) (Hayday et al., 1985; Marrack et al., 1983), these observations paved the way for the contemporary era of immunology. A vast amount of knowledge has been gained since, particularly in regards to the different cell types involved in mediating immunity, the events that lead to their development, how they recognize antigen, and the means by which protective, and in

some instances, non-protective immune responses, are generated (Abbas, Lichtman, & Pober, 2000; Paul, 1999). However, the means by which the immune system discriminates peripheral self from non-self to selectively generate immune responses against foreign antigens remained enigmatic. This central question in immunology is the focus of this thesis work. In order to properly discuss how the immune response potentially discriminates peripheral self from non-self during the generation of primary immune responses, one must consider current wisdom as to how the cells of the immune response function.

1.1.2. A Brief Synopsis of Modern Day Knowledge of the Immune System: Innate and Adaptive

The immune system of higher vertebrates is now known to be composed primarily of three cell types, which are granulocytes, monocytes, and lymphocytes (Abbas et al., 2000; Clark, 1983; Paul, 1999). The immunological protection provided by these cells can be divided in two separate types of immunity that are referred to as innate and adaptive immunity. The innate response provides an individual with constitutive and pre-existing protection (Medzhitov, 2001). Non-antigen-specific immune cells, such as neutrophils, cells of the monocyte lineage, and NK cells mediate this protection (Bendelac, Bonneville, & Kearney, 2001). By contrast, the adaptive response is acquired, and is mediated by lymphocytes that bear clonally distributed, antigen-specific receptors (Abbas et al., 2000; Clark, 1983; Paul, 1999). The antigen-specific cells that mediate adaptive immunity include antibody-producing B-lymphocytes, αβ TCR expressing CD4+ T helper, and CD8+ cytotoxic

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T lymphocytes. As Burnet and Talmage correctly predicted (Burnet & Fenner, 1949; Talmage, 1980; Talmage & Pearlman, 1963), the expansion of antigen-specific cells after a primary encounter with a foreign antigen enables the adaptive immune response to respond more rapidly if the antigen is encountered again.

The cells of the innate immune response lack the capacity to remember a previous encounter with foreign antigen. However, the cells of the innate immune response play an integral role in the defense against infectious organisms (Medzhitov & Janeway, 1998). They are essential for maintaining tissue integrity, lowering infectious burdens, and are key cells involved in maintaining the homeostasis of antigen-specific T lymphocytes of adaptive immune responses (Hoebe, Janssen, & Beutler, 2004; Holt & Sly, 1999; Moretta, 2002). For example, cells such as γδ T lymphocytes (Hoebe et al., 2004; Kaufmann, 1996) are important for mediating the repair of tissue damage caused by inflammation, and antigen-presenting cells are essential for providing homeostatic survival signals to antigen-specific CD4+ and CD8+ T lymphocytes (Dai & Lakkis, 2001; Holt & Sly, 1999).

More important to the initiation of adaptive responses are the antigen-presenting cell functions of monocyte derived macrophages and dendritic cells (Guermonprez, Valladeau, Zitvogel, Thery, & Amigorena, 2002; Steinman, 1991; Unanue, 1984). These cells are the primary antigen-presenting cells that phagocytose and process protein antigens into antigenic peptides. Antigenic peptides are presented on the surface of antigen presenting cells in the context of protein antigens encoded by a region called the major histocompatibility region located on chromosome 14 in mice and chromosome 6 in man (Abbas et al., 2000; Clark, 1983;

Paul, 1999). This region, which contains the immune response genes that can determine whether particular strains of mice can respond to simple or limiting doses of more complex foreign antigens, encodes protein products referred to as Major Histocompatibility Complex antigens (McDevitt & Sela, 1965; Mozes, McDevitt, Jaton, & Sela, 1969; Rosenthal & Shevach, 1973). Antigenic-peptides bound in a groove of MHC Class II or Class I molecules are presented to $\alpha\beta$ CD4+ and CD8+ T cells, respectively (Davis et al., 1998). Inhibition of antigen processing prevents the recognition of antigen by T cells, and prevents the generation of protective immune responses (Guery, Sette, Leighton, Dragomir, & Adorini, 1992; Jenkins & Schwartz, 1987). The homeostatic and antigen presentation roles of antigen presenting cells are thus essential to the initiation and mediation of protective primary and secondary immune responses.

The antigen-specific cells of the adaptive immune response that mediate primary and secondary immune responses are antibody producing B cells, $\alpha\beta$ TCR expressing CD4+ and CD8+ T lymphocytes (Abbas et al., 2000; Clark, 1983; Paul, 1999). As mentioned previously, these lymphocytes display clonally distributed receptors that enable the recognition of a vast array of foreign antigens (Blackman, Kappler, & Marrack, 1988; Litman, Anderson, & Rast, 1999; Reth, 1992). The ability of the adaptive immune response to recognize a universe of potential antigens is the result of genetic recombination as was originally visualized by Lederberg (Lederberg, 1959). During B and T cell development, recombination events, referred to as V (D) J joining, occur in genes encoding the regions of the B and T cell receptors (Abbas et al., 2000; Clark, 1983; Paul, 1999). The resulting lymphocyte

population that develops is composed of an array of cells, each bearing receptors of a unique antigen-specificity.

B cells bear immunoglobulin receptors on their cell surface (Abbas et al., 2000; Clark, 1983; Paul, 1999). Recognition of antigen by this receptor can result in cross-linking and the internalization of receptor-antigen complexes, and in the presence of the appropriate cooperative interactions with thymus dependent lymphocytes, B cells are stimulated to up-regulate surface molecules, produce growth factors called cytokines, and can be stimulated to terminally differentiate into plasma cells that primarily secrete immunoglobulin (Hodgkin & Basten, 1995; Lanzavecchia, 1990; Lanzavecchia & Bove, 1985; Rush & Hodgkin, 2001). The antibody produced by B cells participates in the immunological protection against pathogens that are found extracellularly, of which circulating Influenza virus is a prime example (Lafferty & Oertelis, 1961). Binding of antibody to circulating virus particles can neutralize the ability of the virus to infect cells, and can prevent it from becoming an intracellular pathogen that is inaccessible to circulating antibody. Binding of antibody to extracellular pathogens can also hasten their removal. Phagocytic cells bear Fc receptors, which bind to portions of the antibody not bound to antigen (Abbas et al., 2000; Clark, 1983; Paul, 1999). When antigen becomes adsorbed to surface bound immunoglobulin it can either be internalized and processed or can be transferred to other lymphocytes (Batista, Iber, & Neuberger, 2001; Batista & Neuberger, 2000). Opsinization of foreign antigens by antibody, such as virus, thus hastens the ability of innate immune cells to process and present antigenic peptides to CD4+ and CD8+ T cells.

B cells also function as antigen-presenting cells. However, their primary means of antigen uptake is immunoglobulin receptor mediated (Brocke, Armandola, Garbi, & Hammerling, 2003; Brocke, Garbi, Momburg, & Hammerling, 2002; Bryant & Ploegh, 2004; Hodgkin & Basten, 1995; Lanzavecchia & Bove, 1985). B cells, as compared to antigen presenting cells such as macrophages, have a 100-1000 fold lower capacity to pinocytose, and a 10-50 fold lower capacity to adsorb antigens (Chesnut, Colon, & Grey, 1982). Antigen-specific B cells achieve maximal efficiency in presenting antigen to T cells by a combination of specific antigen-uptake by the B cell receptor, and efficient antigen-targeting of antigens taken up by the B cell receptor to antigen processing and MHC loading compartments (Brocke et al., 2003; Brocke et al., 2002; Bryant & Ploegh, 2004; Watts, 1997). These qualities enable an antigen specific B cell to present antigen to T cells at 1000 fold lower concentrations than non-specific antigen-presenting cells that can acquire antigen by active macropinocytosis of much larger fluid volumes (Sallusto, Cella, Danieli, & Lanzavecchia, 1995; Unanue, 1984; Watts, 1997) or by adsorption of antigen via a range of endocytosis receptors (Steinman, Hawiger, & Nussenzweig, 2003; Watts, 1997). The latter antigen presenting cells are hence the primary antigen presenting cells that endocytose apoptotic cells.

$\alpha\beta$ bearing CD4+ and CD8+ T cells of the adaptive immune response bear clonally distributed T cell receptors that recognize small fragments of degraded proteins (Abbas et al., 2000; Clark, 1983; Paul, 1999). In order for adaptive immune responses to be initiated, these peptide fragments must be presented to T cells in the context of MHC molecules (Goldrath & Bevan, 1999). The $\alpha\beta$ T cell receptors of

CD4+ T cells recognize peptides in the context of MHC class II molecules, and the receptors of CD8+ T cells recognize peptides in the context of MHC Class I (Cantor & Boyse, 1975; Davis et al., 1998; Doherty & Zinkernagel, 1975; Rosenberg, Mizuochi, Sharrow, & Singer, 1987; Swain, Dialynas, Fitch, & English, 1984). All cells of the body posses the processing machinery to present endogenously derived peptides on their cell surface in the context of MHC Class I antigens. It is primarily specialized cells, the antigen presenting cells, briefly introduced, that possess the antigen uptake and processing machinery to present exogenously acquired antigens on MHC Class II antigens (Abbas et al., 2000). Other cells, such as endothelial cells, can be stimulated by inflammatory stimuli to up regulate expression of MHC class II antigens, but their ability to actively acquire antigen is minimal.

Peptides derived from exogenous phagocytosed antigens are primarily presented to CD4+ T cells on MHC Class II, whereas intracellular antigens, such as those produced during virus infection are primarily presented to CD8+ T cells on MHC Class I (Goldrath & Bevan, 1999; Marrack et al., 2001). A cross-presentation pathway of antigen-presentation occurs in some antigen-presenting cells, most commonly in dendritic cells, whereby exogenous antigens taken up by defined endocytic surface receptors, such as FcγR and defined integrins, gain access to the MHC Class I presentation pathway (Bevan, 1976; Kurts, Cannarile, Klebba, & Brocker, 2001; Kurts et al., 1996; Sigal, Crotty, Andino, & Rock, 1999; Srivastava, 2002). Presentation of the exogenously acquired antigens appears to be an activation dependent process (Albert, Jegathesan, & Darnell, 2001; Delamarre, Holcombe, & Mellman, 2003). Recycled endogenous derived peptides presented on MHC class I

can also gain access to the Class II presentation pathway (Adorini et al., 1991; Gromme et al., 1999).

MHC class II restricted, CD4+ T cells have two immunological roles once activated. Upon recognition of MHC Class II presented peptides, they mediate cellular immune responses by producing inflammatory cytokines, such as IFN-γ, which triggers the activation of macrophages (Blanden, Lefford, & Mackaness, 1969; Coffman & Mosmann, 1991; Unanue, 1984; Wheelock, 1965). The activation of macrophages by CD4+ T cells initiates the recruitment of additional non-specific cells of the innate response, and results in the expression of an immune response termed Delayed Type Hypersensitivity (Blanden et al., 1969; Mackaness & Blanden, 1967).

Activated antigen-specific CD4+ T cells also orchestrate the generation of antigen-specific immune cells (Swain, 1999). The ability of CD4+ T cells to orchestrate immune responses by innate and adaptive immune cells is commonly referred to as T cell help (Husmann & Bevan, 1988; Mitchison, 1971). It is now known that CD4+ T cells are the thymus dependent cells required in Claman's experiments for the optimal production of antibody from helper-dependent B cells (Claman et al., 1966b; Miller & Mitchell, 1969). After specific recognition of a peptide-MHC Class II complex presented on surface of a B cell, CD4+ T cells help B cells differentiate into antibody producing cells by producing B cell growth factors or cytokines (Croft & Swain, 1995; Hodgkin & Basten, 1995; Kehry & Hodgkin, 1993; Lanzavecchia, 1990; Rush & Hodgkin, 2001), and via the ligation of surface antigens CD4+ T helper cells trigger the initiation of intracellular signaling pathways

within the B cell that allow for clonal expansion (DeFranco, 1999; Kehry & Hodgkin, 1993; Rush & Hodgkin, 2001).

In addition to providing antigen-specific B cell help, CD4+ T cells also help another type of thymus dependent lymphocyte, the CD8+ T cells, differentiate into cytotoxic T lymphocytes (Cantor & Boyse, 1975; Keene & Forman, 1982; Pilarski, 1977; Pilarski, Al-Adra, & McKenzie, 1980). CD8+ cytotoxic T cells are potent antigen-specific cells of the adaptive immune response that play a large role in the rejection of allogeneic tissue grafts and the defense against viruses (Doherty & Zinkernagel, 1975; Plata, Cerottini, & Brunner, 1975; Plata, Jongeneel, Cerottini, & Brunner, 1976; Rosenberg et al., 1987). Upon recognition of a foreign peptide-MHC Class I complex, activated CD8+ T cells release short-range proteins, such as perforin, that lyse the targeted cell (Lieberman, 2003; Trambas & Griffiths, 2003). Much like the help for B cells, CD4+ T cells provide help for CD8+ T cells by providing essential differentiation factors, such as the cytokine IL-2 (Dai, Konieczny, & Lakkis, 2000; Smith, 1984; Teh & Teh, 1980), and again via the ligation of surface receptors they trigger the initiation of intracellular signaling pathways within the CD8+ T cell that allow for clonal expansion (Weiss, 1999). In contrast to the CD4+ T help for B cell activation that is provided directly, help for CD8+ T cell activation in mice is delivered indirectly through an MHC Class II and Class I expressing, antigen-presenting cell (Bennett, Carbone, Karamalis, Miller, & Heath, 1997; Ridge, Di Rosa, & Matzinger, 1998). The antigen-presenting cell bridge between CD4+ T helper cells and CD8+ T cells is essential for the generation of primary and secondary antigen-specific cellular responses (Shedlock & Shen,

2003; Sun & Bevan, 2003). Some CD8+ T cell responses can be generated in the absence of CD4+ T cells, such as those generated against LCMV, Sendai, and some strains of influenza virus (Homann, McGavern, & Oldstone, 2004; Wong & Pamer, 2003; Wu & Liu, 1994). In general, these responses fail to be sustained in the absence of appropriate CD4+ T cell help (Bourgeois, Veiga-Fernandes, Joret, Rocha, & Tanchot, 2002; Hunziker, Klenerman, Zinkernagel, & Ehl, 2002; Janssen et al., 2003; Shedlock & Shen, 2003; Sun & Bevan, 2003; Zhan, Corbett, Brady, Sutherland, & Lew, 2000), and hence memory CD8+ T cell responses are impaired.

1.1.3. The Anatomical Organization and Development of Cells of the Immune System

In order to provide the best possible defense against a pathogen when the skin or mucosal barriers are broken, immune cells are in sentinel locations beneath the skin and mucosal tissues, and possess the potential to circulate throughout the body via an extensive system of vessels and immune organs (Gowans, 1959; Gowans & Steer, 1980; Merad et al., 2002; Steinman, 1991). This network is called the lymphatic system. It parallels the circulatory system, collecting any fluid that has leaked from the circulatory system into interstitial spaces. The draining lymphatic fluid and the infectious pathogens it potentially contains are collected and delivered to immune related organs (Abbas et al., 2000; Clark, 1983).

The first lymphoid organ encountered is a lymph node. Lymph nodes are distributed throughout the body and are central for the entrapment and recognition of

foreign antigens by cells of the immune response (Clark, 1983; Harris, Harris, Beale, & Smith, 1954; N. J. Warner, A. Szenberg, & F. M. Burnet, 1962). Antigens may enter the lymph nodes in one of two ways, either suspended in lymphatic fluid or as an engulfed antigen harbored inside a migrating antigen presenting cell (Steinman, 1991). Antigen presenting dendritic cells are specialized for this task (Guery & Adorini, 1995). Upon antigen uptake, they undergo a differentiation step that enables them to extravasate from peripheral tissues, and transmigrate through the lymphatic vessels to the draining lymph nodes (Romani et al., 1989; Sallusto & Lanzavecchia, 2000; Steinman et al., 2003). Draining lymph nodes are the first site where the innate and previously unexposed adaptive cells of the immune system come together for the generation of primary antigen-specific immune responses. While antigens are being presented to antigen-specific CD4+ and CD8+ T cells in the lymph nodes, the cells of the innate immune response are recruited to the site of infection and attempt to contain pathogenic microorganisms by creating an inflammatory environment (Rosenberg & Gallin, 1999).

A second site where innate and adaptive immune cells come together is the spleen (Picker & Siegelman, 1999). In contrast to lymph nodes that are fed by the lymphatic vessels, the spleen receives circulating lymphocytes through the blood (Clark, 1983). The spleen is composed of two sections. The red pulp is composed of RBCs that are destined for removal, and the white pulp is composed of lymphocytes (Abbas et al., 2000).

There are additional lymphoid tissues and organs of the immune system; however, unlike the lymph nodes and spleen, two of these organs, the bone marrow

and thymus, are not involved in the entrapment and recognition of foreign antigens for the generation of immune responses. The major function of these organs is the generation and development of immune cells (Claman, Chaperon, & Hayes, 1969; Miller, 1964; Picker & Siegelman, 1999).

All of the red blood cells and white cells found in the blood and lymph are generated from pluripotent, hematopoetic stem cells in the bone marrow (Abbas et al., 2000; Ikuta, Uchida, Friedman, & Weissman, 1992). In addition to being the major site for hematopoesis in higher vertebrates, the bone marrow is also the primary site where B cell development occurs (Hardy & Hayakawa, 2001; Howard, Hunt, & Gowans, 1972). Only one species, the avian species, possesses a separate organ where B cell development occurs. This organ is called the Bursa of Fabricus and it is located at the lower end of the intestine (Glick, Chang, & Jaap, 1956; Warner & Szenberg, 1964). Its removal at the time of birth results in a complete lack of antibody producing cells (Szenberg, 1970); hence the coining of the term B cell.

Another lymphoid organ, the thymus, is similar to the Bursa of Fabricus in that it also involutes with age (Picker & Siegelman, 1999). However, the thymus is the site of T lymphocyte development rather than B cell development (Miller, 1964). The unique environment of the thymus positively selects progenitor T cells that bear T cell receptors that can recognize self-MHC complexes (Jameson, Hogquist, & Bevan, 1995). The recognition of Class II complexes leads to the development of self-MHC restricted CD4+ T cells, and the recognition of Class I MHC complexes leads to the development of self-MHC restricted CD8+ T cells from CD4+CD8+

double positive thymocytes. Positive selection of self-restricted CD4+ and CD8+ T cells occurs in the cortical regions of the thymus (Anderson, Moore, Owen, & Jenkinson, 1996; Laufer, DeKoning, Markowitz, Lo, & Glimcher, 1996). When these thymocytes migrate into the medullary regions of the thymus, cells that bear T cell receptors that are specific for, and possess high affinity for, peptides derived from ubiquitous self-antigens seen in the context of self-MHC are removed from the developing lymphocyte pool by the induction of apoptosis (Palmer, 2003). The result of T cell development in the thymus is thus a pool of single positive, self-MHC restricted, CD4+ and CD8+ T cells that express a diverse array of $\alpha\beta$ T cell receptors that primarily recognize peptides derived from foreign antigens (Bevan, Hogquist, & Jameson, 1994). While originally describing tolerance induction of antibody producing cells, Burnet and Lederberg correctly predicted that the removal of a vast array of self-reactive lymphocytes, $\alpha\beta$ TCR bearing CD4+ and CD8+ T cells in today's terminology, is essential for tolerance towards self (Burnet, 1959; Lederberg, 1959). The deletion of self-reactive clones in the thymus during T cell development is referred to as central tolerance. However, this mechanism of self-tolerance induction does not remove all of the CD4+ and CD8+ T cells capable of recognizing self-antigens. A number of T cells with specificity for organ specific antigens that are not abundantly expressed in the thymus escape clonal deletion and are present in the peripheral T cell pool (Bouneaud, Kourilsky, & Bousso, 2000; Fowell & Mason, 1993; Goldrath & Bevan, 1999; Jiang et al., 2003; Kuchroo et al., 2002; Lesage et al., 2002).

Developing B cells are also subject to self-tolerance mechanisms (Goodnow, Adelstein, & Basten, 1990). However, unlike T cells that must recognize self-peptide in the context of MHC antigens, helper cell dependent B cells undergo apoptosis when immunoglobulin receptor ligation by antigen occurs in the absence of T cell help (Bretscher, 1975; Fulcher et al., 1996; Goodnow, Crosbie, Jorgensen, Brink, & Basten, 1989). This can occur in the bone marrow, or in the periphery (Chung, Silverman, & Monroe, 2003). The majority of B cells with immunoglobulin receptors for ubiquitous self-antigens are lacking because of the absence of CD4+ T helper cells with self-specificity (Goodnow et al., 1989). A minority of B cells escape clonal deletion because they express immunoglobulin receptors specific for self-antigens inaccessible to the B cell (Ferry, Jones, Vaux, Roberts, & Cornall, 2003). These B cells include those specific for self-antigens that are sequestered inside of cells, which are also normally expressed at very low levels. The existence of such cells in the periphery, and the possibility that cognate peripheral self antigen-specific CD4+ T helper cells are also present leads one to question how the immune system prevents their activation (Cook, Basten, & Fazekas de St Groth, 1998; Lesage et al., 2002) and exemplifies the need for a mechanism to regulate T cell activation in the periphery.

Thus, the immunological question that was a concern even in the days of Ehrlich remains (Ehrlich, 1900); how are immune responses against foreign antigens/pathogens selectively generated and responses against self, in modern terms, peripheral self, circumvented?

1.2. CD4+ T Helper Cells

1.2.1. CD4+ T cell Subsets

CD4+ T helper cells are the orchestrators of the antigen-specific immune responses. Modern knowledge that protective cellular and humoral immunity tend to be mutually exclusive allows the division of activated CD4+ T helper cells into distinct subsets (Mosmann & Coffman, 1989; Swain et al., 1991). While many responses are mixed, CD4+ T cells that help in the generation of cytotoxic CD8+ T cell responses, and delayed-type-hypersensitivity reactions generally belong to the Th1 subset (Cher & Mosmann, 1987; Wong & Pamer, 2003). Th1 responses are effective at containing intracellular pathogens, of which *M. tuberculosis* and *Leishmania* species are prime examples (Bretscher, Hamilton, & Ogunremi, 2002; Sacks & Noben-Trauth, 2002).   Th1-type cytokines include IL-2, IFN-γ, LT (lymphotoxin), IL-12, and IL-18 (Lewkowich & HayGlass, 2002; Mosmann & Coffman, 1989; Nakanishi, Yoshimoto, Tsutsui, & Okamura, 2001; Rempel, Wang, & HayGlass, 1997; Seder, Gazzinelli, Sher, & Paul, 1993; Smeltz, Chen, Ehrhardt, & Shevach, 2002). In addition to aiding in the differentiation of CTLs, the secretion of Th1-type cytokines activates macrophages, and natural killer cells of the innate immune response (Hoebe et al., 2004). On a molecular level, the commitment to the

Th1 lineage is associated with the expression of the transcription factor T-bet (Mullen et al., 2001; Robinson & O'Garra, 2002)

CD4+ T cells that help in the generation of humoral responses generally belong to the Th2 subset (Mosmann & Coffman, 1989). These responses are effective in the defense against pathogens that are extracellular or have a significant extracellular phase, which includes helminth parasites and circulating viruses, respectively (Ahmed & Biron, 1999; Pearce, Scott, & Sher, 1999; Sher, Fiorentino, Caspar, Pearce, & Mosmann, 1991). Th2-type cytokines include IL-4, IL-5, IL-6, IL-13, IL-9, and in some situations IL-10 (Mosmann & Coffman, 1989; Sher et al., 1991; Swain et al., 1991; Swain, Weinberg, English, & Huston, 1990). IL-4 is a growth factor that promotes the proliferation of antibody-producing B cells, and promotes mast cell activation (Pfeifer, McKenzie, Swain, & Dutton, 1987). Other Th2-type cytokines influence the class of antibody produced (Abbas et al., 2000). Th2 lineage commitment is typically associated with the expression of the transcription factor GATA-3 (Farrar et al., 2001; Robinson & O'Garra, 2002; Zhang, Cohn, Ray, Bottomly, & Ray, 1997).

1.2.2. T cell Differentiation into Th1/Th2 Type Subsets

The more general knowledge that CD4+ T cells have different functions depending on their cytokine profile led to their separation into distinct Th1 and Th2 subsets (Mosmann & Coffman, 1989). In some cases, segregation of subsets correlates with either resistance or susceptibility to infection (Bretscher, Ogunremi,

& Menon, 1997; Hailu et al., 2001). In general terms, intracellular pathogens are best controlled by Th1 responses, whereas, extracellular pathogens are best controlled by Th2 responses (Bretscher et al., 2002). The realization that separate subsets correlate with protective immunity spurred the development of a number of experimental systems to delineate what factor(s) influence CD4+ T cell Th1/Th2 lineage commitment (Constant & Bottomly, 1997; Del Prete, 1998). The delineation of such factors is important for an understanding of how CD4+ T cell differentiation occurs in response to antigenic challenge.

Although the exact mechanisms governing the initial lineage instruction of naïve CD4+ T cells is still unknown, a number of factors are believed to influence lineage commitment. The factors influence both *in vitro* and *in vivo* CD4+ T cell differentiation (Murphy & Reiner, 2002; Seder & Paul, 1994). They include the cytokine milieu present at the initiation of responses, the dose of antigen used to stimulate naïve CD4+ T cells, and the nature and the type of antigen presenting cell that presents foreign antigen-derived peptides to naïve CD4+ T cells (Constant & Bottomly, 1997; Hosken, Shibuya, Heath, Murphy, & O'Garra, 1995; Maldonado-Lopez, Maliszewski, Urbain, & Moser, 2001; Moser & Murphy, 2000; Seder & Paul, 1994). Initial research dealing with CD4+ T cell lineage commitment largely dealt with how the cytokine milieu present at priming influences naïve CD4+ T cell differentiation (HayGlass, Wang, Gieni, Ellison, & Gartner, 1996; Mosmann & Coffman, 1989; Swain, Weinberg, English et al., 1990; Yang & HayGlass, 1993).

The influence of cytokines on the development of CD4+ T cell lineage commitment, which follows the initial activation events, is well established. The

presence of IFN-γ, IL-12, or IL-18 favors the generation of Th1 type responses, whereas the presence of IL-4, IL-5, or IL-13 favors the generation of Th2 responses (Bradley, Yoshimoto, & Swain, 1995; Seder, Paul, Davis, & Fazekas de St Groth, 1992; Smeltz et al., 2002; Swain, Weinberg, English et al., 1990). Cross-regulation is the modulation of established responses by the presence of cytokines. The presence of IL-12 stimulates the up regulation and maintenance of IFN-γ receptors on precursors destined to be Th1 type effectors, and suppresses the expression of IL-4 (So, Park, & Lee, 2000; Trinchieri, 1995). The presence of IL-4 down-regulates the production of IL-12 by APC, such as dendritic cells, and suppresses IFN-γ production in precursors destined to become Th2 effectors (Murphy & Reiner, 2002; Nelms, Keegan, Zamorano, Ryan, & Paul, 1999).

The demonstration of the importance of the cytokine milieu leads to the question of how the differential production of Th1 and Th2 cytokines occurs when naïve precursor CD4+ T cells encounter a foreign antigen. It is evident from a number of studies that the nature and dose of antigen plays a large role in T cell activation and Th1 or Th2 lineage commitment (Adkins, Jones, Bu, & Levy, 2004; Boonstra et al., 2003; Bretscher, Wei, Menon, & Bielefeldt-Ohmann, 1992; Hosken et al., 1995; Menon & Bretscher, 1998; Parish, 1971; Power, Wei, & Bretscher, 1998; Sarzotti, Robbins, & Hoffman, 1996; Taylor-Robinson & Phillips, 1998). However, there are some conflicting findings as to which dose of antigen preferentially induces the production of a Th1 type or Th2 type cells. Some report that low doses of antigen preferentially stimulate the generation of Th2 type responses, moderate doses preferentially stimulate Th1 type responses, while very

high does favor the generation of Th2 type cells (Boonstra et al., 2003; Constant & Bottomly, 1997; Hosken et al., 1995; Rogers & Croft, 1999). Other studies provide evidence that low doses of antigen, or infectious agents, lead to the generation of Th1-type responses, and high doses the generation of Th2 responses (Adkins et al., 2004; Bretscher et al., 1992; Menon & Bretscher, 1998; Parish, 1971; Power et al., 1998; Sarzotti et al., 1996; Taylor-Robinson & Phillips, 1998). The varied conclusions made in regards to influence of different antigen doses may be reflective of the marked differences in the experimental systems employed, such as the use of normal versus TCR transgenic mice as host animals.

Precursor cell number can also influence the Th1/Th2-type nature of the immune responses upon stimulation with antigen (Bretscher, 1983a; Harris et al., 1954; Ismail & Bretscher, 2001; Liew, Millott, Lelchuk, Cobbold, & Waldmann, 1989; Titus, Ceredig, Cerottini, & Louis, 1985). For instance, in an *in vivo* model of Leishmania, the partial depletion of CD4+ cells switches the immune response normally generated in susceptible mice, which is Th2 in nature, to a protective one that is predominately Th1 (Liew et al., 1989; Titus et al., 1985). Further findings demonstrate that the number of cells adoptively transferred to lethally irradiated mice determines the Th1/Th2 phenotype of the response generated against SRBC (Bretscher, 1983b; Ismail & Bretscher, 2001). A threshold number of adoptively transferred precursor cells is required for immune responses to be generated, and once this threshold is reached, a low number of cells favors the development of Th1 responses, whereas, a high number favors the generation of Th2 type responses. Hence, the number of naïve CD4+ T cells present upon antigen encounter, or

alternatively, the number stimulated to proliferate upon exposure to antigen can also influence the generation of immune responses and the subsequent Th1/Th2 lineage commitment of the antigen-specific precursor, CD4+ T cells (Bretscher, 1983a; Gerloni et al., 2000; Strutt & Bretscher, 2005; Tucker & Bretscher, 1982).

The proper regulation of CD4+ T cell lineage commitment to Th1/Th2 subsets is central to the development of protective immunity. Although the exact mechanisms underlying lineage commitment and regulation of immune responses are unknown, the decisive events are likely tightly associated with the events involved in primary T cell activation.

1.2.3. Regulation of Immune Responses and T Regulatory Cells

A number of regulatory mechanisms brought about by the presence of cytokines and inhibitory signals are known to influence T cell survival and differentiation. These regulatory mechanisms include those that indirectly inhibit T cell proliferative responses by down-regulating cytokine synthesis and accessory cell functions of antigen presenting cells, such as the pleiotrophic effects of the auto-regulated cytokine IL-10 (Fiorentino, Zlotnik, Mosmann, Howard, & O'Garra, 1991; Moore, O'Garra, de Waal Malefyt, Vieira, & Mosmann, 1993; Sher et al., 1991). They also include those mechanisms that regulate the phenotype of established Th1/Th2 type responses, which includes the cytokines IL-12 and IL-4 (Mosmann et al., 1991), whose affects were discussed in the previous section. The effects of inhibitory signals such as those induced by ligation of cell surface receptors, such as

CTLA-4-B7 ligation, can also influence the generation of immune responses (Egen, Kuhns, & Allison, 2002; Thompson & Allison, 1997). Ligation of these surface molecules, which will be discussed in more depth in section 1.3, can induce the metabolism of tryptophan by the enzyme indolamine 2,3-dioxygenase, which is produced by appropriately triggered antigen presenting cells, to result in the release of metabolites that induce apoptosis in T cells (Grohmann et al., 2002; Leibson, 2004; Steinman et al., 2003).

Immune responses can also be regulated by distinct subsets of T cells that can influence both the phenotype and magnitude of established immune responses (Asherson, Colizzi, & Zembala, 1986; Chess & Jiang, 2004; Ramshaw, McKenzie, Bretscher, & Parish, 1977; Shevach, 2002). These cells were originally termed suppressor cells, but in the modern literature, they are known as T regulatory cells. A recently identified subset of regulatory cells, the CD25+ CD4+ T cell subset, may also participate in the distinction of self from non-self (Fulcher et al., 1996; Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995; Shevach, McHugh, Piccirillo, & Thornton, 2001).

At least three distinct subsets of CD4+ T regulatory cells exist. Tr-1 regulatory cells are associated with the secretion of high levels of IL-10 and TGF-β, which again are cytokines that suppress a number of cell functions including the indirect inhibition of T cell proliferation through suppression of the production of cytokines and several accessory cell functions of antigen-presenting cells (Annacker et al., 2001; Barrat et al., 2002; Fowler & Powrie, 1999; von Boehmer, 2003). In addition to the secretion of IL-10 and TGF-β, the second Th3 regulatory cells subset

is also associated with the secretion of IL-4 (Seddon & Mason, 1999; von Boehmer, 2003). The CD25+ CD4+ T cell subset is distinct from Tr-1 and Th3 cells in that the repeated stimulation of CD4+ T cells with antigen in the presence of IL-10 and TGF-β fails to lead to their generation (Jonuleit, Schmitt, Schuler, Knop, & Enk, 2000; Shevach, 2002; von Boehmer, 2003). CD25+ CD4+ T regulatory cells are thought to be generated in the thymus, require cell contact to mediate their suppressor functions, and to date they have not been solidly associated with the secretion of cytokines (Apostolou, Sarukhan, Klein, & von Boehmer, 2002; Jordan et al., 2001; Nakamura, Kitani, & Strober, 2001; Shevach, 2002). The most reliable marker for CD25+CD4+ T cells is the expression of the transcription factor FOXP3 (Chen et al., 2003; Fontenot, Gavin, & Rudensky, 2003; Hori, Nomura, & Sakaguchi, 2003).

The presence of CD25+ CD4+ T regulatory cells inhibits the generation of autoimmune responses in experimentally generated lymphopenic environments (Fowell & Mason, 1993; Sakaguchi et al., 1995). The possibility now exists that CD25+ CD4+ T cells may play an essential role in the maintenance of peripheral self-tolerance possibly through CTLA-4 triggered tryptophan metabolism (Grohmann et al., 2002; Sakaguchi, 2004). Results indicate that the generation of CD25+CD4+ T regulatory cells may occur by an unknown mechanism in the thymus (Apostolou et al., 2002; Jordan et al., 2001). However, in light of the fact that there is uncertainty in the means by which these cells are activated and regulated in the periphery, it may be premature to conclude that these cells participate in the discrimination of immunological self from non-self (Belkaid, Piccirillo, Mendez, Shevach, & Sacks, 2002; Fulcher et al., 1996; Pasare & Medzhitov, 2003; Yamazaki

et al., 2003). The observation that the removal of such cells from normal mice fails to result in the generation of autoimmune disease reinforces this concern (McHugh & Shevach, 2002; Shevach, 2002). It may be reasonable to speculate that CD25+ CD4+ T cells function to regulate the homeostasis of peripheral naïve and memory T cells (Annacker, Burlen-Defranoux, Pimenta-Araujo, Cumano, & Bandeira, 2000; Bhandoola et al., 2002; Gavin & Rudensky, 2003; Ge, Palliser, Eisen, & Chen, 2002; King, Ilic, Koelsch, & Sarvetnick, 2004; Murakami, Sakamoto, Bender, Kappler, & Marrack, 2002)

1.2.4. T cell Differentiation into Memory Cells

Immunological memory is a hallmark of the adaptive immune response. CD4+ T helper cells play a large role in the maintenance of these responses. CD4+ T memory cells hasten the rate by which specific immune responses are orchestrated upon a secondary encounter with a pathogen (Dutton, Bradley, & Swain, 1998).

The rapidity of memory responses is largely attributed to the clonal expansion of antigen-specific CD4+ and CD8+ T cells, and antigen-specific B cells during the generation of primary immune responses (Bradley, Duncan, Yoshimoto, & Swain, 1993; Leduc, Coons, & Connolly, 1955; Plata et al., 1975). The majority of clonally expanded and activated cells will die of exhaustion, competition for antigen presented by antigen presenting cells, or because of withdrawal of required growth factors after the clearance of antigen (Belkaid et al., 2001; Hunziker et al., 2002;

Kedl et al., 2000; Kundig et al., 1996; Sprent & Tough, 1994; Uzonna, Wei, Yurkowski, & Bretscher, 2001). A small population of the antigen-specific cells, normally those that express high affinity receptors for the antigen in question, survives as a memory subset (Kedl et al., 2000). One sub-type of memory cells, the effector memory cells, can traffic throughout the body through the lymphatic system, and another, the central memory subset, is centrally located within the lymphoid organs (Blander et al., 2003; Huehn et al., 2004; Sallusto, Lenig, Forster, Lipp, & Lanzavecchia, 1999).

There is some debate in the literature as to whether the generation of memory cells follows a linear path from naïve to effector to memory cell, or whether naïve T cells directory differentiate into memory cell subsets (Kaech, Wherry, & Ahmed, 2002; Lanzavecchia & Sallusto, 2002; Swain, Agrewala, Brown, & Roman, 2002). The most recent decisive factor reported to influence the memory cell fate of an activated T cell is the activation status of antigen presenting cells (de Heusch et al., 2004). However, it remains possible that both the linear and direct pathways lead to memory T cell generation. In order for the more revealing evidence to be obtained, definitive surface markers on all subsets of T memory cells, particularly resting memory T cells, must be identified (Ahmadzadeh, Hussain, & Farber, 2001; Birkeland, Johnson, Trowbridge, & Pure, 1989; Goldrath, Bogatzki, & Bevan, 2000; Hengel et al., 2003; Kelly & Scollay, 1990). While their definitive identification and means of generation remain a topic of debate, memory CD4+ T helper cells are undoubtedly essential for the mediation of protective cellular and humoral immunological memory against infectious organisms.

1.3. Naive T cell Activation

1.3.1. The Signals Involved in T cell Activation

The activation of CD4+ T cells must be tightly regulated because these cells are, for the most part, the guardians over the activation and establishment of antigen specific immune responses, immunological memory, and tolerance towards peripheral self. As mentioned previously, Burnet, Talmage, and Lederberg have all formulated models to provide a rationale for how the immune system discriminates self-from non-self (Burnet, 1959; Lederberg, 1959; Talmage & Pearlman, 1963). In the context of modern knowledge of central tolerance and the importance of CD4+ T cells in the generation and mediation of B cell and CD8+ T cell immune responses, it is clear that the mechanisms that regulate the activation of CD4+ T cells are central to the maintenance of tolerance towards peripheral self-antigens (Goldrath & Bevan, 1999; Hanahan, 1998).

The frequency of naïve T cells that exit the thymus and are specific for any one antigen is initially small, in the order of magnitude of one in 10^4-10^5 cells (Blattman et al., 2002; Goldrath & Bevan, 1999). In order to orchestrate protective immune responses, naïve T cells must selectively increase in number. Since the seminal observations of Jenkins and Schwartz, the initial expansion and activation of T cells to generate effector T cells is now known to involve several complex interactions between the T cell and the antigen-presenting cell presenting the relevant

antigen (Abbas et al., 2000; Jenkins & Schwartz, 1987). For CD4+ T cells, activation results in the acquisition of the ability to secrete cytokines, replicate, and differentiate into competent cells that help in the mediation of cellular and humoral immune responses (Weiss, 1999). For CD8+ T cells, activation results in the ability to secrete cytokines, replicate, and differentiate into mature cytolytically active cells. The lack of the receipt of all of the appropriate signals during T cell activation can lead to a period where the recently expanded T cells, CD4 or CD8+ T cells, are refractory to further activation signals and are susceptible to activation induced cell death (Kishimoto & Sprent, 1999; Kurts, Carbone et al., 1997; Kurts, Kosaka, Carbone, Miller, & Heath, 1997; Nossal & Pike, 1980; Schwartz et al., 1989).

Recognition of peptide-MHC molecules, presented on the surface of antigen-presenting cells, by the T cell receptor of a naïve T cell results in the generation of the first signal required for T cell activation (Bretscher & Cohn, 1970; Jenkins & Schwartz, 1987). This signal results in the activation of various kinases and phosphatases within the T cell and within the antigen-presenting cell (Schwartz et al., 1989; Weiss, 1999). The receipt of signal 1, which causes an influx in intracellular calcium, results in the translocation of DNA-binding proteins into the nucleus and the initiation of gene transcription (Abbas et al., 2000). In a resting T cell, signal 1 results in the expression a number of genes encoding immune related encoding proteins including IL-2, the primary growth factor for most T cells (Smith, 1984). Activation to cell division requires additional receptor-ligand interactions, which were originally thought to be generated by the provision of IL-1 or recognition of IL-2 by the IL-2 receptor (Malek, Ashwell, Germain, Shevach, & Miller, 1986;

Manger, Weiss, Weyand, Goronzy, & Stobo, 1985; Smith, 1984). It is now generally accepted that a large number of accessory molecules participate in costimulating the intracellular signals triggered by T cell receptor stimulation, in either the presence or absence of IL-2 (Coyle & Gutierrez-Ramos, 2001; Croft & Dubey, 1997; Weiss, 1999).

However, it remains unclear which accessory or costimulatory molecules are essential for the generation of T cell responses because a number perform redundant roles, act in concert, or function in a temporal fashion (Boussiotis, Freeman, Gribben, & Nadler, 1996; Coyle & Gutierrez-Ramos, 2001; Croft & Dubey, 1997; Lanier et al., 1995; Lenschow, Walunas, & Bluestone, 1996; Watanabe et al., 2003). For example, the costimulatory molecules B7-1/CD80 and B7-2/CD86 that are differentially expressed on antigen-presenting cells can both bind CD28 (Lanier et al., 1995), a molecule constitutively expressed on the surface of T cells (Lenschow et al., 1996). The B7 molecules can also bind alternate receptors, such as CTLA4, which is maximally expressed 48 to 72 hours after the initial activation of T cells (Linsley, 1995). Ligation of CTLA4 or alternative receptors that also bind B7, such as PD-1 and BTLA, also negatively regulate the generation of T cell responses by halting T cell proliferation and/or by inducing T cell apoptosis, possibly by inducing the metabolism of tryptophan as introduced in section 1.2.3. (Boussiotis et al., 1996; Carreno & Collins, 2002; Egen et al., 2002; Grohmann et al., 2002; Okazaki, Iwai, & Honjo, 2002; Watanabe et al., 2003). Members of the TNF/TNFR family of surface molecules expressed on antigen-presenting cells have also been implicated as being essential co-receptors for surface molecules on T cells, and these include CD40, 4-

1BB, CD27, and Ox-40 (Croft & Dubey, 1997; Johnson-Leger, Christensen, & Klaus, 1998; Ohshima et al., 1998; Rogers & Croft, 2000; Wen, Bukczynski, & Watts, 2002; Wu et al., 1995). In general, the B7-CD28 costimulatory interactions, in addition to those interactions that are involved in stabilizing T cell-APC contact, such as the ICAM-1-LFA ligation, are essential for primary CD4+ T cell activation (Dubey, Croft, & Swain, 1995; Green et al., 1994; Harding, McArthur, Gross, Raulet, & Allison, 1992; Jenkins, Taylor, Norton, & Urdahl, 1991), and CD40-CD40L interactions are essential for CD8+ T cell activation (Bennett et al., 1998; Borrow et al., 1998; Lanier et al., 1995; Shepherd & Kerkvliet, 1999; Sigal, Reiser, & Rock, 1998). Other interactions possibly function to allow responses to proceed and develop efficiently once responses are initiated (Banchereau et al., 1994; Lee, Haynes, Eaton, Swain, & Randall, 2002; Maxwell, Campbell, Kim, & Vella, 1999; Wallin, Liang, Bakardjiev, & Sha, 2001).

T cell activation is thus a multi-step process, involving a cascade of costimulatory events initiated after antigen-dependent T cell receptor ligation (Carreno & Collins, 2002; Coyle & Gutierrez-Ramos, 2001; Croft & Dubey, 1997; Lenschow et al., 1996). Several phenotypic changes accompany the process of T cell activation. These include the up-modulation on the surface of the T cell the expression of CD40L, the IL-2 receptor, CD69, and CD44 (Budd et al., 1987; Dutton et al., 1998; Jaiswal, Dubey, Swain, & Croft, 1996; Minami, Kono, Miyazaki, & Taniguchi, 1993; Swain et al., 1991), and the down-modulation of such surface ligands as CD62L and CD45RB (Birkeland et al., 1989; Dutton et al., 1998; Gallatin, Weissman, & Butcher, 1983). The same molecules that are up/down modulated

during activation can be down/up modulated during the differentiation pathway to resting memory cells (Ahmadzadeh et al., 2001; Hengel et al., 2003; Kassiotis & Stockinger, 2004; Ponta, Wainwright, & Herrlich, 1998). The early realization of transitory expression of such markers has lead some to suggest "that there are no markers of [T cell] memory, but only markers of activation" (Mitchison, 1992).

Of the antigen presenting cells of the immune system, being macrophages, dendritic cells, and B cells, the dendritic cell is the most favored cell implicated in participating in the initial activation of naïve T cells (Banchereau & Steinman, 1998; Metlay, Pure, & Steinman, 1989; Unanue, 1984). Primed CD4+ T cells can help resting B cells become efficient stimulator cells of naïve T cells (Ho, Cooke, Goodnow, & Davis, 1994). However, on a per cell basis they are generally considered inferior in their capacity to stimulate naïve T cells (Cassell & Schwartz, 1994; Constant, Schweitzer, West, Ranney, & Bottomly, 1995; Lassila, Vainio, & Matzinger, 1988; Steinman & Witmer, 1978; Tzehoval et al., 1983). This view is primarily based on the fact that B cells specific for any given antigen are relatively rare, even in immunized animals.

Dendritic cells are the most favored cell implicated in participating in the initial activation of naïve T cells because of their ability to sample peripheral antigens, their ability to migrate into the T cell zones of the lymph nodes (Itano & Jenkins, 2003; Reis e Sousa et al., 1997), and their constitutive expression of low levels, and up-modulated expression of high levels of the costimulatory molecules B7-1, B7-2, ICAM, LFA-3 and CD40 (Inaba et al., 1994; Larsen et al., 1994; Sporri & Reis e Sousa, 2003). To date, five different dendritic cell subtypes have been

defined (Henri et al., 2001; Shortman & Liu, 2002; Vremec, Pooley, Hochrein, Wu, & Shortman, 2000; Vremec & Shortman, 1997), and all are now believed to originate from precursors found in the bone marrow (Shortman & Liu, 2002; Traver et al., 2000; Wu et al., 2001). In general, all subtypes are believed to have a capacity for initiating immune responses in their activated state (Shortman & Heath, 2001; Shortman & Liu, 2002; Vremec et al., 2000), and the two major subtypes, the CD4-CD8α+, and the CD4-CD8α- dendritic cells, have been demonstrated to be functionally plastic in the stimulation of Th1/Th2 responses (Boonstra et al., 2003; Guermonprez et al., 2002). The spleen harbors three subtypes, which are the CD4+CD8α-, the CD4-CD8α+, and the CD4-CD8α- dendritic cells (Vremec et al., 2000; Vremec & Shortman, 1997). The lymph nodes contain two additional subtypes that are not normally found in the spleen, and these include the CD4-CD8α-CD11b+ dendritic cells that also express moderate levels of CD205, and a subtype of dendritic cells that express an array of myeloid markers including CD11b, low CD8α, and high levels of CD205 that can be found in the skin-draining lymph nodes (Henri et al., 2001; Shortman & Liu, 2002). The latter is distinguished from other subtypes of dendritic cells by high expression of langerin, a characteristic marker of epidermal Langerhans cells (Henri et al., 2001). Those dendritic cells in an immature state, in general, fail to initiate immune responses and it has been proposed that they have the potential to inactivate T cells. One subtype, specifically the CD8α expressing dendritic cells, may be specialized for this task. However, to date, a consensus has not been reached on this matter (Belz et al., 2002; Belz et al.,

2004; Grohmann et al., 2003; Grohmann, Fallarino, Bianchi et al., 2001; Shortman &

Heath, 2001; Smith et al., 2003; Winkel, Kronin, Krummel, & Shortman, 1997).

1.3.2. Contemporary Models for the Control of the Delivery of Signal Two

All contemporary models that attempt to describe the nature of events

involved in naive T cell activation agree that two signals are required (Bretscher,

1999; Janeway, 1989; Matzinger, 1994). The provision of signal one alone, through

recognition of antigen by the T cell receptor without costimulation, fails to result in

the subsequent activation of the T cell. The receipt of signal 1 alone can result in the

induction of a state of anergy, which may or may not be broken by the provision of

IL-2, or it may result in the induction of apoptosis (Guinan et al., 1999; Jenkins &

Schwartz, 1987; Kishimoto & Sprent, 1999; Schwartz, 2003). Provision of signal

one in conjunction with a costimulatory signal two results in T cell activation

(Fulcher et al., 1996; Jenkins et al., 1991; Lenschow et al., 1996; Sperling et al.,

1996; Tao, Constant, Jorritsma, & Bottomly, 1997). Hence, the regulation of the

expression of costimulatory molecules on antigen-presenting cells that collectively

result in the generation of signal two is of central importance to naive T cell

activation. Competing contemporary models of naive T cell activation differ greatly

in the mechanisms by which the up-regulation of signal two is initiated and

regulated. Each model possesses particular strengths and weaknesses, which will be discussed.

1.3.3. The Infectious Non-Self Model

To account for a need to preferentially activate T helper cells with foreign antigen-specificity, C. Janeway Jr. proposed that the immune system has evolved to specifically recognize the presence of infectious non-self (Janeway, 1989). C. Janeway Jr. postulated that the requirement in a number of experimental systems for the presence of bacterial adjuvant to efficiently induce the generation of immune responses against simple protein antigens is directly related to the generation of signal two (Janeway, 1989). He rationalized that recognition of bacterial products, present in adjuvant, by a receptor present on the antigen presenting cells stimulates the up-regulation of the molecules mediating the delivery of signal two. In modern terms, the recognition of pathogen-derived products by antigen-presenting cells occurs through ligation of receptors more commonly referred to as Toll-like receptors (Iwasaki & Medzhitov, 2004; Takeda, Kaisho, & Akira, 2003). The intracellular signaling pathways triggered by Toll-like receptor ligation result in the up-regulation of costimulatory molecules B7.1 and B7.2 on the antigen-presenting cell surface, and the expression and release of inflammatory mediators IL-1, IL-6, and IL-8 (Agrawal et al., 2003; Fulcher et al., 1996; Iwasaki & Medzhitov, 2004; Takeda et al., 2003).

C. Janeway Jr. rationalized that self-antigens would fail to trigger the up-modulation of costimulatory molecules on antigen-presenting cells, and therefore the activation of any self-reactive T cells that escaped central tolerance mechanisms in the thymus would fail to occur (Janeway, 1989). He also rationalized that dendritic cells, which constitutively express costimulatory molecules on their surface (Inaba et al., 1994; Larsen, Ritchie, Pearson, Linsley, & Lowry, 1992), pose a particular threat to self-tolerance, but dismissed these cells as participants in T cell activation because of their inefficient antigen uptake (Janeway, 1989). In light of modern knowledge that dendritic cells are in fact very potent antigen-presenting cells that take up antigen in a differentiation dependent manner, mature activated dendritic cells have in general lost their ability to acquire antigen (Banchereau & Steinman, 1998; Romani et al., 1989; Steinman, 1991), it is difficult to rationalize a mechanism within the context of this model that results in the preferential activation of foreign antigen-specific T cells. Of late, C. Janeway Jr. has stated "My personal opinion is that autoimmunity would be nearly inevitable were it not for the action of regulatory or suppressor T cells" (Janeway, 2001). However, he also has stated "…virtually always this distinction (self versus non-self) is made by the innate immune system, which primes the adaptive immune system when a pathogen is present, but fails to prime such responses in the absence of infection" (Janeway, 2001; Medzhitov & Janeway, 2002). These statements bring to the fore two questions. The first is which mechanism, T regulatory cells or Toll-like receptor ligation, ultimately regulates tolerance towards self-antigens in light of the recent knowledge that inflammatory stimuli have been reported to bypass the action of suppressor cells (Grohmann,

Fallarino, Bianchi et al., 2001; Pasare & Medzhitov, 2003; Yang, Huang, Huang, & Pardoll, 2004), and the second is how immune responses against non-infectious foreign antigens, such as RBCs and some allergens, are generated in the absence of Toll-like receptor ligation?

1.3.4. The Danger Model

In order to account for the generation of immune responses in the absence of products derived from infectious organisms, P. Matzinger proposed a model in which the activation of antigen-presenting cells to express the costimulatory molecules required for the generation of signal two occurs through the recognition of stressed self (Matzinger, 1994). The decision of whether or not to up-regulate signal two is, according to P. Matzinger, in the hands of tissues. She hypothesizes that the innate immune response has evolved to respond to danger signals produced by tissue cells, which are then relayed to cells of the adaptive immune response (Matzinger, 1994, 2002). The release of products from stressed or necrotic cells, which are released during inflammatory or infectious tissue damage, activates non-specific antigen presenting cells to release pro-inflammatory cytokines, and up-regulate the expression of costimulatory molecules (Gallucci, Lolkema, & Matzinger, 1999). She states "there is no reason why antigen presenting cells should not respond to both endogenous and exogenous signals" (Matzinger, 2002).

The Danger Model proposes that the generation of signal one through TCR recognition of antigen on antigen-presenting cells, and the provision of signal two by

ligation of "danger" induced costimulatory molecules, results in the subsequent activation of naïve, antigen specific CD4+ T cells (Matzinger, 1994, 2002).

A concern raised regarding the infectious non-self model of primary CD4+ T cell activation is also applicable to the Danger Model. It is not immediately evident how the activation of peripheral self-reactive T cells is prevented. P. Matzinger has proposed that one does not need to account for a mechanism to prevent the activation of peripheral self-reactive cells because they do not exist in the periphery (Matzinger & Guerder, 1989; Ridge, Fuchs, & Matzinger, 1996). She has proposed that the majority of self-reactive T cells are eliminated in the thymus by central tolerance mechanisms, either by recognition of ubiquitous self-antigens on resident thymic antigen-presenting cells, or by recognition of peripheral self-antigens presented on dendritic cell immigrants (Ridge et al., 1996). It is now clear that a subset of organ-specific self-antigens are expressed and presented on the thymic antigen-presenting cells involved in negative selection (Anderson et al., 2002; Hanahan, 1998; Liston, Lesage, Wilson, Peltonen, & Goodnow, 2003). However, the delivery of organ-specific self-antigens to the thymus by dendritic cells that have sampled peripheral tissues has not been adequately substantiated. It is generally accepted that dendritic cells travel from tissues to draining lymph nodes via the lymphatic system (Sallusto & Lanzavecchia, 2000; Scheinecker, McHugh, Shevach, & Germain, 2002). To date, there is little evidence to support that they travel through the lymph nodes and gain access to the circulation system that supplies the thymus, and it has recently been suggested that thymic dendritic cells develop from precursors within the thymus (Ardavin, Wu, Li, & Shortman, 1993; Kamath, Henri, Battye, Tough, &

Shortman, 2002). How the activation of precursor T cells is regulated in the presence of danger to result in the preferential activation of foreign-antigen specific cells in the presence of peripheral "danger" is thus somewhat unclear.

1.3.5. The Two-Step, Two-Signal Model

An alternative model of primary T cell activation that attempts to describe a mechanism for the preferential activation of foreign-antigen-specific T cells is the Two-step, Two-signal Model (Bretscher, 1999). This model differs from the previous models in that the ultimate gatekeepers of the up-regulation of costimulatory molecules that lead to the generation of the final activating signals are antigen-specific immune cells rather than non-discriminatory, antigen-presenting cells of the innate immune response (Janeway, 1989; Matzinger, 1994).

According to the tenets of this model, the generation of signal 1 by TCR stimulation, and the generation of a signal 2 by recognition of costimulatory molecules on non-antigen specific antigen presenting cells, such as dendritic cells, results only in the initiation of precursor CD4 T cell proliferation (Bretscher, 1999). This first step, which is all that is required for full naïve CD4 T cell activation in the previous models, is postulated to only result in the clonal expansion of antigen-specific T cells.

In order to prevent the generation of autoimmune responses, the Two-step, Two-signal Model argues that an additional step, involving other antigen-specific cells of the adaptive immune responses is required for the full activation of expanded

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CD4+ T helper cells (Bretscher, 1999). The antigen-specific cells required for the second step of naïve CD4 T cell activation include a population of effector CD4+ T helper cells, and cognate, antigen-specific B cells acting as antigen presenting cells. The effector CD4+ T helper cells are required to activate the cognate, antigen-specific B cells, which in turn, are postulated to deliver the required second costimulatory signals to recently expanded CD4+ precursor T helper cells (Bretscher, 1999). The provision of a second set of costimulatory signals and/or cytokines from B cells rescues recently expanded precursor CD4+ T helper cells from a fate of apoptotic death or anergy, and results in the full activation of CD4+ T helper cells. The most obvious question in regards to this model is where do the first foreign antigen-specific effector CD4+ T helper cells arise. This question, in addition to the nature of the particular cells envisaged to be involved in the separate steps of this model, will be discussed below.

1.3.6. The Cells Participating in the Two-Steps of Primary T Cell Activation: Dendritic Cells

When they were first identified, dendritic cells were originally described as a stellate population poor in antigen processing because of their lack of adequate lysosomes and an apparent inability to phagocytose and present antigen (Kelly, Balfour, Armstrong, & Griffiths, 1978; Klinkert, LaBadie, & Bowers, 1982; Steinman, Adams, & Cohn, 1975; Steinman & Cohn, 1973; Wolff & Schreiner,

1970). These findings were substantiated in a number of systems (Hart & McKenzie, 1988; Pugh, MacPherson, & Steer, 1983; Schuler & Steinman, 1985; Van Voorhis, Hair, Steinman, & Kaplan, 1982). Once it was discovered that the ability of dendritic cells to take up and present antigen is generally a function of maturity, only immature/un-activated dendritic cells efficiently take up and present antigen in a manner that is dependent upon MHC class II synthesis, it was quickly realized that dendritic cells are very potent stimulators of T cell proliferation and cytokine production (Cella, Sallusto, & Lanzavecchia, 1997; Crowley, Inaba, & Steinman, 1990; Girolomoni, Simon, Bergstresser, & Cruz, 1990; Inaba et al., 1986; Kampgen et al., 1991; Koch et al., 1995; Pure et al., 1990; Romani et al., 1989; Sallusto et al., 1995; Schuler & Steinman, 1985; Steinman & Witmer, 1978; Wilson et al., 2003). On a per cell basis, they are believed to be the most efficient antigen-presenting cells at stimulating proliferation of both naïve and memory T cells, and as such have been labeled "nature's adjuvant" (Banchereau & Steinman, 1998; Cassell & Schwartz, 1994; Crowley et al., 1990; Steinman, 1991; Steinman & Witmer, 1978).

The discovery of Toll-like receptors and the ability of dendritic cells to respond to inflammatory environments provide support for the position that the Infectious Non-self and Danger Models of naïve T cell activation describe the cellular events involved in primary T cell activation (Iwasaki & Medzhitov, 2004). However, "maturation (of dendritic cells) creates a problem with respect to self/non-self discrimination" (Steinman & Nussenzweig, 2002) because activation of dendritic cells results in presentation of both foreign and self-antigens on surface MHC molecules (Guery & Adorini, 1995). The discoverer of dendritic cells, R.

Steinman, has therefore proposed his own model of peripheral tolerance (Hawiger et al., 2001; Steinman et al., 2003). He postulates that peripheral self-specific T cells that encounter antigen on immature/non-activated dendritic cells that have not encountered infectious non-self or "danger" are subject to deletion, and that such deletion induces a steady state of peripheral self-tolerance (Steinman et al., 2003). R. Steinman proposes that these two opposing functions of dendritic cells can be rationalized by the deletion of self-reactive cells in the steady state, and the activation of immune cells in the context of inflammation or infection. In light of observations showing that dendritic cells need to be activated to induce tolerance in naïve T cells (Albert et al., 2001), and that CD25+ CD4+ T cell suppression can be overcome upon dendritic cell activation or the presence of inflammatory cytokines (Pasare & Medzhitov, 2003; Yang et al., 2004), one again has to ponder the question of how the activation of peripheral self-specific precursor T cells is prevented when "stranger" or "dangerous" antigens are presented on dendritic cells that also present self-derived antigens.

The Two-step, Two-signal Model incorporates the view that the immune system must contain intricate control mechanisms to regulate the activation of naive T cells (Bretscher, 1999). Placing the regulation of naïve T cell activation in the hands of a non-specific cell, such as the dendritic cell, which has potent stimulatory capacity, does not provide a way of definitively discriminating self from foreign antigens (Guery & Adorini, 1995; Steinman et al., 2003), nor a way of ensuring that only foreign antigens are presented to precursor T cells in a way that results in their activation, opens up the likelihood that immune responses towards peripheral self

can be generated and sustained. It must be clarified, however, that the predictions of this model do not deny the importance of non-antigen specific immune cells, such as activated dendritic cells, in activation of naïve T cells. They do however suggest that the role of dendritic cells is primarily to stimulate the clonal expansion of antigen-specific T cells (Bretscher, 1999), and provide an antigen-bridge for the helper-dependent activation of CD8+ cytotoxic T cells. In order to fully rationalize the stance of this model one must consider the fact that a number of experimental systems demonstrate that dendritic cells are potent stimulators of naïve T cells, which are normally separated from previously activated cells via the use of T cell surface markers (Alpan, Bachelder, Isil, Arnheiter, & Matzinger, 2004; Croft, Duncan, & Swain, 1992; Steinman & Witmer, 1978). In light of uncertainties in the reliability of the use of surface T cell activation markers to distinguish naïve T cells from resting memory cells that have down-modulated expression of such markers (Ahmadzadeh et al., 2001; Birkeland et al., 1989; Hengel et al., 2003; Murali-Krishna & Ahmed, 2000), it may be reasonable to question whether the cells employed as naïve responding T cells in a number of studies are in fact free of contaminating transitional or resting memory T cells. The uncertainty as to whether dendritic cells truly possess the potential to activate naïve T cells in the absence of other antigen-presenting cells is exemplified by the possibility that the dendritic cells used in a number of studies may also contain low numbers of other lymphocytes (Coligan, Kruisbeek, Shevach, & Stober, 1998; Izon et al., 2001). Despite optimized experimental tools and techniques, the following statement previously made by Ashwell et. al may still hold, "attempts to completely purify these cells

[macrophages and dendritic cells] away from surface immunoglobulin-positive cells have so far been only partially successful" (Ashwell, DeFranco, Paul, & Schwartz, 1984).

1.3.7. The Cells Participating in the Two-Steps of Primary T Cell Activation: The Effector CD4+ T Helper Cells

The effector CD4+ T helper cells, which are postulated to activate cognate antigen-presenting B cells, are one of the lymphocyte populations believed to regulate the up-modulation of the final second signal required for full naïve T cell activation (Bretscher, 1999). Experimental evidence in the literature indirectly supports the existence of such a cell. For example, CD4+ T cells that possess radiation resistant helper effector function, and hence can mediate the generation of responses without undergoing proliferation, are present in the spleens of normal un-manipulated mice (Pilarski, 1977). A number of *in vivo* studies estimate that radiation resistant CD4+ T cells compose approximately 10% of the peripheral CD4+ T cell pool (Cederbom, Bandeira, Coutinho, & Ivars, 1998; Pereira, Larsson, Forni, Bandeira, & Coutinho, 1985; Williams, Patchen, Darden, & Jackson, 1994).

The postulate of a requirement for an effector CD4+ T cell that potentially regulates the up-modulation of costimulatory molecules critical for the generation and delivery of signal two, upon challenge with a foreign antigen, generates a conceptual problem of how the first effector CD4+ T cells arise (Bretscher, 1999).

As discussed above, evidence to support the functional and phenotypic existence of such cells is strong (Cederbom et al., 1998; Pereira et al., 1985; Pilarski, 1977; Williams et al., 1994), but the means by which they are initially activated is purely speculative. One possibility is that the initial activation of the effector CD4+ T helper cells occurs during homeostatic processes. During homeostatic proliferation, a fraction of the T cells that proliferate acquire and maintain an activated T cell phenotype (Cho, Rao, Ge, Eisen, & Chen, 2000; Min et al., 2003; Murali-Krishna & Ahmed, 2000; Schuler, Hammerling, & Arnold, 2004; Tanchot, Le Campion, Leaument, Dautigny, & Lucas, 2001). This proliferation, and subsequent acquisition of an activated status, of T cells is an IL-7 and CD28 costimulation dependent phenomenon that is somehow triggered by the lymphopenic environment (Tan et al., 2001), which is typical of newborns, lethally irradiated mice, or of genetically modified mice deficient in lymphocytes (Le Campion et al., 2002; Paul, 1999; Williams et al., 1994). The Two-step, Two-signal Model postulates that the effector CD4+ T helper cells that exist, and which may have been activated during homeostatic proliferation, must be specific for foreign but not self-antigens (Bretscher, 1999). Because the majority of self-specific CD4+ T cells are subject to central tolerance mechanisms in the thymus, or are subject to deletion upon the receipt of signal one in the periphery in the absence of signal two (Bevan et al., 1994), the number of self-antigen specific effector CD4+ T helper cells is predicted to be a number that is insufficient to result in the activation of further self-antigen-specific CD4+ T cells (Bretscher, 1999).

1.3.8. The Cells Participating in the Two-Steps of Primary T Cell Activation: The Role of Antigen-specific B Cells as Antigen-Presenting Cells

As discussed, activated B cells are postulated in the two-step, two-signal model to play a central role in naive T cell activation (Bretscher, 1999). They are the favored antigen presenting cells believed to deliver survival cytokines and/or costimulatory second signals to recently expanded precursor CD4+ T helper cells because of their specific means of antigen-uptake/presentation, and CD4+ T helper cell dependent expression of costimulatory molecules.

Many studies have tried to address whether the generation of immune responses *in vitro* and *in vivo* requires immunoglobulin bearing B cells, but the conclusions drawn have been contradictory (Cassell & Schwartz, 1994; Chan, Hannum, Haberman, Madaio, & Shlomchik, 1999; Epstein, Di Rosa, Jankovic, Sher, & Matzinger, 1995; Greeley et al., 2001; Hayglass, Naides, Scott, Benacerraf, & Sy, 1986; Homann et al., 1998; Janeway, Ron, & Katz, 1987; Kurt-Jones et al., 1988; Liu et al., 1995; Macaulay, DeKruyff, & Umetsu, 1998; Rivera, Chen, Ron, Dougherty, & Ron, 2001; Ron & Sprent, 1987; Ronchese & Hausmann, 1993; Schultz, Klarnet, Gieni, HayGlass, & Greenberg, 1990; Schultz, Paquet, Bader, & HayGlass, 1995; van der Heyde, Huszar, Woodhouse, Manning, & Weidanz, 1994; Williams, Oxenius, Hengartner, Benoist, & Mathis, 1998). The two most widely used contemporary murine models of *in vivo* B cell deficiency are the μMT and the

JhD B cell knockout mouse strains. The development of B cells has been prevented in both of these strains of mice by the removal of gene segments essential for immunoglobulin receptor rearrangement.

Observations from studies employing μMT B cell deficient mice have led to the conclusion that B cells are not required for the induction of primary cellular and humoral responses (Epstein et al., 1995; Leef, Elkins, Barbic, & Shahin, 2000; Rivera et al., 2001). However, it is now apparent that an abnormal genetic recombination event in μMT mice, at least on the BALB/c background, enables the survival of some B cells (Fulcher et al., 1996; Hasan, Polic, Bralic, Jonjic, & Rajewsky, 2002; Melamed, Miri, Leider, & Nemazee, 2000). Other studies, employing a less "leaky" model of B cell deficiency, JhD B cell knock-out mice, have led to the conclusion that B cells are essential for the activation of T cell responses (Chan, Hannum et al., 1999; Leef et al., 2000). These studies suggest that the involvement of B cells in T cell activation involves a mechanism other than the production of secreted antibody. It has been argued that the requirement for B cells in such studies is either for the development of proper lymphoid architecture, or for the enhancement of immune responses once established (Croft & Dubey, 1997; Golovkina, Shlomchik, Hannum, & Chervonsky, 1999; Ngo, Cornall, & Cyster, 2001).

The postulates of the Two-step, Two-signal Model state that B cells, and not dendritic cells, are the antigen-specific antigen-presenting cells involved in delivering required cytokines and/or costimulatory signals to naïve T cells during step two of primary activation of T cells (Bretscher, 1999). The reason that B cells

are favored over dendritic cells is their antigen-specificity. It is well established that B cells primarily endocytose exogenous antigens via their antigen-specific surface immunoglobulin receptors in an efficient manner (Lanzavecchia, 1990). They also primarily present the antigens taken up by receptor-mediated endocytosis on their surface MHC molecules (Brocke et al., 2003; Bryant & Ploegh, 2004). By contrast, dendritic cells take up antigen by macropinocytosis or via clathrin-coated pits utilizing adsorptive endocytosis receptors. They present all antigens endocytosed in their immature state indiscriminately on their surface MHC Class II molecules (Cella, Sallusto et al., 1997). The combination of the lack of the majority of B cells with self-specificity, and the ability of B cells to take up and present antigen in an antigen-specific manner, makes them prime candidates for being the antigen-presenting cell that delivers the final activating signal two to precursor CD4+ T helper cells during primary T cell activation.

In summary, the two-step, two-signal model postulates that placing the regulation of the up-modulation of the final second signal needed for primary T cell activation in the hands of antigen-specific CD4+ effector T helper cells, and cognate antigen-specific B cells, both of which are subject to self-tolerance mechanisms, provides a mechanism whereby primary immune responses can preferentially be generated against foreign antigens (Bretscher, 1973; Bretscher & Cohn, 1970; Bretscher, 1999).

1.4 Theoretical Basis of Thesis Objectives

At the present time, an immunological mechanism to account for the maintenance of tolerance towards peripheral self during primary CD4+ T cell activation is not complete. Several models have been proposed and discussed, and these include the Infectious Non-self, Danger, and Two-step, Two-signal Models (Bretscher, 1999; Janeway, 1989; Matzinger, 1994). The preferential activation of CD4+ T cells that bear $\alpha\beta$ TCR reactive against foreign antigens, and the subversion of the activation of those with specificity towards self is essential to the avoidance of autoimmunity, and must be considered within a model that best describes the events involved in primary CD4+ T cell activation.

The central question that must be addressed in order to discriminate between the competing models is can non-specific antigen-presenting cells, such as dendritic cells, stimulate the generation of immune responses from naïve CD4+ T cells, or is the presence of an antigen-presenting B cell required? The experimental work of this thesis is focused on establishing an experimental system to address this question.

As mentioned previously, a general approach to determine the requirements for the activation of naïve T cells requires a highly purified population of such cells. Naïve T cells are typically characterized as $CD44^{low}$, $CD45RB^{hi}$, and $CD62L^{hi}$ and activated effector T cells as $CD44^{hi}$, $CD45RB^{lo}$, and $CD62L^{lo}$ (Dutton et al., 1998). The use of these markers is useful for the identification of effector T cells, but there is some uncertainty as to whether a population of $CD44^{low}$, $CD45RB^{hi}$, and $CD62L^{hi}$ T cells, purified from the secondary lymphoid organs of normal or genetically

modified TCR transgenic mice, constitutes a population of naïve T cells uncontaminated with transitional or resting memory T cells (Ahmadzadeh et al., 2001; Hengel et al., 2003). This uncertainty is exemplified by the possibility that previously activated T cells possess the potential to revert to, or maintain, the expression of surface antigens often regarded as defining naïve cells (Blander et al., 2003). Impurity in purified T cell populations may lead to misleading observations on the requirements to activate naïve T cells. Hence, obtaining a population of naïve responding lymphocytes free of previously activated cells is central to an experimental approach designed to address whether non-specific antigen-presenting cells, such as dendritic cells, efficiently stimulate the generation of immune responses from naïve T cells, or whether the presence of antigen-presenting B cells is also required. Attempts were made in this work to study the antigen presenting cell requirements involved in primary T cell activation employing naïve responding T cells uncontaminated with previously activated peripheral T cells. The experimental system employed will be discussed in full in the beginning of the results section.

2. Research Objectives

The objectives of this work were as follows:

(1) To devise an experimental system to test whether the Two-step, Two-signal Model validly describes the cellular interactions involved in naïve CD4+ T cell activation. The primary goal of this Ph.D. thesis is thus to attempt to understand and delineate the cellular interactions involved in the primary activation of naïve CD4+ T cells employing such a system, which as for any experimental system, caveats must be accepted.

(2) According to the model of immune CD4+ T cell activation around which the hypotheses of this thesis are centered, the Two-step, Two-signal model, an interaction between a naïve T cell and a non-specific APC, such as a dendritic cell, is insufficient to result in the primary activation of naive CD4+ T helper cells. Such an interaction is likely sufficient to initiate cellular proliferation, which occurs upon completion of step one of activation. Step one, primed CD4+ T cells have to undergo a second cellular interaction, step two, with other lymphocytes in order for full activation. One critical lymphocyte population involved in the secondary cellular

interaction is a naturally acquired reservoir of foreign, antigen-specific effector CD4+ T helper cells. Only those with specificity for foreign antigens are believed to be at numbers sufficient to participate in primary T cell activation; those specific for self-antigens are postulated to be at numbers insufficient to result in full naïve T cell activation. The other essential lymphocytes envisaged to be required for primary T cell activation are antigen presenting B cells. Once antigen-specific effector CD4+ T helper cells activate cognate B cells, they are capable of delivering essential costimulatory and/or cytokine survival signals to recently expanded step one primed, CD4+ T helper cells. Without such a signal from a B cell, a recently expanded population of precursor CD4+ T helper cells is postulated to succumb to apoptotic cell death. Thus, the second objective of this thesis work is to test whether non-specific antigen-presenting cells, such as dendritic cells are able/unable to stimulate the generation of primary immune responses from naïve CD4+ T cells when antigen-specific effector CD4+ T helper cells and/or cognate B cell antigen-presenting cells are absent. It is hypothesized that B cells are the antigen-presenting cells that deliver essential activation signals to naïve, precursor CD4+ T helper cells.

(3) Several predictions can be made if the Two-step, Two-signal Model of primary T cell activation holds true. The primary objectives of this thesis are not to test the adequacy of these predictions. However, they warrant discussion:

    a. After naïve T cells encounter specific antigen, a state of antigen-specific CD4+ and/or CD8+ immune unresponsiveness will be generated if either one or both of the lymphoid populations involved in the critical second cellular interaction are absent, the effector CD4+ T helper cells and/or B cells.

    b. The unresponsive state generated in (a) is antigen-specific; immune responsiveness towards a second antigen will be unaffected by the generation of unresponsiveness towards the first antigen.

    c. The presence of activated, cognate B cells can prevent the unresponsiveness generated by antigen stimulation with non-specific antigen presenting cells, such as dendritic cells, if provided within a sufficient period of time.

    d. Alternatively, the unresponsiveness generated by the stimulation of naïve T cells with non-specific antigen-presenting cells is preventable by the provision of an analogue of the critical survival factors normally produced by B cells activated by functional CD4+ T helper cells.

3.0. MATERIALS AND METHODS

3.1. Mice

CBA/J (H-2^k), C57BL/6 (H-2^b), or BALB/c (H-2^d) mice were obtained either from the animal colony of the College of Medicine, University of Saskatchewan (Canada) or from Charles River (Montreal, Quebec). Mice were housed under specific pathogen free conditions. Routine screening ensured that mice were free of certain sub-clinical viral and bacterial infections. The mice employed within each experiment were of the same sex and were typically 5 to 8 weeks of age, unless otherwise indicated. Thymus donor mice were younger than splenic donors and were between 4 to 6 weeks of age. All experiments were performed with ethical approval from the Canadian Council on Animal Care.

3.2. Media

RPMI 1640 supplemented with L-glutamine and Leibovitz medium (Gibco Laboratories, Grand Island, NY) were prepared from powdered stocks as per manufacture's instructions. Solutions were sterilized by filtration through 0.22 μm

filters (Millipore, Molsheim, France) and sterility assessed by overnight incubation at 37 C.

3.3. Preparation of Single Cell Suspensions

Mice were killed by cervical dislocation and their spleen or thymus aseptically removed. Spleens or thymi were placed in 10 mL of Leibovitz media and single cell suspensions were prepared by gentle disruption and passage through stainless steel mesh. Suspensions were left for 2-3 minutes to allow debris to settle, and the supernatant collected. Single cell suspensions were then washed twice with Leibovitz media via centrifugation for 10 minutes at 280-x g. After the final wash, splenocytes or thymocytes were resuspended in complete RPMI media (RPMI 1640 supplemented with L-glutamine containing 10% fetal bovine serum, penicillin-streptomycin (100 U/mL), 0.8 mM sodium pyruvate, and 5×10^{-5} M β-mercaptoethanol). Viable leukocytes were counted by trypan blue exclusion using a haemocytometer. Cells were kept on ice until aliquoted either in culture or in the ELISPOT plates. Thymi were harvested from mice less than or equal to 6 weeks of age.

3.4. The Generation of Immune Responses against allo-MHC Antigens in Vitro

CBA/J thymocytes were plated in Falcon 24 well tissue culture plates (Becton Dickinson & Co., Franklin Lakes NJ, USA) such that 2×10^6 cells/well were stimulated with 2×10^6 cells/well BALB/c or C57BL spleen cells, which had received 1500 rads of γ irradiation from a Co^{60} source (Pilarski, 1977). Similarly irradiated CBA/J spleens cells were added to the indicated cultures at a number of 1×10^7 cells/well. For some experiments, 2×10^6 cells/well CBA/J splenocytes were stimulated with 2×10^6 cells/well irradiated BALB/c or C57BL spleen cells. Five identical wells of each culture condition were plated and maintained at 37 C and 5% CO_2 for 5-7 days. The generation of CTL and cytokine responses of pooled wells, representing a standard 1×10^7 input thymocytes, was then determined using the standard ^{51}Cr CTL assay and a modified ELISPOT assay, respectively. In some experiments, the number of thymocytes, the number of stimulators, or the number of irradiated syngeneic spleen cells were varied as indicated in the respective figures. For some experiments, different cell subsets were depleted by antibody dependent complement mediated lysis or separated via procedures to be discussed in the following sections. In all experiments where cells that are normally irradiated were treated or sorted, cells were irradiated following the completion of the treatment. For the majority of experiments presented, Thy1.2+ cells were depleted from allogeneic spleen stimulators by antibody dependent complement-mediated lysis as described in

section 3.7.1. Unless otherwise stated, experiments were repeated at least three times.

3.5. Assessment of the Generation of Immune Responses against Allo-MHC Antigens in Vitro

3.5.1. Detection of Antigen-Specific CD8+ Cytotoxic T Cells

Specific cytolytic activity was measured using sodium chromate, $Na_2^{51}CrO_4$, (Perkin Elmer, Boston, MA) labeled P815 mastocytoma ($H-2^d$) or EL4 lymphoma ($H-2^b$) target cells (ATCC, Manassas, VA) (Dunkley, Miller, & Shortman, 1974). Target cells were incubated for 2hrs at 37 C with 200 μCi per 2 x 10^6 cells after an equal volume of FCS was added; the desired volume of sodium chromate was determined separately for each experiment and was corrected for radioactive decay. Labeled cells were then washed three times and resuspended in warm, complete RPMI at 1 x 10^5 cells/mL.

Effector cells were harvested from primary cultures, pooled, washed, and resuspended in 2 mL of RPMI complete media warmed to 37 C. To ensure that CTL responses were normalized for a constant number of input thymocytes, serial dilutions, starting at 5% of culture by volume or 5 x 10^5 input thymocytes, were made in triplicate in 96-well Nunclon flat-bottom micro titer plates (Nalge Nunc International, Denmark). For responsive thymocyte cultures given irradiated

syngeneic spleen cells, 5% of culture corresponds to an approximate effector:target ratio of 10:1. Serially diluted cells were incubated with 1 x 10^4 labeled target cells/well for 4 hrs at 37 C at 5% CO_2. Maximal release of ^{51}Cr sodium chromate from the target cells without effectors was induced by the presence of 10% w/v Sarcosine. Spontaneous release of the radioactive label from the target cells without effectors was assessed in the presence of RPMI complete media. Estimates of maximal and spontaneous release from the target cells were obtained from the average of six identical wells. The presence of ^{51}Cr sodium chromate in 0.1 mL of supernatants was determined using a LS 5000TD Liquid Scintillation System (Beckman Instruments, Fullerton, CA) after all volume had evaporated, and scintillation fluid (4 g/L Omnifluor (Packard Instrument Company Inc., Meriden, CT) in scintillation grade Toluene) was added. The percent specific lysis was calculated as follows (Coligan et al., 1998):

$$\% \text{ lysis} = 100 \text{ x } \frac{[(\text{cpm experimental release} - \text{cpm spontaneous release})]}{[(\text{cpm maximal release}) - (\text{cpm spontaneous release})]}. \quad (3.1)$$

For experiments with multiple experimental conditions, specific lysis values were converted to lytic unit values in order for linear comparisons to be made. The number of lytic units was calculated as follows (Miller & Dunkley, 1974; Sambhara, Upadhya, & Miller, 1990):

$$\text{Lytic Units} = -100 \text{ x } [\ln (1 - p)] \quad (3.2)$$

$$\text{where } p = \frac{[(\text{cpm experimental release} - \text{cpm spontaneous release})]}{[(\text{cpm maximal release}) - (\text{cpm spontaneous release})]}. \quad (3.3)$$

3.5.2. Detection of Antigen-Specific Cytokine Producing Cells

Antigen dependent cytokine production was assessed with the ELISPOT assay (Coligan et al., 1998; Power et al., 1999). Ninety-six-well, unifilter 350 nitrocellulose-flat bottom plates (Whatman-Polyfiltronics, Clifton, NJ, USA) were prepared as follows: plates were coated by adding 100 μl/well of purified anti-IFN-γ (R4-6A2), anti-IL-2 (JES6-1A12) or anti-IL-4 (11B11) monoclonal Abs (Pharmingen, San Diego, CA) at 1.25 μg/ml in 1M bicarbonate buffer at a pH of 9.6. Coated plates were incubated overnight at 4 C. The plates were subsequently washed once with RPMI, and blocked with complete-RPMI for at least 2 h before the addition of effector cells.

Effector cells were harvested from primary cultures, pooled, washed, and resuspended in 2 mL of warm RPMI complete media. After seeding in the CTL assay, effector cells were resuspended in 2mL of complete RPMI and dead cells were removed by density gradient centrifugation for 20 minutes at 400-x g on Ficoll ® 400 (Pharmacia Biotech AB, Uppsala, Sweden). Cells at the interface were harvested and washed twice with RPMI complete media to remove residual Ficoll ® 400. CBA/J thymocyte derived effector cells were resuspended in 1 mL of complete RPMI and the number of viable cells determined by trypan blue exclusion. Effector cells were seeded in the ELISPOT at a concentration of 1 x 10^4 cells/well with or without 2.5-5 x 10^5 cells/well irradiated BALB/c or C57BL/6 spleen cells. At this number of

irradiated BALB/c or C57BL/6 spleen cells, spots generated in the ELISPOT by unprimed CBA/J anti BALB/c or C75BL/6 spleen cells are undetectable.

After the addition of cells, ELISPOT plates were incubated overnight at 37°C in 5% CO_2. Cells were then lysed with ddH20, and cellular debris washed away with PBST. After at least five PBST washes, 100 µl of biotinylated anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-5H4) or anti-IL-4 (BVD6-24G2) (BD PharMingen (Mississauga, ON, Canada)) at a concentration of 1.25 µg/ml PBST was added to the appropriate wells. ELISPOT plates were then incubated overnight at 4°C, or alternatively for 4 hrs at room temperature. Biotinylated antibodies were washed away with PBST and 100 µl of alkaline-phosphatase strepavidin at a concentration of 0.2 µg/mL in PBST was added to each well. After an incubation period of 1.5 hrs at room temperature, plates were extensively washed with ddH$_2$0 and spots developed by the addition of 100 µl/well of NBT/BCIP Stock Solution (Nitro blue tetrazolium chloride/5-Bromo-4-Chloro-3-indolyl phosphate, toluidine salt, (Boehringer Mannheim, Germany) diluted 1:50 in 0.1 M Tris-Hcl, pH 9.5, 0.1 M NaCl, and 0.05M $MgCl_2$). ELISPOT plates were allowed to dry and spots were enumerated using a dissecting microscope with the assistance of an eyepiece grid.

3.6. Monoclonal Antibodies

Monoclonal antibodies were prepared from culture supernatants or ascitis fluid as described (Coligan et al., 1998). Hybridomas producing monoclonal antibody were obtained from the American Type Culture Collection (Rockville, MD) or were

generous gifts: TIB-207 (GK1.5) anti-CD4, TIB-99 (HO-13-4) anti-Thy1.2, TIB-211 (3.155) anti-CD8, TIB-235 (IM7.8.1) anti-CD44, TIB 146 (RA3-3A/6.1) anti-B220, HB-253 (GL1) anti-CD86, HB-301 (16-10A1) anti-B7.1, and HB-290 (NLDC-145) anti-Dec-205 were obtained from ATCC; anti-CD62L (MEL14.D54) and anti-CD45RB (MB23G2) were gifts from I. Weissman and E. Pure, respectively. Purified hamster anti-mouse CD40 ligand (CD154, MR1) and anti-mouse CD40 (HM40-3) monoclonal antibodies were obtained from BD PharMingen (Mississauga, ON, Canada).

3.7. Cell Separation Procedures

3.7.1. Antibody-Dependent Complement Mediated Lysis

Thymocytes or spleen cells were suspended at 10^7 cells/ml in complete RPMI. Ascitis fluid containing the anti-Thy-1.2, anti-CD4, or anti-CD8 antibody was added to the cell suspensions at a dilution previously determined to be optimal for effective depletion, generally 1/200, and the cells incubated on ice. Similarly, effective concentrations of monoclonal antibodies from hybridoma supernatant containing anti-CD44, anti-CD62L, anti-CD45RB, or anti-B220 were added to cell suspensions, generally undiluted or 1/2 dilutions of hybridoma supernatants were employed. After 1.5 hrs, cells were washed and resuspended in either media for cell controls, or 1 mL per 10^7 cells of rabbit complement (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) diluted 1:20 in complete RPMI. Prior to its addition to

cells, the rabbit complement was passed through a 0.22 μm Nalgene Syringe Filter (Nalge Company, Rochester, NY, USA), and was subsequently incubated with a minimum of 2 x 10^7 thymocytes on ice for 20 min to reduce toxicity. The sterile-adsorbed complement was centrifuged for 10 minutes at 280-x g and the supernatant used for antibody dependent complement mediated depletion. After the addition of complement, control and antibody tagged cells were incubated at 37 C for 1 hr, with gentle mixing every 20 minutes. At this time, cells were washed twice with complete RPMI and resuspended in fresh medium. The number of viable white cells was again determined by trypan blue exclusion to calculate the percentage cell lysis. In procedures where antibody dependent complement mediated lysis of cells was performed, cells were added to cultures or the ELISPOT assay based on cell counts obtained prior to depletion. The efficiency of antibody-dependent complement mediated depletion was determined by flow cytometry as described in section 3.9.1.

3.7.2. MACS sorting of CD45R+ (B220+) and CD45R- Cells from Normal BALB/c Splenocytes

Single cell suspensions were prepared from BALB/c splenocytes and CD45R+ (B220+) and CD45R- cells (B220-) separated by magnetic cell sorting with MACs Microbeads (Miltenyi Biotech GmbH, Germany). B220 is described as a pan B cell marker (Coffman & Weissman, 1981). Briefly, after the concentration of spleen cells was determined by trypan blue exclusion, red blood cells in the desired number of splenocytes resuspended in 1-2 mL were lysed by a 3 minute incubation

at room temperature with 5 mL/spleen of 0.2 μm filter sterilized, ACK lysing buffer

(0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1mM Na_2EDTA in ddH20, pH 7.2-7.4). Cells

were washed once with complete RPMI, resuspended in MACs buffer (5% FCS in

PBS). Cellular debris was removed by passage through 30 μm nylon mesh. Filtered

cells were centrifuged for 10 minutes at 280-x g and resuspended in 90 μL MACS

buffer per 10^7 cells. Ten μL of MACs CD45R (B220) Microbeads per 10^7 total cells,

were then added as per the manufacture's instructions. Cells were mixed and

incubated at 6-12 C for 15 minutes. Cells were washed with 10-20X the labeling

volume of buffer and centrifuged at 280-x g for 10 minutes. The cell pellet was

resuspended in 1 mL of MACs buffer per 10^8 cells. CD45R+/- cells were separated

on washed LS Selection Columns (Miltenyi). CD45R- cells were collected from the

column effluent while the LS column was in the magnetic field of a MidiMacs

separator magnet (Miltenyi). CD45R+ cells were collected from the expulsed

column effluent after the column was removed from the magnetic field of the

MidiMacs separator magnet (Miltenyi). Counts from trypan blue exclusion indicate

that the total cell recovery from the procedure ranged from 60 to 85%. Results from

flow cytometry that was performed as described in section 3.7.1 indicate that the

purity of the sorted CD45R+ B220+ and CD19+ double positive population was

98%. It was also confirmed that CD11c expressing cells were absent.

3.7.3. The Removal of Immunoglobulin Bearing Cells by Panning on Antibody-Coated Plates

MACs sorted CD45R+ cells from BALB/c mice were depleted of immunoglobulin bearing cells by panning on antibody-coated Petri plates (Coligan et al., 1998). Falcon 100mmx15mm plates (Becton Dickinson & Co.) were coated overnight at 4°C with 9 mL of 100 µg/mL polyspecific rabbit anti-mouse immunoglobulin (Cedarlane Laboratories Ltd.). The following day, the coating solution was poured off and the plates gently washed 4-5 times with 5ml PBS. Cell suspensions in complete RPMI were then plated at $\leq 2x10^8$ cells/plate for 30 minutes at room temperature. After 30 minutes, the plates were gently swirled and the nonadherent immunoglobulin- cells collected and plated onto a second coated and washed Petri plate. Cells were allowed to adsorb for another 30 minutes at room temperature. The procedure was repeated once more. Nonadherent immunoglobulin- cells were collected, washed, and resuspended in complete RPMI. When cell recovery from the procedure was assessed by trypan blue exclusion, 95% of the CD45R+ cells were removed. Flow cytometry that was performed as described in section 3.9.1. confirmed that 95.4% of the B220+ cells were removed. The cell counts determined before the panning procedure were used to calculate the number of BALB/c cells to plate in culture. Nonadherent, immunoglobulin- BALB/c splenocytes were also depleted of Thy1.2+ cells by antibody-dependent complement mediated depletion prior to addition to culture as described in section 3.7.1. This

result was again confirmed by flow cytometry that was performed as described in section 3.9.1, and such treatment removed 97.3% of αβ TCR+ cells.

3.8. The Generation and Purification of Dendritic Cells from Bone Marrow, and their Isolation from Spleen

3.8.1. Generation of Dendritic cells from Proliferating Mouse Bone Marrow Progenitor Cells

Bone marrow derived dendritic cells were prepared as described (Coligan et al., 1998) employing a protocol based on a previously described method (Inaba et al., 1992). Femurs and tibias were aseptically removed from sacrificed 6-8 week old female mice. Muscle tissue was removed and the bones placed in a Falcon 60mmx15mm Petri plates (Becton Dickinson & Co.) containing cold RPMI-1640 (Gibco Laboratories). Residual tissue was rinsed away with cold RPMI-1640. Intact and cleaned bones were sterilized by soaking for 2 minutes in 70% ethanol. Sterilized femurs and tibias were subsequently washed 3 times with ice-cold RPMI-1640.

Both epiphyses (bone ends) were separated from femur and tibia shafts with sterile scissors and transferred to a separate Petri dish. The ends were minced and large particles removed by passage through a stainless steel mesh. Marrow plugs were flushed from the femur and tibia shafts with an RPMI-1640 containing, latex-free 10 mL B-D syringe with an attached B-D PrecisionGlide 26-G, 1/2 in. needle

(Becton Dickinson & Co.). Bone marrow cells were collected into a 50 mL centrifuge tube and washed by centrifugation for 10 minutes at 280-x g. Cells were resuspended in 2 mL of cold RPMI-1640 and red blood cells lysed by a 3-minute incubation at room temperature with 5 mL ACK lysing buffer. Bone marrow cells were washed twice with 20 mL of RPMI-1640 and resuspended in 10 mL of complete RPMI media containing 1 ng/mL recombinant mouse GM-CSF (BD PharMingen). Cells were counted by trypan blue exclusion to calculate bone marrow progenitor cell recovery.

After the concentration of cells was determined, bone marrow progenitors were plated in Falcon 24-well tissue culture plates (Becton Dickinson & Co.) containing 800 Units/well recombinant mouse GM-CSF (BD PharMingen). Bone marrow cells from one mouse were plated in one 24-well tissue culture plate. Plates were maintained at 37 C and 5% CO_2 for 9-15 days. Every 48 hrs, the supernatant was removed and the nonadherent, contaminating lymphocytes were washed away by the addition and removal of 1-1.5 mL of RPMI-1640 warmed to 37 C. Fresh, complete RPMI media containing 800 Units/well of recombinant GM-CSF (BD PharMingen) was added. After 9-15 days, dendritic cell aggregates were dislodged by pipetting and collected. Dislodged cells were pooled and centrifuged for 10 minutes at 280-x g. Following centrifugation, dendritic cells were resuspended in complete-RPMI without GM-CSF and subcultured at 37 C and 5% CO_2 for 24 hrs in Falcon 100mmx15mm Petri plates (Becton Dickinson & Co.) to promote full differentiation (Coligan et al., 1998). Nonadherent, nonproliferating differentiated dendritic cells were collected from the subcultures by gentle collection of the

supernatant after swirling. After washing, the number of viable cells was determined by trypan blue exclusion.

For some experiments, as indicated in the relevant figure legends, bone marrow derived dendritic cells were exposed to exogenous activation stimuli. Control bone marrow cultures contained complete RPMI without GM-CSF. Dendritic cells activated by exogenous stimuli were sub-cultured at 37 C in 5% CO_2 for 24, or for 48 hrs in a replicate experiments to confirm efficient dendritic cell activation, in the presence of complete-RPMI without GM-CSF containing either 2.5 µg/mL anti-CD40 mab (HM40-3, BD PharMingen), or 1 µg/mL LPS (Sigma-Aldrich, St. Louis, MO). After activation in the presence of exogenous stimuli, mature dendritic cells were harvested as described and washed twice to remove excess anti-CD40 mab or LPS (Coligan et al., 1998; Inaba et al., 1992). Cells were resuspended in 2-5 mL of complete RPMI and the number of viable cells determined by trypan blue exclusion.

For some experiments, the number of dendritic cells added to culture was varied as indicated. When the number was not varied, the number of allogeneic dendritic cells used to stimulate thymocytes was a physiological dose derived from the number that would normally be found in 2×10^6 BALB/c splenocytes, being 0.1-0.5% of the total splenocytes (Coligan et al., 1998). Using the highest percentage, thymocytes were stimulated with 1×10^4 allogeneic dendritic cells/well. During the repetition of some experiments, thymocytes were also stimulated with a two fold higher number of dendritic cells, 2×10^4 cells/well, representative of 1% of the total

splenocytes. Similar findings were obtained with both dendritic cell doses, as noted in the results section.

3.8.2. Antigen Presentation to BALB/c Ovalbumin-Specific CD4+ T cell Clones by Bone Marrow Derived Dendritic Cells

The ability of mature and activated bone marrow derived dendritic cells to present antigen to ovalbumin specific CD4+ T cell clones was determined in a standard proliferation assay (Coligan et al., 1998). Th1, IFN-γ secreting, CD4+ T cell clones obtained from K.K. McKinstry were plated in 96-well Nunclon flat-bottom micro titer plates (Nalge Nunc International) at 1×10^4 cells/well in complete RPMI in the presence or absence of 1×10^4 cells/well unstimulated, or LPS-stimulated bone marrow derived dendritic cells. Ovalbumin (Grade V, Sigma-Aldrich) or Hen Egg Lysozyme (Grade VI, Sigma-Aldrich) was added to the appropriate wells containing either un-stimulated or 24 hour-LPS stimulated dendritic cells at a concentration of 1.25 mg/mL, which was previously found by K. McKinstry to be an optimal concentration of antigen for *in vitro* expansion and cytokine elicitation in the ELISPOT assay.

Proliferation was measured by the addition of 1 μCi/well of ^{3}H-thymidine (Mandel Scientific Co. Ltd., Guelph, ON, Canada) for the last 18 hours of 4-day culture. Cells were harvested 18 hrs after the addition of ^{3}H-thymidine using a PHD Cell Harvester (Cambridge Technology Inc., Cambridge, MA, USA). The presence of incorporated ^{3}H-thymidine was determined using a LS 5000TD Liquid

Scintillation System (Beckman Instruments, Fullerton, CA) after all volume had evaporated, and scintillation fluid (4 g/L Omnifluor (Packard Instrument Company Inc., Meriden, CT) in scintillation grade Toluene) was added. The ability of the different dendritic cell populations to present antigen was determined from the amount of ^{3}H-thymidine uptake above the background observed in the absence of antigen.

3.8.3. Enrichment of Non-T and Non-B Cells by removal of CD45R+ and Thy1.2+ Cells from BALB/c Splenocytes

Single cell suspensions were prepared from BALB/c splenocytes. CD45R+ (B220+) and CD45R- (B220-) cells were fractionated by magnetic cell sorting with MACs Microbeads. The CD45R- population was collected and depleted of Thy1.2+ T cells by antibody-dependent complement mediated lysis as previously described in section 3.7.1. The percentage of cells removed by the procedure employed to deplete CD45R+ and Thy1.2+ cells was determined to be 88% by trypan blue exclusion. To prevent subsequent proliferation in culture, the CD45R- Thy1.2- cells, which contains macrophages and most dendritic cell subsets, were exposed to 1500 rads of γ irradiation from a Co^{60} source, and plated. The cell counts determined prior to MACs sorting, adjusted for total cell loss, were used to calculate the concentration of cells plated in culture.

Separated CD45R+ and CD45R- Thy1.2- cells that had not been exposed to γ irradiation were plated overnight in Corning 25 cm^2 Tissue Culture Flasks (Corning

Glass Works, Corning, NY, USA) containing complete RPMI media supplemented with 1 ng/mL recombinant mouse GM-CSF (BD PharMingen). Flow cytometry performed as described in section 3.9.1 before culture confirmed that unseparated spleen cell populations contained 1% of cells with low side scatter as described for dendritic cells (Girolomoni et al., 1990) and were enriched to 9% after the sorting procedure. CD11c and MHC Class II expressing cells were also found to be present. The number of cells with dendritic cell morphology after overnight culture derived from CD45+ and Thy1.2+ T cell depleted spleen was repeatedly 0.2% of the original number of spleen cells, which is within the range of the expected number of dendritic cells (Coligan et al., 1998).

3.9. Flow Cytometric Analysis

3.9.1. Identification of Cell Subsets and Characterization of Surface Marker Expression by Flow Cytometric Analysis

Flow cytometric analysis was performed on normal cell populations (Butcher, Scollay, & Weissman, 1980), cell populations depleted by antibody-dependent complement mediated cell lysis, on cell populations positively or negatively selected by MACs sorting, and on cell populations depleted by anti-immunoglobulin panning (Coligan et al., 1998). Expression of costimulatory and dendritic cell markers was also assessed on cells derived from bone marrow cultures matured in the presence of complete RPMI or those matured in the presence of additional maturation stimuli. The indirect detection of cell surface markers was

carried out as follows: between 1-10 x 10^5 cells, in complete RPMI, were centrifuged for 10 minutes at 280-x g and resuspended in an amount of supernatant previously determined to be saturating, generally 1 mL of undiluted to 1/2 diluted hybridoma supernatants. Cells were stained with antibodies for 1.5 hrs at 4°C and then flooded with FACs solution (2% FCS in PBS). Cells were again centrifuged for 10 minutes at 280-x g and resuspended in 100 μl of FACS solution. The appropriate FITC conjugated goat anti-rat, anti-kappa chain, or anti-hamster IgG antibody (Cedarlane Laboratories Ltd.) or isotype antibody control was then added at the manufacturer's recommended concentration, normally 10 μl per sample. Cells were also directly stained for expression of CD4 (L3/T4), CD8 (Ly2), Thy1.2 (clone 5a-8), αβ TCR (clone H57-597), B220 (Ly5), or CD19 (B4) with FITC or PE conjugated antibodies (Cedarlane Laboratories Ltd.). When cells were stained after antibody dependent complement mediated depletion, the removal of cells was assessed with FITC conjugated antibodies that recognize different epitopes than the depleting antibody, for example FITC anti-mouse CD4+ (RM4-4) (BD PharMingen) was employed when cells were were tagged with GK1.5 anti-CD4 antibody, or the appropriate FITC conjugated goat anti-rat IgG, anti-rat kappa chain, or anti-hamster IgG antibodies were employed to detect antibody tagged cells. Cells were washed with FACs solution, resuspended in 100 μl of the same solution, and stained with the manufacturer's recommended concentration, normally 2-10 μl, of the appropriate fluorochrome conjugated antibody. Labeled cells were incubated in the dark for 30 minutes at 4°C. In experiments where indirectly stained cells were double stained, cells were stained sequentially, with a wash in between incubations, with either FITC

or PE conjugated antibodies. After staining, cells were washed once with 1 mL of FACS solution and cell pellets were resuspended in 100 μL of FACS solution. Once cells were in suspension, an equal volume of fixative (PBS + 2% formaldehyde) was added. Stained and fixed cells were analyzed by flow cytometry using a Beckman Coulter Epics Flow Cytometer, and Expo32v1.2 analysis software (Beckman Coulter, Mississauga, ON, Canada).

Removal of cell populations by antibody dependent complement mediated depletion generally resulted in 92-97% depletion of the desired population. Purity of sorted CD45R (B220+) cells was found to be 98%. MACs sorted CD45R (B220-) cells were found to still contain a population of cells expressing low levels of the B cell marker B220+ in the range of 5-9%. Panning of Macs sorted CD45R (B220+) cells on immunoglobulin coated plates resulted in the loss of 94-96% of the B220+ cells.

3.9.2. Detection of Cell Proliferation with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)

Proliferation of thymocytes stimulated with irradiated, allogeneic antigen presenting cells in the absence or presence of syngeneic, irradiated help was assessed by flow cytometry using CFSE. CFSE is a stable, intracellular dye that is diluted two fold with each cell division (Lyons & Parish, 1994). The number of viable cells in single cell suspensions in complete RPMI was determined. Cells were flooded with PBS and centrifuged for 10 minutes at 280-x g. A total of 5 x 10^7 cells/mL,

resuspended in sterile PBS, was stained with a final concentration of 5 µM CFSE (Molecular Probes, Eugene, OR, USA). CFSE powder was dissolved in anhydrous Dimethyl Sulfoxide (DMSO) to make a 5 mM stock solution that was stored at –70 C for long term storage, or desiccated at –20 C, as per manufacture's instructions. Cells were stained with CFSE for 30 minutes at 37 C in 5% CO_2. At this time, cells were washed twice with complete RPMI to remove any residual dye. After staining, cells were plated and stimulated in culture as described in section 3.4. Once harvested, dead cells were removed by density gradient centrifugation for 20 minutes at 400-x g on Ficoll ® 400 (Pharmacia Biotech AB). Cell division of single cells was determined by viable cell expression of CFSE, which was determined by flow cytometry using a Beckman Coulter Epics Flow Cytometer, and Expo32v1.2 analysis software (Beckman Coulter).

3.10. *In Vitro* Generation of CBA/J CD4+ T cell Clones Specific for BALB/c Alloantigen

CBA/J CD4+ T cell clones were derived from effector cells harvested from thymocyte cultures stimulated with irradiated BALB/c T cell depleted spleen cells in the presence of irradiated syngeneic CBA/J splenic helper cells (Coligan et al., 1998; Sredni, Tse, Chen, & Schwartz, 1981). Thymocyte derived effector cells were plated in limiting dilutions ranging from 1 x 10^4 to 10 cells/well in 96-well Nunclon flat-bottom micro titer plates (Nalge Nunc International) in complete RPMI containing a standard amount, 10 Units/mL of recombinant mouse IL-2, which was collected

from the supernatant of L630 and titrated as described (Coligan et al., 1998). Effector cells were cultured in the presence or absence of 0.5 x 10^6 cells/well irradiated BALB/c spleen cells. Wells positive for T cell clusters were harvested and washed by centrifugation for 10 minutes at 280-x g. Cell pellets were resuspended in complete RPMI and sub-cultured in Falcon 24 well tissue culture plates (Becton Dickinson & Co.). Wells positive for T cell clusters were again harvested and replated in limiting dilutions in 96-well Nunclon flat-bottom micro titer plates (Nalge Nunc International). Alternating rounds of re-stimulation with irradiated BALB/c splenocytes in the presence or absence of IL-2 resulted in the isolation of T cell clones. Alloantigen specificity and the cytokine profile of each cloned line were determined in the ELISPOT assay as described in section 3.7.2. The CD4+ T cell phenotype of each line was determined by flow cytometry as described in section 3.9.1. Once the phenotype, antigen-specificity, and cytokine profile of each clone was determined, samples resuspended in complete RPMI with 20% FCS were prepared for freezing by the slow addition, while mixing in an ice slurry, of 10% DMSO. Small aliquots were transferred to cryovials and placed at –70 C for 2 hrs, after which, the vials were stored in liquid nitrogen.

4. RESULTS

4.1. Introduction to the Experimental System

In order to delineate the cell interactions involved in naive T cell activation, we chose to use an *in vitro* culture system relying on thymocytes as the responding lymphocyte population and alloantigen as the stimulating antigen. The former lymphocyte population was chosen because it was recognized that an incisive study of the initiation and generation of immune responses by naïve T cells can be undermined by the presence of previously activated lymphocytes among the responding naïve T cells. The reliance on the use of an experimental system employing alloantigen as the stimulating antigen has allowed us to assess the generation of immune response from naïve thymocytes after primary culture. While the frequency of alloreactive cells is higher than that of protein antigen-specific T cells, in the range of 1/100 to 1/1000 versus $1/10^4$ to $1/10^5$ for other antigen-specific T cells, this response is still an antigen-specific T cell response; the response generated upon stimulation with alloantigens is a compilation of a number of T cell specificities, all of which possess a TCR that can cross-react with the MHC and peptide complexes presented on allogeneic cells.

Naive T cells can theoretically be isolated from secondary lymphoid organs, such as lymph nodes and the spleen (Dutton et al., 1998). However, the reliability of current separation methods has recently been questioned in light of observations

showing, for example, that one of the standard surface markers of naïve T cells, namely CD62L or L-selectin, is also present on resting memory T cells (Ahmadzadeh et al., 2001; Hengel et al., 2003; Kassiotis & Stockinger, 2004). An experimental system relying on the use of thymocytes may be an ideal model for naïve T cells of the periphery. It is recognized that some propose thymocytes may receive additional maturation signals upon emigration into the periphery (Boursalian, Golob, Soper, Cooper, & Fink, 2004). Advantages and disadvantages in regards to the use of thymocytes, and experimental approaches to delineate the potential differences between competent thymocytes and naïve T cells isolated from the periphery, will be addressed further in the discussion.

A small fraction of thymocytes found among the mature single positive cells found in the medullary regions of the thymus, and those mature T cells that have recently emigrated from the thymus are capable of generating immune responses (Bennink & Doherty, 1981; Fink, Gallatin, Reichert, Butcher, & Weissman, 1985; Pilarski, 1977; Ramsdell, Jenkins, Dinh, & Fowlkes, 1991; Scollay, Chen, & Shortman, 1984; Wagner, Hardt, Bartlett, Rollinghoff, & Pfizenmaier, 1980; Widmer, MacDonald, & Cerottini, 1981). Of importance to this thesis work are the findings of L. Pilarski that the generation of antigen-specific CTL responses from CBA/J thymocytes stimulated with irradiated, allogeneic BALB/c splenocytes, is dependent upon the presence of additional CD4+ T helper cells, provided in the form of irradiated, syngeneic CBA/J splenocytes (Pilarski, 1977). The radiation-resistant nature of the CD4+ T cell help is important. This property indicates that the helper

activity of these cells is independent of cell proliferation (Kettman & Dutton, 1971; Sprent, Anderson, & Miller, 1974).

The initial observations made by L. Pilarski support the proposal that effector CD4+ T helper cells that can "help" in the generation of immune responses from naïve thymic T cells exist in the spleens of normal, un-primed mice (Pilarski, 1977). She also demonstrated, employing spleen cells from CBA/J into CBA/J X BALB/c chimeras, that the CD4+ T cell in the irradiated spleen that "helps" thymocytes generate allo-MHC specific CTL responses is tolerant towards self; spleen cells from such mice fail to "help" in the generation of responses against BALB/c MHC-antigens but do "help' in the generation of responses against third party MHC-antigens (Pilarski, 1977; Pilarski et al., 1980). The latter findings provide strong evidence that the radiation resistant effector CD4+ T cells found in the spleens of normal mice are tolerant to self and are antigen specific. Radiation-resistant CD4+ effector T helper cells generated *in vitro* from normal spleen cells similarly display allo-MHC specificity (Baum & Pilarski, 1978).

We have devised a simpler, but similar, experimental system to the one of L. Pilarski to delineate the cell interactions involved in naive T cell activation. Her major experimental observations that the activation of thymocytes, to generate allo-MHC specific CTLs, is dependent upon radiation-resistant syngeneic spleen cells have been repeated.

In addition to the generation of CTLs, the generation of cytokine producing cells from thymocytes has been assessed. In the experiments discussed, unless otherwise indicated, the irradiated-allogeneic spleen used to stimulate thymocytes has been

depleted of Thy1.2+ T cells, because the presence of such cells may not allow a clear analysis of the cellular events involved in the generation of immune responses, as we shall show.

4.2. The Basic Experimental System

The experimental conditions required to generate optimal responses from thymocytes were determined via titration of the number of thymocytes, and the number of irradiated syngeneic spleen cells, using the findings of L. Pilarski as a starting point.   Employing conditions giving rise to consistent responses, experiments were carried out to determine the kinetics of the *in vitro* generation of allo-MHC specific CTL and cytokine producing cells from thymocytes stimulated with alloantigen in the presence of irradiated syngeneic spleen cells. Using the standard chromium release assay to assess CTL activation and the ELISPOT assay to enumerate alloantigen-specific cytokine producing cells (Dunkley et al., 1974; Power et al., 1999), the generation of alloantigen-specific CTL, and cytokine producing cells from thymocytes has been found to depend upon the presence of irradiated-allogeneic spleen cells, see Figures 4.2.1 and 4.2.2. The generation of IFN-γ- and IL-2- producing cells peaks on day 4 of culture and thereafter, on day 5, cytotoxic activity is evident. The cell that possesses CTL activity has been determined to be an antigen-specific, CD8+ T cell, see Figure 4.2.3. The phenotype of the IFN-γ and IL-2 producing cells has also been determined. They have been found to be CD8+ and CD4+ T cells, respectively, see Figure 4.2.4. Additionally, the detection of both cytokine producing cells has been demonstrated to be dependent upon both the

number of thymocyte derived effector phase cells seeded, and the number of irradiated BALB/c spleen cells used to re-stimulate the effectors in the ELISPOT assay, see Figure 4.2.5.

The observations that the generation of allo-MHC specific CD8+ CTL and IFN-γ producing, and CD4+ IL-2 producing cells from thymocytes are dependent upon syngeneic irradiation resistant "helper" cells confirms previous experimental findings (Pilarski, 1977), and also provides experimental support for the choice of using competent thymocytes as responding naïve T cells *in vitro* to delineate the cellular interactions involved in naive T cell activation
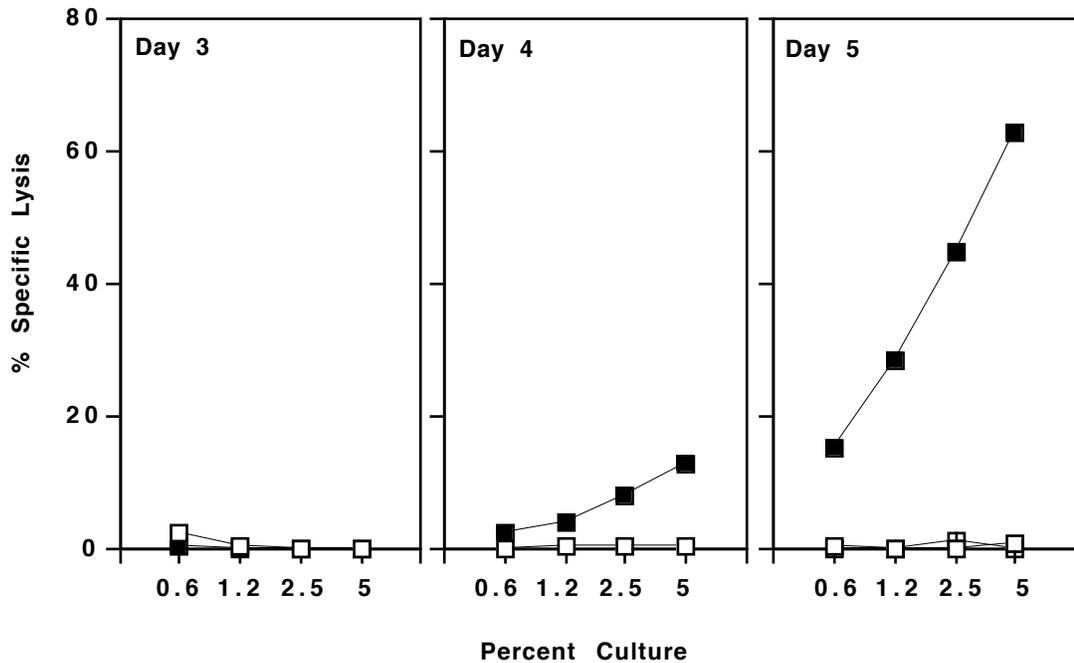
Figure 4.2.1. *The Kinetics of the Generation of CTLs from Thymocytes Stimulated with Allo-MHC Spleen Cells in the Presence of Syngeneic Irradiated Spleen Cells.* CBA/J thymocytes were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence (□, ■, respectively) of irradiated CBA/J spleen cells as described in section 3.4. The response of the syngeneic irradiated spleen cells stimulated with BALB/c spleen cells is represented by the hatched square, and lysis of the BALB/c syngeneic target was ≤ 3% upon incubation with all corresponding fractions of the culture. The generation of CTLs was assessed on the indicated days using the standard ^{51}Cr CTL assay against P815 (H-2^d) target cells as described in section 3.5.1.

Figure 4.2.2. *The Kinetics of the Generation of IFN-γ and IL-2 Producing Cells from Thymocytes Stimulated with Allo-MHC Spleen Cells in the Presence of Syngeneic Irradiated Spleen Cells.* CBA/J thymocytes were stimulated with irradiated, T cell depleted BALB/c spleen cells in the absence or presence (open and filled symbols, respectively) of irradiated CBA/J spleen cells as described in section 3.4. Cytokine producing cells were detected on the indicated days in the ELISPOT assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. The detection of a low number of antigen-dependent IL-2 producing cells from thymocyte cultures stimulated with allogeneic spleen cells in the absence of irradiated syngeneic spleen was found to be erratic and the highest number detected from replicate experiments is shown.

84
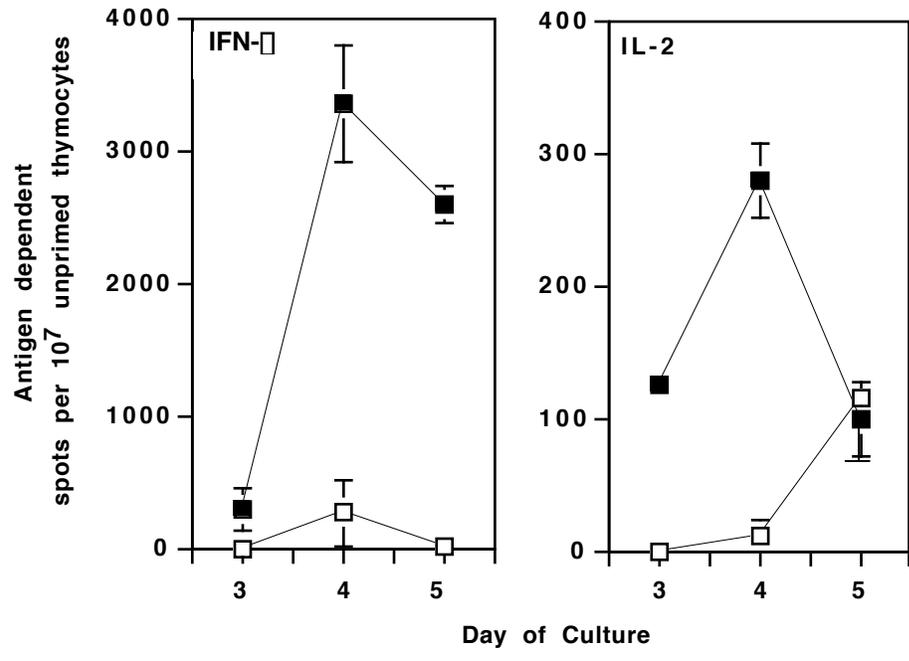
Figure 4.2.3. *The Allo-MHC Specific CTL is a CD8+ T cell.* CBA/J thymocytes, **T**, were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells or **H**-Help as described in section 3.4. Prior to seeding in the CTL assay, T cell subsets were depleted from day 6 effector phase cells by antibody dependent C'-mediated depletion. Cells were untreated, treated (Rx) with C' alone, or with C' and anti-CD4 (GK1.5), or anti-CD8 (TIB 211), or anti-Thy1.2 (TIB 99) hybridoma supernatants as described in section 3.7.1. Adjustments were not made to cell numbers to compensate for cell loss. The presence of CTLs was assessed on day 6 using the standard ^{51}Cr CTL assay against P815 (H-2^d-left panel) or EL-4 (H-2^b- right panel) target cells as described in section 3.5.1. The response of the syngeneic irradiated spleen cells stimulated with BALB/c spleen cells against both targets represented by the hatched square was $\leq$ 5% upon incubation with all corresponding fractions of the culture and is hidden by the other symbols.
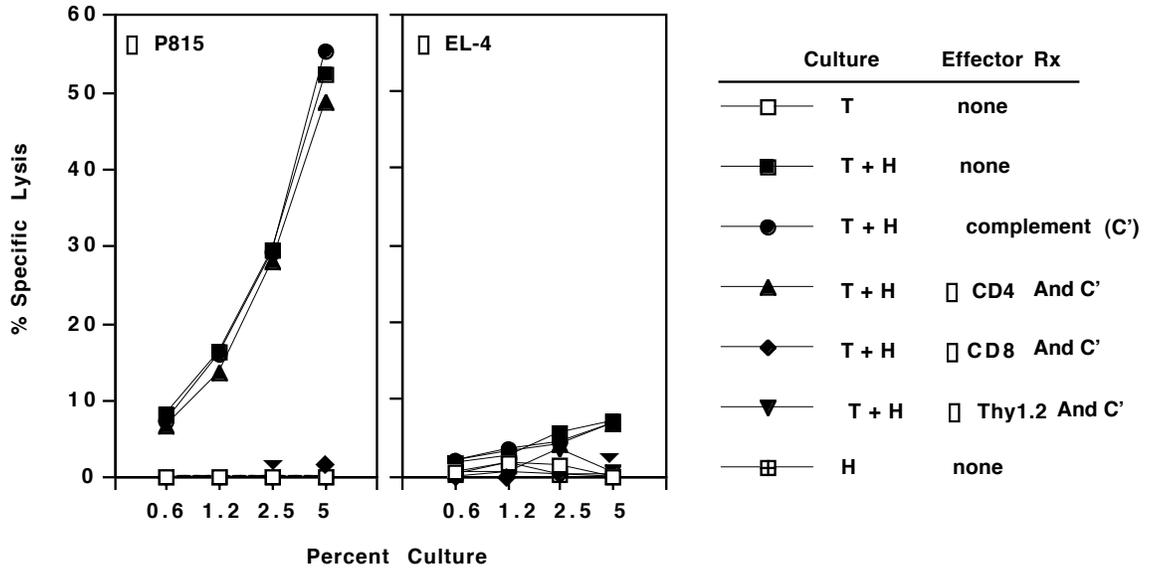
Figure 4.2.4. *The CD4/CD8 Phenotype of the Allo-MHC Specific IFN-γ and IL-2 Producing Cells*. IFN-γ and IL-2 producing cells generated as described in section 3.4 by stimulation of thymocytes, **T**, with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells or **H**-help, were detected in the ELISPOT assay as described in 3.5.2. Before seeding, T cell subsets were depleted from day 5 effector phase cells by antibody dependent C'-mediated depletion as described in section 3.7.1. Cells were untreated (1, 2, 7), treated (Rx) with C' alone (3), treated with C' and anti-CD4 (GK1.5), anti-CD8 (TIB 211), or anti-Thy1.2 (TIB 99) hybridoma supernatants (4-6, respectively). Adjustments were not made to cell numbers to compensate for cell loss. The average percentage of single positive CD4+ and CD8+ T cells present in cultures as assessed by flow cytometry performed as described in section 3.9.1 on Day 0 were 8.7 and 3.8%, respectively, and on Day 5, for responsive cultures (T+H), 26.8 and 22.1%, respectively. The error bars represent the standard deviation of the number of antigen dependent spots. Similar results were obtained in three separate experiments.

Rx of Effectors/Culture

88
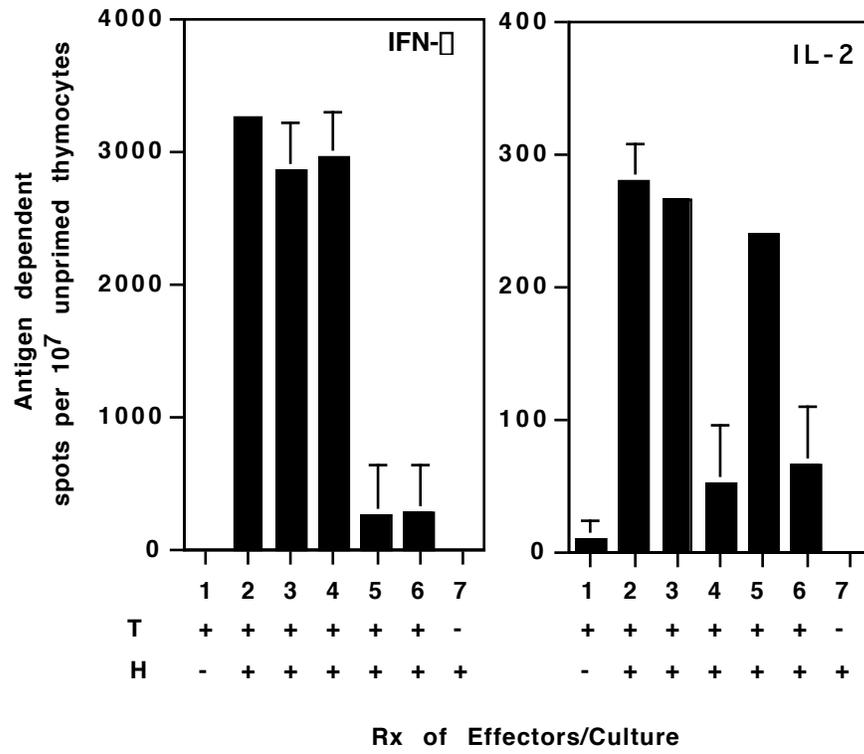
Figure 4.2.5. *The Number of Antigen Dependent IFN-γ and IL-2 Producing Cells Detected in the ELISPOT Assay is Dependent upon the Number of Effector Cells and Allo-MHC Stimulators Plated*. CBA/J thymocytes were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the presence of irradiated CBA/J spleen cells as described in section 3.4. The ability to detect the generation of IFN-γ (upper row) or IL-2 (lower row) producing cells in the ELISPOT assay was assessed by varying either the number of day 6 effector phase cells (left panels) or the number of irradiated BALB/c stimulating spleen cells plated (right panels). The ELISPOT assay was performed as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar results were obtained in three separate experiments.
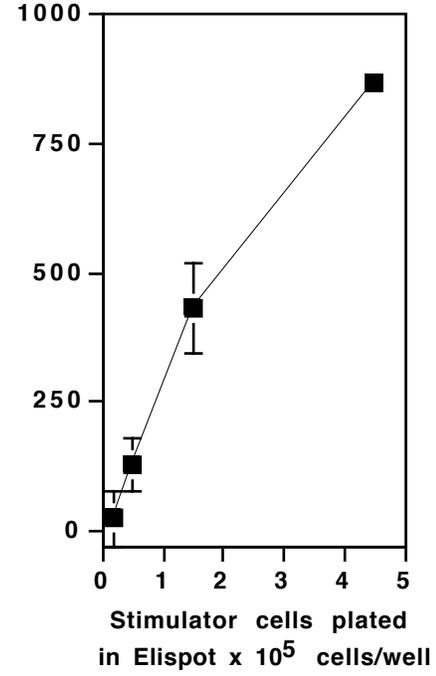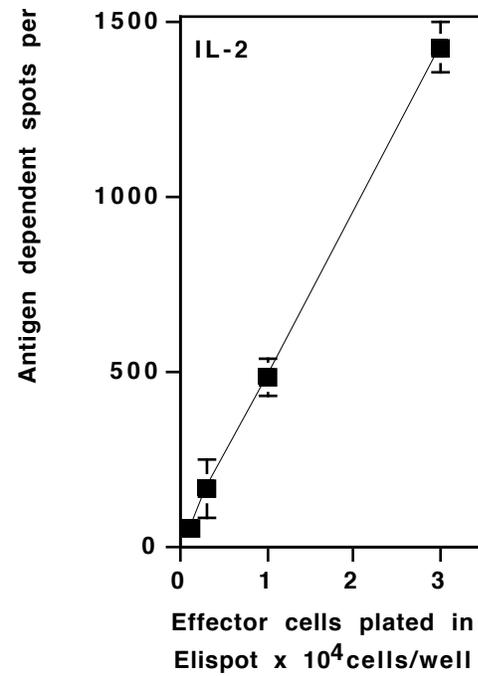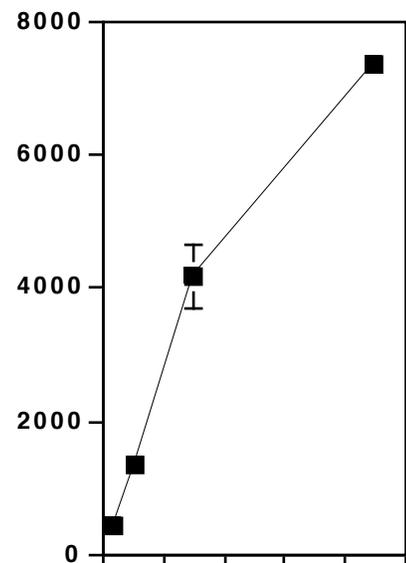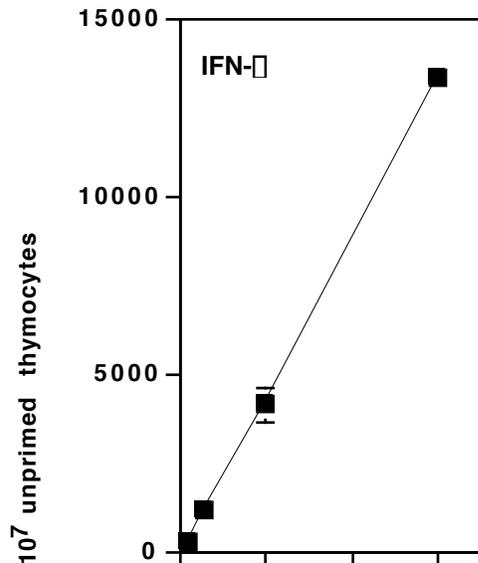
4.3. Dependence of the *In Vitro* Generation of Primary Immune Responses from Thymocytes upon the Density of Thymocytes and "Initiator T Helper" Cells

To ensure optimal sensitivity for subsequent depletion studies, the number of thymocytes or number of irradiated syngeneic spleen cells added to culture was varied and the generation of CTL and cytokine producing cells from thymocytes upon stimulation with alloantigen, in either the absence or presence of irradiated syngeneic spleen cells, assessed.

It is evident that when the density of thymic precursors is varied, and the number of irradiated syngeneic spleen cells and stimulators held constant, the generation of allo-MHC specific CD8+ CTLs is dependent upon the number of thymocytes plated as one would expect, see the left panel of Figure 4.3.1. When the density of thymocytes and irradiated stimulators were constant, and the density of irradiated syngeneic spleen cells varied, the generation of allo-MHC specific CD8+ CTLs is similarly dependent upon the number of irradiated syngeneic spleen cells plated, see right panel of Figure 4.3.1.

A cooperative trend for the generation of antigen-dependent IFN-γ producing cells from thymocytes is evident from the normalized cytokine responses, see Figure 4.3.1. Cytokine responses were normalized to consider the varied number of responding cells present at the initiation of culture. A comparison of the efficiency with which responses are generated at different cell densities must take account of

the different number of precursor cells present under different conditions. We therefore compared the responses generated after correcting for the differences in responder cell density. Normalization or expressing responses per constant number of input cells/precursors ensures that different efficiencies in the generation of responses reflects density dependence, and not differences in the number of responding lymphocytes present at the onset of culture.   The cooperative trend that is evident for the generation of IFN-γ producing cells from thymocytes is not evident for the generation of CTLs when the density of thymocytes is varied. It is difficult to validly assess the presence or absence of a cooperative effect for the generation of CTLs within this experiment because of the need to rely on low specific lysis values. However, if the generation of CTLs from thymocytes is in fact a non-cooperative phenomenon, as is indicated from the horizontal line of normalized lytic responses and the results to be discussed in section 4.4, the discrepancy between the generation of CTLs and IFN-γ producing cells may be related to the independent regulation of cytotoxicity and IFN-γ secretion in CD8+ T cells (Snyder et al., 2003). Enumeration of the number of cytolytic and IFN-γ releasing cells simultaneously, as is possible in the LYSISPOT assay (Snyder et al., 2003), may be required to adequately assess whether cooperation among thymocytes is involved in the generation of CTLs.

The influence of the presence of T cells in the stimulating BALB/c alloantigen was determined in a separate experiment, in which the number of radiation-resistant CBA/J spleen cells and allogeneic stimulators were again constant, and the number of thymocytes plated in culture varied five-fold. The number of radiation-resistant CBA/J spleen cells used in this experiment, 4×10^6

cells/well, differed from that normally used, 1 x 10^7 cells/well, in that it was a number known to be limiting for providing "help" under these conditions. When T cells are present in the allogeneic stimulators, high numbers of thymocytes generate CTL responses in a manner not critically dependent on the presence of radiation-resistant CBA/J spleen cells, see the left panel of Figure 4.3.2, whereas a lower number of thymocytes is dependent upon the presence of irradiated spleen cells for the generation of CTL responses. When T cells are depleted from the allogeneic stimulators by antibody dependent complement mediated lysis, only cultures containing a high number of thymocytes support the generation of CTL responses in a manner that is partially dependent upon the presence of radiation-resistant CBA/J spleen cells, see the left panel of Figure 4.3.2. It is possible that a small population of approximately 3 to 5% residual T cells that normally escape antibody dependent complement mediated depletion as assessed by flow cytometry are present in the irradiated allogeneic stimulators, and that this residual population contains cells that can participate in the generation of responses when a sufficient number of responding thymocytes is present. To minimize the influence of the residual T cells in the allogeneic stimulators in preceding experiments, a density of thymocytes, 2 x 10^6 cells/well, that fails to generate CTL and IFN-γ responses unless an optimal number of irradiated spleen cells, 1 x 10^7 cells/well, are provided was employed.

We conclude from our findings that a threshold number of cooperative T cell interactions must occur between thymocytes and radiation-resistant CBA/J spleen cells for the efficient generation of primary CTL and IFN-γ immune responses when thymocytes are cultured at lower densities and are stimulated with allogeneic spleen

cells depleted of T cells by antibody dependent complement mediated depletion. Some experimental evidence supports the suggestion that T cell cooperation between CD4+ T cells is required to generate responses *in vivo* and *in vitro* (Bretscher, 1979, 1986; Gerloni et al., 2000; Ismail & Bretscher, 2001; Strutt & Bretscher, 2005; Tucker & Bretscher, 1982). The thymocyte cells involved in the cooperative interactions with radiation-resistant CBA/J spleen cells will be explored further in the following section.

Figure 4.3.1. *The Generation of CTL and IFN-γ Producing cells from Thymocytes Stimulated with Allo-MHC Stimulators in the Presence of Syngeneic Irradiated Spleen Cells is Dependent upon both the Thymocyte and Syngeneic Spleen Cell Number*. CBA/J thymocytes were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence (open and filled symbols, respectively) of irradiated CBA/J spleen cells as described in section 3.4. Either the number of thymocytes (left panel) or the number of irradiated syngeneic helper cells (right panel) plated in culture was varied as follows: **Thymocytes** (left panel), ▼- 0.25 x 10^6 cells/well; ● - 0.50 x 10^6 cells/well; ▲ - 1.0 x 10^6 cells/well; and ■ - 2.0 x 10^6 cells/well all stimulated in the presence of 1.0 x 10^7 irradiated spleen cells; or **Splenocytes** (right panel): 2 x 10^6 thymocytes/well stimulated in the presence of ▼- 0.4 x 10^7 cells/well; ●- 0.6 x 10^7 cells/well; ▲ - 0.8 x 10^7 cells/well; ■ - 1.0 x 10^7 cells/well irradiated syngeneic spleen cells. The generation of CTLs was assessed on day 6 using the standard ^{51}Cr CTL assay against P815 (H-2^d) target cells as described in section 3.5.1. The corresponding number of antigen-dependent IFN-γ producing cells/10^7 input thymocytes given "help" detected in the ELISPOT performed as described in section 3.5.2 for each condition is also presented ± the standard deviation. The normalized CTL response of 1.25 x 10^4 input stimulated thymocytes given irradiated syngeneic help is also shown. Similar observations were obtained in three separate experiments.

Top left panel (Thymocytes)

% Specific Lysis (y-axis, 0–50)

Thymocytes

IFN-γ Spots

9038+/-825

2853+/-473

1933+/-257

1246+/-170

αThy1.2 Rx Stimulators

Percent Culture

0.6 1.2 2.5 5

Top right panel (Splenocytes)

Splenocytes

IFN-γ Spots

8063+/-289

5680+/-656

5192+/-260

3884+/-219

0.6 1.2 2.5 5

Percent Culture

Bottom panel

Normalized % Specific Lysis (y-axis, 0–30)

x-axis: 0 0.5 1 1.5 2 2.5

Thymocyte Culture Density x 10^6 cells/well
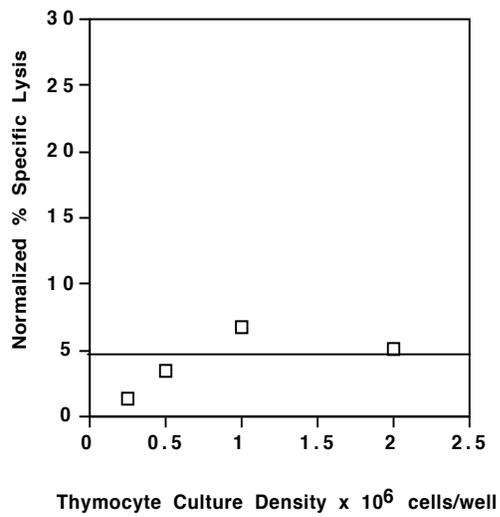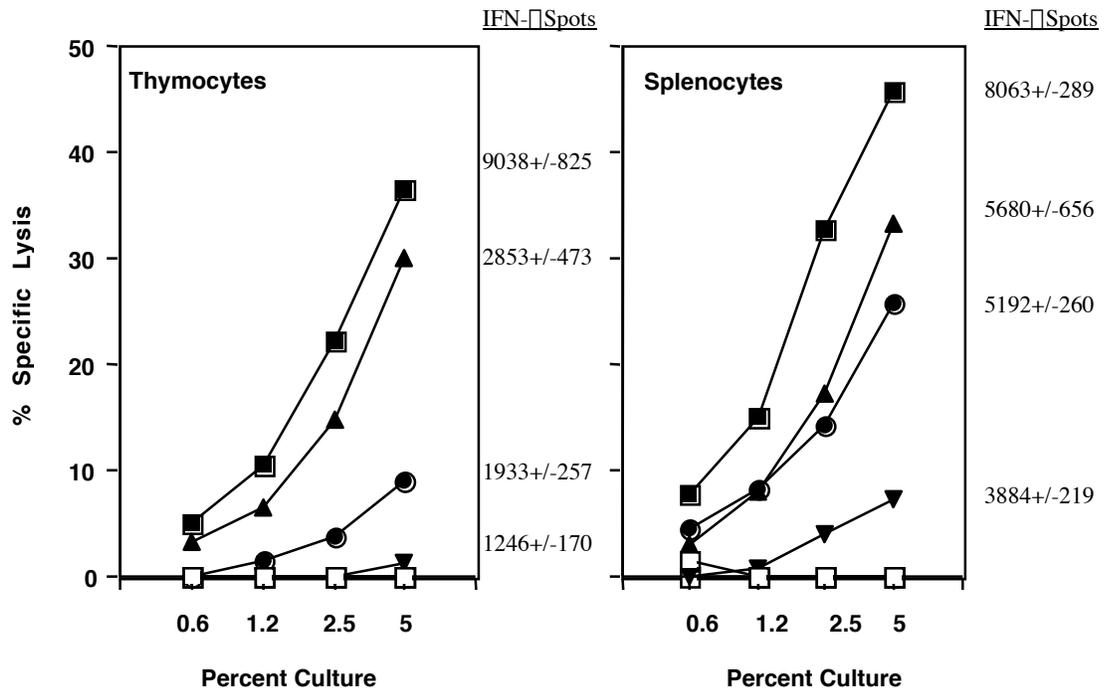
Figure 4.3.2. *The Generation of CTL Responses from Thymocytes Stimulated with Allo-MHC Stimulators is not Critically Dependent on Syngeneic Irradiated Spleen Cells at High Thymocyte Densities when the Allo-MHC Stimulators Contain Thy1.2+ T cells*. A high number, ■ - 1x 10^7 cells/well, or a moderate number, ● - 2x 10^6 cells/well, of CBA/J thymocytes was stimulated with irradiated BALB/c spleen cells in the absence or presence (open and filled symbols, respectively) of a limiting number, 4 x 10^6 cells/well, of irradiated CBA/J spleen cells as described in section 3.4. The BALB/c stimulator cells were either untreated (left panel) or depleted of Thy1.2+ T cells (right panel) by antibody dependent complement mediated depletion prior to addition to culture as described in section 3.7.1. Adjustments were not made to cell numbers to compensate for cell loss. The generation of CTLs was assessed on day 6 using the standard ^{51}Cr CTL assay against P815 (H-2^d) target cells as described in section 3.5.1. The response of the syngeneic irradiated spleen cells stimulated with BALB/c spleen cells against syngeneic targets is represented by the hatched square and was $\leq$ 5% upon incubation with all corresponding fractions of the culture and is hidden by the other symbols. The numbers to the right of each symbol represent the normalized percent specific lysis of the different conditions per 5 x 10^4 input thymocytes. Similar observations were obtained in two separate experiments.

Untreated Stimulators

αThy1.2 Rx Stimulators

% Specific Lysis

100

75

50

25

0

40

30

29

2.1

15

5.4

1.4

0.2

0.6   1.2   2.5   5

0.6   1.2   2.5   5

Percent Culture

98
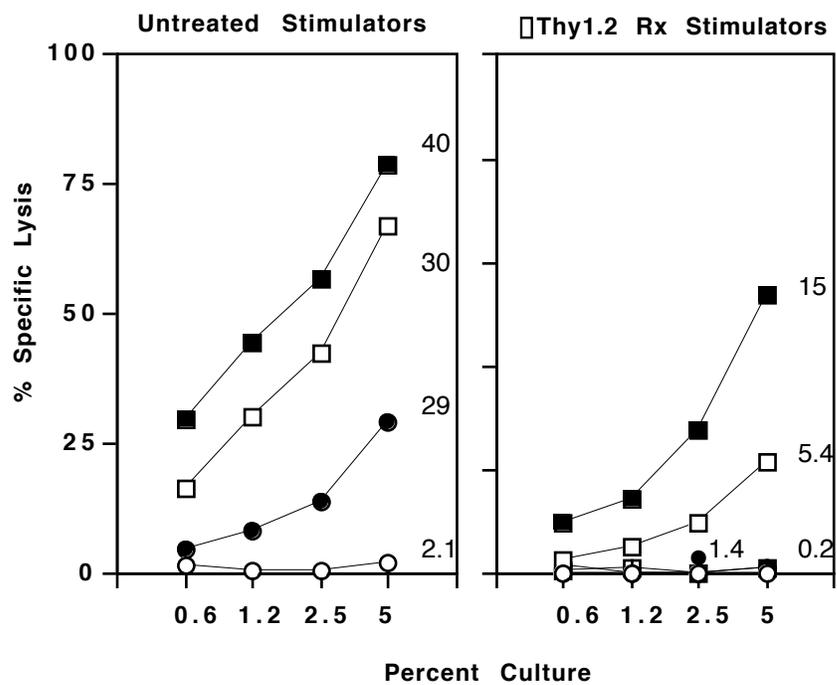
4.4. CD4+ T cell Cooperation in the Generation of Primary Immune Responses *In Vitro*

The experimental findings just described indicate that a threshold number of T cell cooperative events are potentially involved in the generation of primary immune responses. Cooperative interactions may occur between CD4+ T cells and intermediary antigen-presenting cells. For example, it is possible that the radiation-resistant, effector CD4+ T helper cells present in the irradiated syngeneic spleen provides "help" for thymocyte precursor CD8+ CTLs by interacting with and activating an antigen presenting cell (Bennett et al., 1998; Lutz & Fitch, 1979; Ridge et al., 1998). It is also conceivable that the effector CD4+ T helper cells present in the irradiated syngeneic spleen are cooperating with, and activating or licensing antigen-presenting cells that then activate a population of naive precursor CD4+ thymocyte T helper cells, which also participate in "helping" in the generation of CD8+ CTL and cytokine responses (Alpan et al., 2004; Creusot et al., 2003).

Two different experiments were performed to discriminate between the two possibilities. In the first, the effect of removing different thymocyte T cell subsets was determined. CD4+, CD8+, or Thy1.2 positive cells were depleted from CBA/J thymocytes by antibody dependent complement mediated lysis before the onset of culture, and the ability of the different thymocyte populations to generate CTL and cytokine responses was assessed upon stimulation with alloantigen or allogeneic spleen cells depleted of T cells in the presence of radiation-resistant CBA/J spleen cells. CTLs and IFN-γ producing cells fail to be generated in the absence of cells

bearing CD8+ and Thy1.2+ surface markers, see Figures 4.4.1. a and b. The generation of IL-4 producing cells was increased by the removal of CD8+ cells, see Figure 4.4.1 b. This increase in the detection of IL-4 producing cells may be the result of increased CD4+ T cell expansion, which may favor the generation of Th2 type responses, or alternatively, an element that suppresses IL-4 production may be absent. When CD4+ T cells were removed from the responding thymocytes before the onset of culture, IL-4 producing cells were no longer generated, and a slight decrease in both the generation of CTLs and IFN-γ producing cells was evident, again see Figures 4.4.1 a and b.

In the second experiment, complement treated and CD4 depleted thymocytes were cultured at different densities and stimulated with allogeneic spleen cells depleted of T cells in the presence of sufficient numbers of irradiated syngeneic spleen cells to ascertain the influence of CD4+ thymocytes on the generation of CTL and normalized cytokine responses. The generation of CD8+ CTLs was found to decrease when thymocyte cultures are devoid of CD4+ T cells, see Figure 4.4.2. a. As was indicated in previous experiments, the generation of CTLs from thymocytes appears to be a non-cooperative phenomenon, and supports the suggestion that the different effector functions of CD8+ T cells are independently regulated (Snyder et al., 2003). The generation of IFN-γ producing cells was also markedly lower in cultures that were devoid of CD4+ thymocyte precursor T helper cells, see Figure 4.4.2. b. As one would expect from the CD4+ phenotype of the IL-2 producing cells, the removal of CD4+ T cells before cell culture abolished the ability to detect the generation of IL-2 producing cells.

We conclude from these findings that thymocyte CD4+ precursor T helper cells can act in conjunction with radiation-resistant CBA/J spleen cells in the generation of antigen-specific CTL and IFN-γ producing cells. CD4+ precursor thymocytes can potentially "help" in the generation of responses from CD8+ thymocytes by either directly licensing additional APC, and/or by providing additional growth cytokines, such as IL-2 and/or IL-4. Naïve CD4+ thymocytes, therefore, possess the potential, if appropriately stimulated, to "help" in the *in vitro* generation of antigen-specific immune responses.

Figure 4.4.1. a. *The Generation of CTLs from Thymocytes after the Removal of different T cell Subsets at the Initiation of Culture.* A standard number of male CBA/J thymocytes, **T**, were stimulated with irradiated, T cell depleted, female BALB/c spleen cells in the absence or presence of irradiated male CBA/J spleen cells or **H**-Help as described in section 3.4. At the initiation of culture, T cell subsets were depleted from input thymocytes by antibody dependent C'-mediated depletion as described in section 3.7.1. Cells were untreated, treated (Rx) with C' alone, or with C' and anti-CD4 (GK1.5), or anti-CD8 (TIB 211), or anti-Thy1.2 (TIB 99) hybridoma supernatants. Adjustments were not made to cell numbers to compensate for cell loss. The generation of CTLs was assessed on day 6 using the standard ^{51}Cr CTL assay against P815 (H-2^d) target cells as described in section 3.5.1. The response of the syngeneic irradiated spleen cells stimulated with BALB/c spleen cells against syngeneic targets is represented by the hatched square was $\leq$ 5% upon incubation with all corresponding fractions of the culture and is hidden by the other symbols. Similar observations were obtained in two independent experiments in which male CBA/J mice, the only mice available at the time, were employed.

% Specific Lysis

80

60

40

20

0

0.6 1.2 2.5 5.0

Percent Culture

Culture/Thymocyte Rx

	Culture	Thymocyte Rx
□	T	-
■	T + H	-
●	T + H	C'
▲	T + H	α CD4 And C'
◆	T + H	αCD8 And C'
▶	T + H	α Thy1.2 And C'
⊞	H	-

103
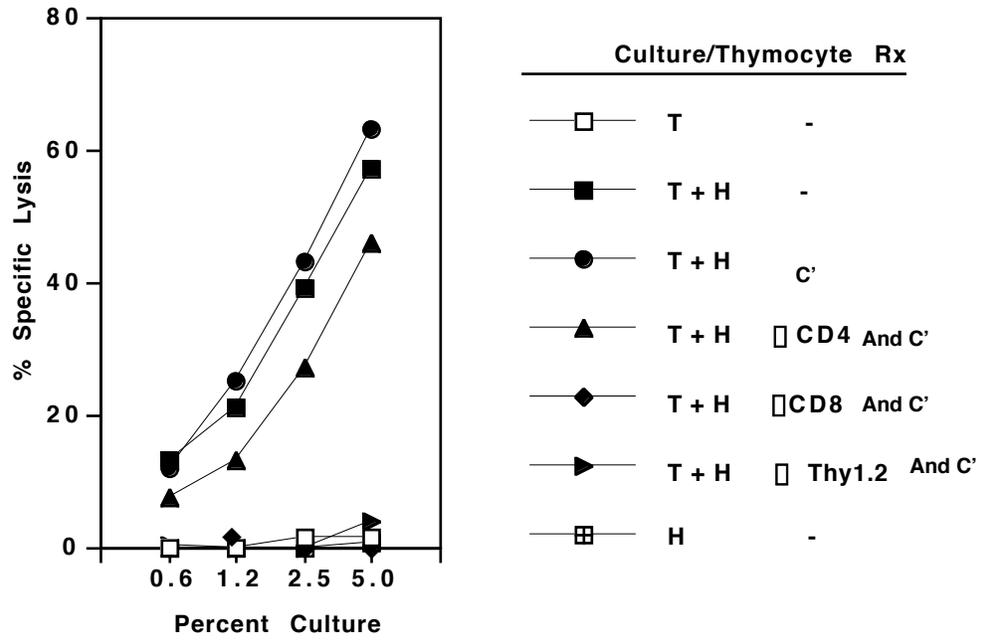
Figure 4.4.1 b. *The Generation of Allo-MHC Specific IFN-γ, IL-2, and IL-4 Cytokine Producing Cells from Thymocytes after the Removal of Different T cell Subsets at the Initiation of Culture*. CBA/J thymocytes, **T**, were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells or **H**-Help as described in section 3.4. At the initiation of culture, T cell subsets were depleted from thymocytes by antibody dependent C'-mediated depletion as described in section 3.7.1. Cells were untreated (1, 2, 7), treated (Rx) with C' alone (3), treated with C' and anti-CD4 (GK1.5), anti-CD8 (TIB 211), or anti-Thy1.2 (TIB 99) hybridoma supernatants (4-6, respectively). Adjustments were not made to cell numbers to compensate for cell loss. The number of IFN-γ and IL-4 producing cells generated by day 6 was enumerated in the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar observations were obtained in two independent experiments.
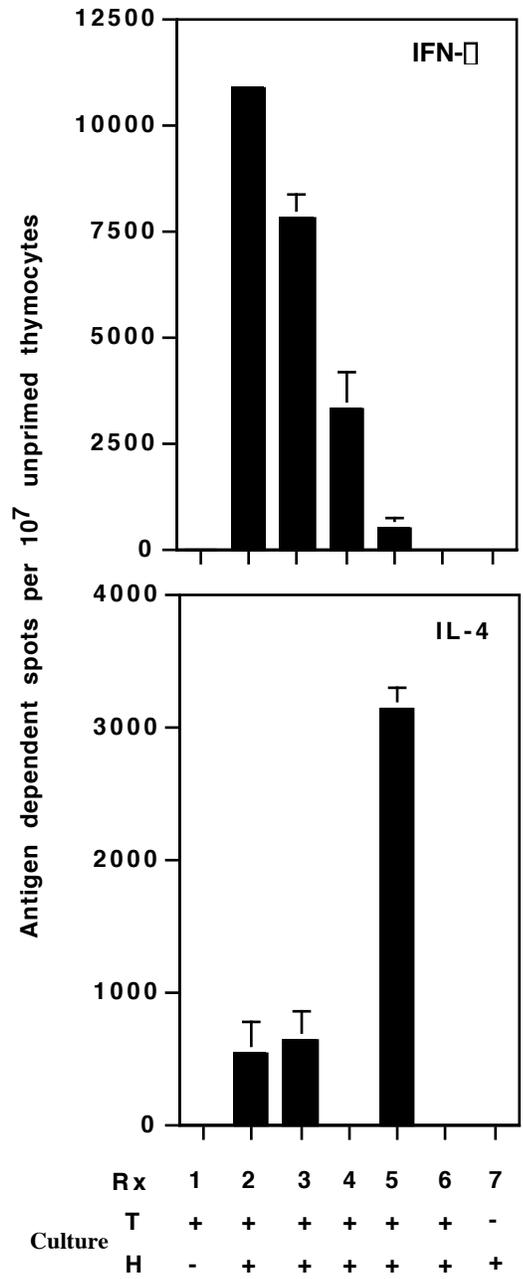
Figure 4.4.2. a. *CD4+ Thymic Precursor T Helper Cells act in Conjunction with the Syngeneic Irradiated Spleen Cells in the Generation of Allo-MHC Specific CTLs*. CBA/J thymocytes were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence (open and filled symbols, respectively) of irradiated CBA/J spleen cells as described in section 3.4. At the initiation of culture, input thymocytes were either ■ - C'-treated, or ● - depleted of CD4+ cells by antibody dependent complement mediated depletion as described in section 3.7.1. Adjustments were not made to cell numbers to compensate for cell loss. Thymocytes were plated at the indicated densities, and on day 6, the generation of CTLs was determined using the standard ^{51}Cr CTL assay against P815 (H-2^d) or EL-4 (H-2^b- inset) target cells as described in section 3.5.1. Similar observations were obtained in two independent experiments. The normalized CTL response of 5 x 10^4 input thymocytes is also shown.

1 x 10^6 Thymocytes/well
2 x 10^6 Thymocytes/well
4 x 10^6 Thymocytes/well

% Specific Lysis

H-2^b

Percent Culture

Normalized % Specific Lysis

CD4 Replete

CD4 Depleted

Thymocyte Culture Density x 10^6 cells/well

107
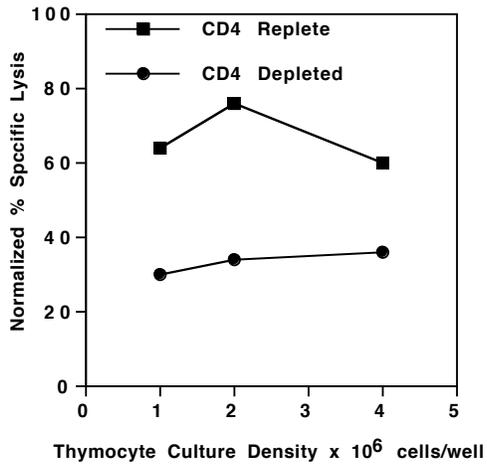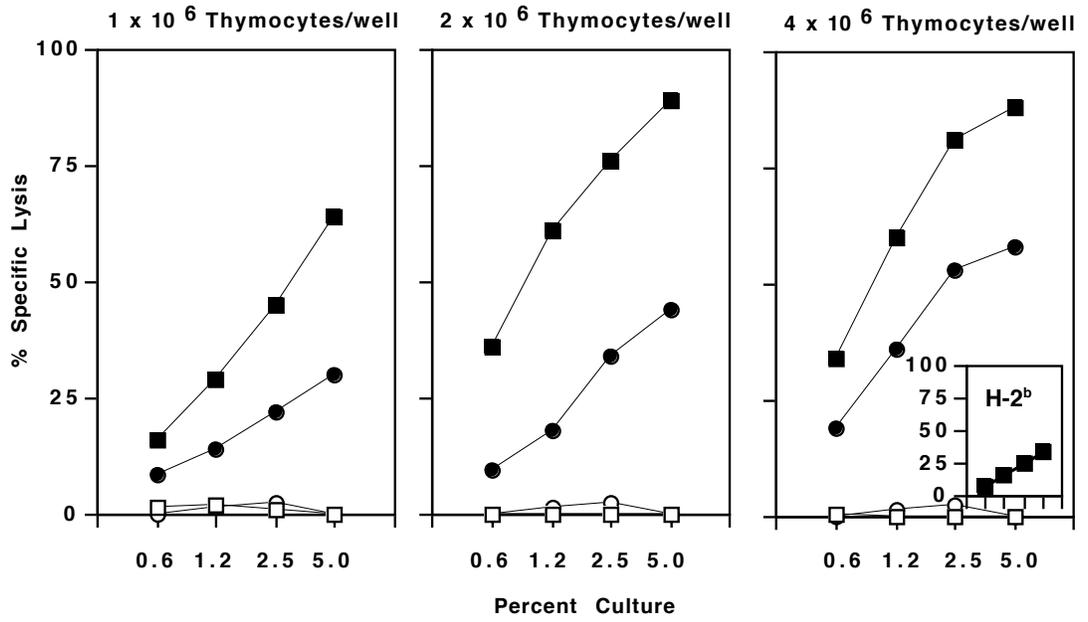
Figure 4.4.2. b. *CD4+ Thymic Precursor T Cells act in Conjunction with the Syngeneic Irradiated Spleen Cells in the Generation of Allo-MHC Specific IFN-γ, and IL-2 Cytokine Producing Cells*. CBA/J thymocytes, **T**, were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells or **H**-Help as described in section 3.4. At the initiation of culture, thymocytes were either C'-treated (filled bars), or depleted of CD4+ cells (open bars) by antibody dependent complement mediated depletion as described in section 3.7.1. Adjustments were not made to cell numbers to compensate for cell loss. Thymocytes were plated at the indicated densities, and on day 6, the number of IFN-γ and IL-2 producing cells generated was enumerated in the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar observations were obtained in two independent experiments.
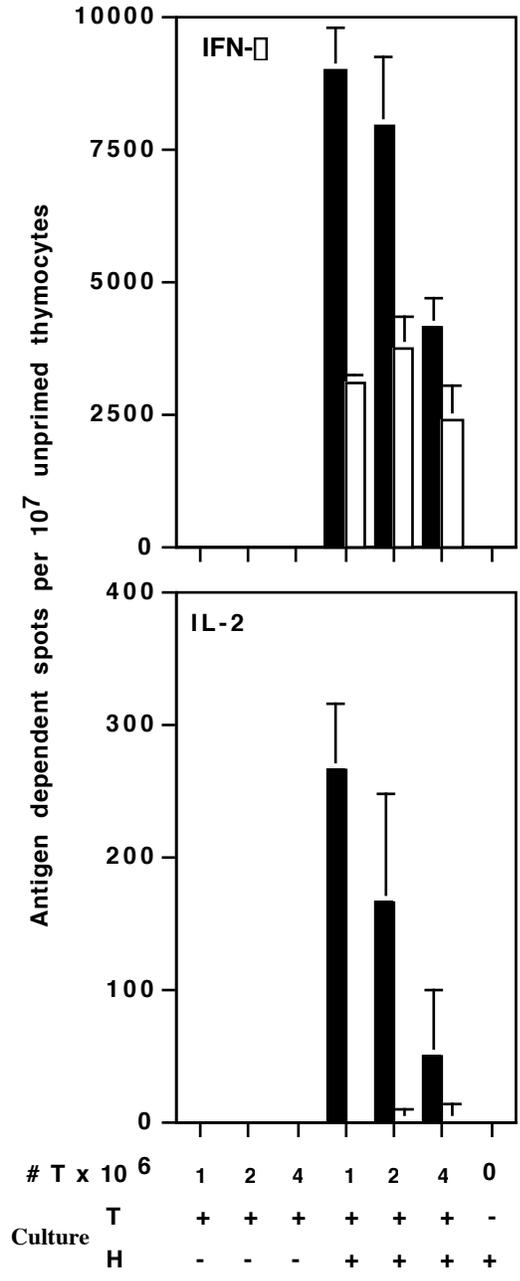
The inference that CD4+ thymocyte precursor T helper cells participate in the generation of primary allo-MHC specific CTL and cytokine responses would be further validated by the demonstration that an allo-MHC specific effector CD4+ T helper cell is generated from naïve thymocytes. It has been previously demonstrated by L. Pilarski with mice with disparate Thy1 alleles, but shared MHC haplotypes, that the CTLs generated upon stimulation of thymocytes with allo-MHC antigens *in vitro,* in the presence of irradiated syngeneic spleen, are derived from the thymocyte precursors (Pilarski, 1977). She was also able to show that effector CD4+ T cells generated *in vitro* from thymocytes possess the ability to "help" in an antigen-specific manner in the generation of CTL responses, albeit at a lower efficiency than irradiated syngeneic spleen cells (Pilarski & McKenzie, 1985).

Experiments were performed to elaborate upon these findings. Thymocyte derived effector populations were harvested and used as a source of irradiated-syngeneic "help" for additional thymocyte cultures. The ELISPOT assay, being a more sensitive than the CTL assay, was employed to determine if the *in vitro* generated effector CD4+ thymocytes could "help" in the generation of CD8+ IFN-γ producing cells from thymocytes upon stimulation with alloantigen. Irradiated-thymocytes harvested from cultures stimulated with alloantigen in the absence of irradiation-resistant syngeneic spleen cells could not "help" in the generation of cytokine producing cells, whereas, irradiated effector thymocytes generated by stimulation with alloantigen in the presence of irradiated syngeneic spleen "helped" thymocytes generate CD8+ IFN-γ producing cells (data not shown).

Thymocyte derived, CD4+ effector T helper cell clones were generated from the stimulated cultures discussed in the previous paragraph. Thymic effector phase cells were repeatedly stimulated with irradiated BALB/c spleen cells, with alternate rounds of stimulation in the presence of recombinant IL-2. All of the thymocyte derived effector T cell clones isolated, including the clones C4 and F6 used in the experiments presented below, express CD4, and primarily produce IL-4 upon stimulation with allo-MHC antigens in the ELISPOT assay, see Table 1.

Experiments were performed to determine whether the thymocyte derived, CD4+ T cell clones generated could operationally provide radiation resistant syngeneic "help", and whether the "help" provided is mediated in an antigen-specific manner. Such a demonstration would indicate that the effector CD4+ T cell clones express antigen-specific TCRs. Separate CBA/J thymocyte cultures were stimulated with either irradiated BALB/c or C57BL/6 spleen cells in the absence or presence of normal irradiated syngeneic CBA/J spleen cells, CBA/J thymocyte derived clone C4, or clone F6. The number of irradiated cloned T cells required to "help" in the generation of responses from thymocytes was previously determined (data not shown). The results in Figure 4.4.3 a and b show the thymocyte derived effector CD4 T cell clones preferentially "help" thymocytes generate CTL and cytokine responses against BALB/c allo-MHC antigens, the allo-MHC antigen employed for their derivation. Similar results were obtained in separate experiments where the ability of CBA/J thymocyte derived clone C4 to "help" thymocytes generate allo-MHC specific responses was assessed in the presence of both irradiated BALB/c and C57BL/6 spleen cells, see Figure 4.4.3. c and d. While these observations suggest

that, the thymocyte derived CD4+ T cell clones operationally provide antigen-specific "help" for thymocytes, the exact nature of this "help" remains to be determined. Nevertheless, these findings allow one to infer that CD4+ thymocyte precursor T helper cells possess the potential to differentiate into functional CD4+ T helper cells.

Table 1. Characterization of the Cytokine Profile and Specificity of CBA/J Thymocyte Derived CD4+ T cell Clones C4 and F6 in the ELISPOT Assay*.

| Clone | None | Antigen Stimulation in ELISPOT | |
		BALB/c	C57BL/6
C4	-	IFN-γ -, IL-2 –, IL-4 ++	IFN-γ -, IL-2 –, IL-4 +
F6	-	IFN-γ -, IL-2 –, IL-4 ++	-

*CBA/J T cell clones were generated as described in section 3.10 and cytokine production was assessed in the ELISPOT as described in section 3.5.2. – and + symbols refer to the absence and presence of detectable cytokine producing cells, respectively. The percentage of cloned T cells plated that produce cytokines ranged from 3-5%. Cytokine production of each clone was assessed in three separate experiments.

BALB/c Stimulators

C57BL/6 Stimulators

% Specific Lysis

Percent Culture
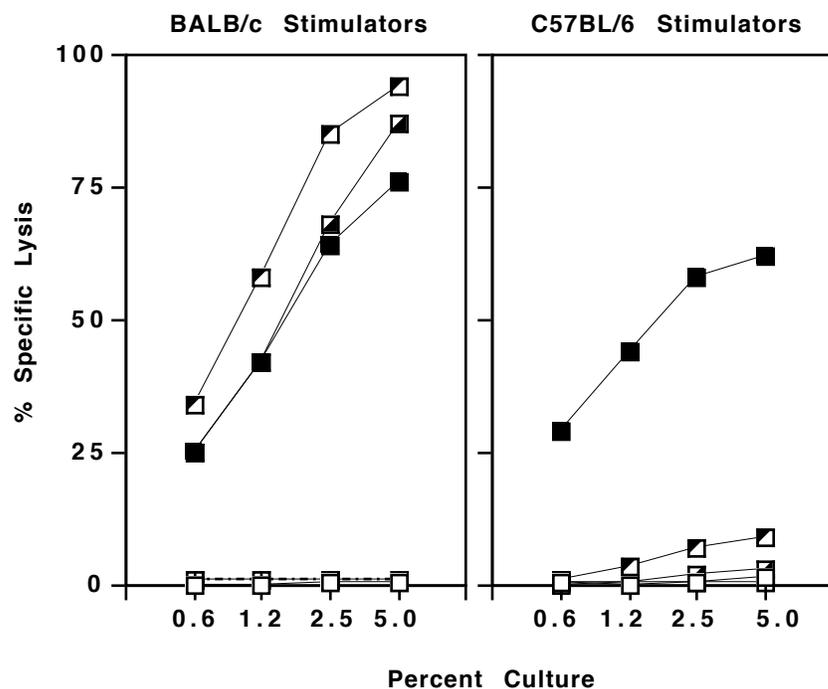
Figure 4.4.3. a. *Thymocyte Derived Effector CD4+ T Helper Cell Clones Help Thymocytes Generate Allo-MHC Specific CTL Responses.* A standard number of CBA/J thymocytes were stimulated with irradiated, T cell depleted, BALB/c (left panel) or C57BL/6 (right panel) spleen cells in the absence (open symbols) or presence of irradiated CBA/J spleen cells (■), 1×10^4 cells/well irradiated CBA/J CD4+ T cell Clone C4 (▨), or Clone F6 (▨) as described in section 3.4. The generation of CTLs was assessed on day 6 using the standard ^{51}Cr CTL assay against P815 (H-2^d- left panel) target cells or EL-4 (H-2^b- right panel) target cells as described in section 3.5.1. Similar observations were obtained in two independent experiments.

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Figure 4.4.3. b. *Thymocyte Derived Effector CD4+ T Helper Cell Clones Help Thymocytes Generate Allo-MHC Specific IFN-γ, IL-2, and IL-4 Cytokine Producing Cells*. CBA/J thymocytes, **T**, were stimulated with irradiated, T cell depleted, BALB/c (filled bars) or C57BL/6 (open bars) spleen cells in the absence or presence of irradiated CBA/J spleen cells, **Spl H**-Help, or 1 x 10^4 cells/well irradiated CBA/J CD4+ T cell Clone **C4** or Clone **F6** as described in section 3.4. The number of IFN-γ, IL-2, and IL-4 producing cells generated by Day 6 was enumerated in the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar observations were obtained in two independent experiments.

ELISPOT Ag

116
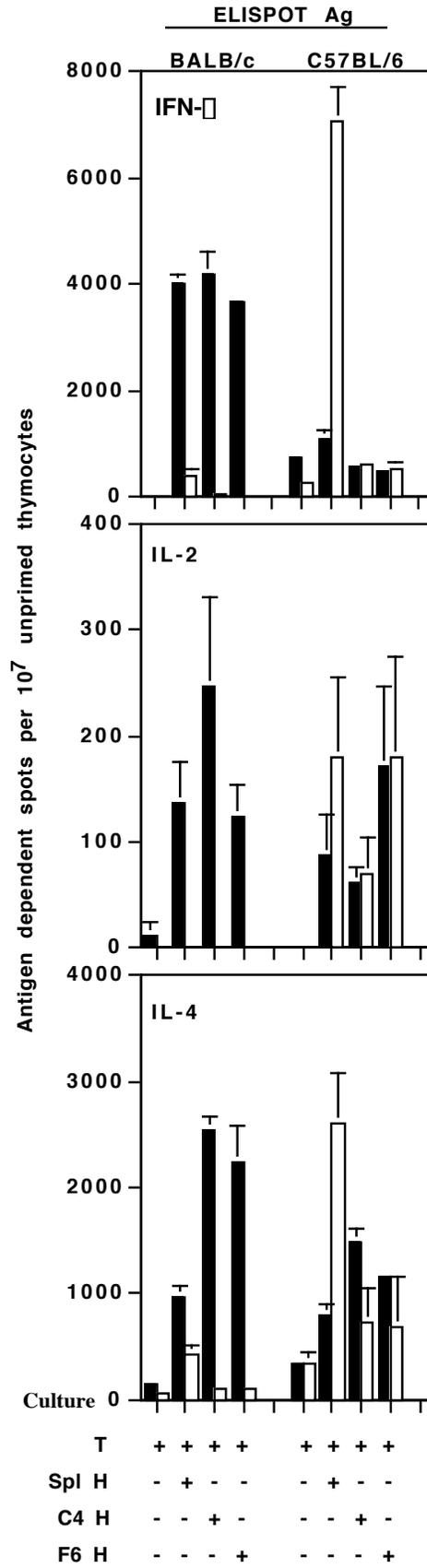
Figure 4.4.3. c. *Thymocyte Derived Effector CD4+ T Helper Cell Clones Help Thymocytes Generate Allo-MHC Specific CTL Responses in an Antigen-Specific Manner*. CBA/J thymocytes were stimulated with irradiated BALB/c (left panel), C57BL/6 (middle panel), or a combination of both (right panel) spleen cells in the absence (open symbols) or presence of 5 x 10^4 cells/well irradiated CBA/J CD4+ T cell Clone C4 (■) as described in section 3.4. The generation of CTLs was assessed on Day 6 using the standard ^{51}Cr CTL assay against P815 (H-2^d) target cells or EL-4 (H-2^b) target cells as described in section 3.5.2. Lysis of third party target cells by cells harvested from cultures stimulated with either BALB/c or C57BL/6 spleen cells is represented by the squares with Xs. The response of the syngeneic irradiated clones stimulated with irradiated, T cell depleted, BALB/c or C57BL/6 spleen cells against syngeneic targets is represented by the hatched square was $\leq$ 5% upon incubation with all corresponding fractions of the culture and is hidden by the other symbols.

BALB/c Stimulators

C57BL/6 Stimulators

**BALB/c +
C57Bl/6 Stimulators**

% Specific Lysis

α P815

α EL-4

α EL-4

α P815

α P815

α EL-4

100

75

50

25

0

0.6 1.2 2.5 5.0

Percent Culture
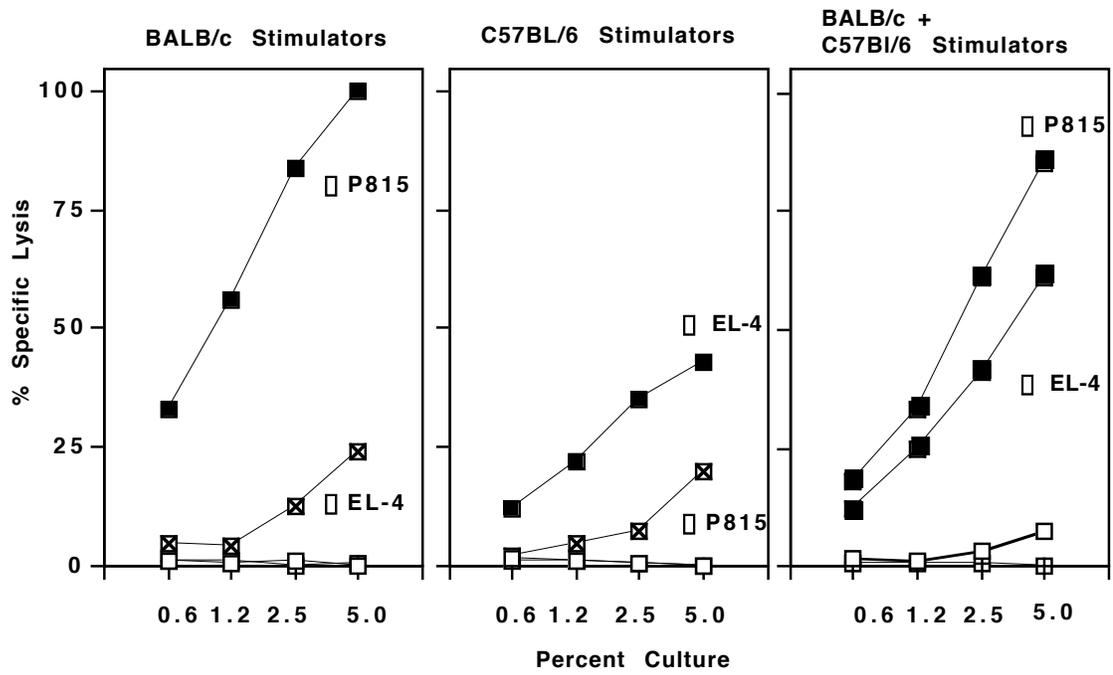
Figure 4.4.3. d. *Thymocyte Derived Effector CD4+ T helper Cell Clones Help Thymocytes Generate Allo-MHC Specific IFN-γ, IL-2, and IL-4 Cytokine Producing Cells in an Antigen-Specific Manner*. CBA/J thymocytes, **T**, were stimulated with irradiated, T cell depleted, BALB/c (filled bars), C57BL/6 (open bars), or a combination of both (hatched bars) spleen cells in the absence or presence of 1 x 10^5 cells/well irradiated CBA/J CD4+ T cell Clone C4 Help or **H** as described in section 3.4. The number of IFN-γ, IL-2, and IL-4 producing cells generated by day 6 was enumerated in the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots.
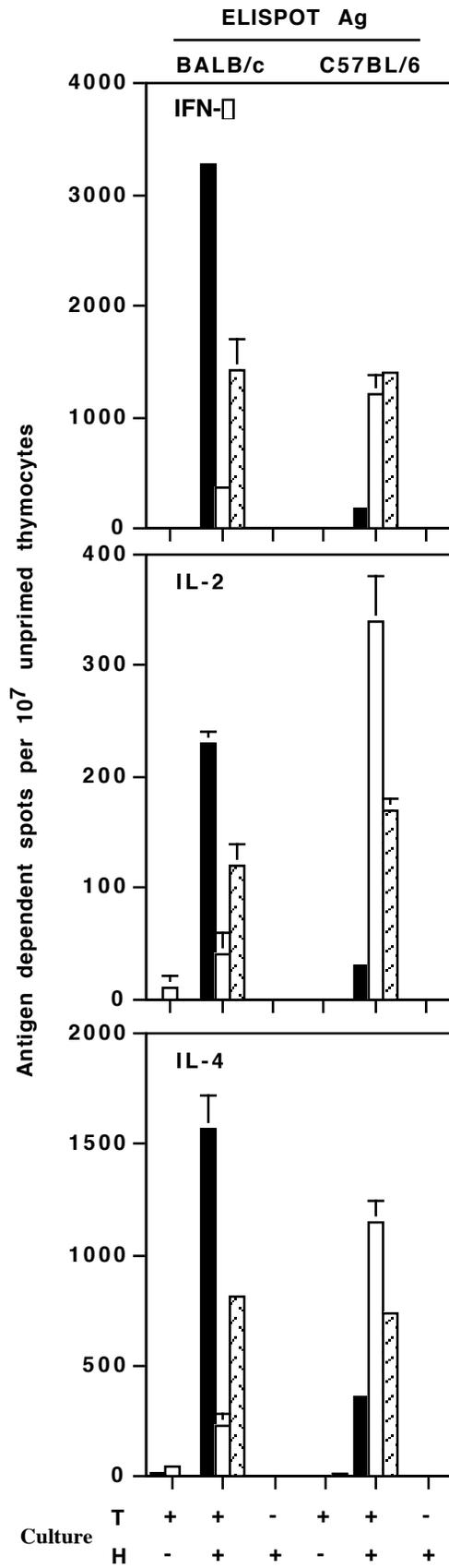
The demonstration that a cell that can act as a radiation resistant helper cell can be generated from thymocyte precursor cells supports the possibility that the radiation resistant CD4+ T spleen cells in normal mice that can "help" thymocytes generate alloresponses are of thymic origin. The demonstration that mice thymectomized within 24 hours of birth lack peripheral radiation resistant CD4+ T cells that can "help" thymocytes generate responses in an antigen specific manner would further support this position.

The observation that effector CD4+ T cell clones that primarily produce the Th2 associated cytokine IL-4 "help" in the generation of cellular responses from naïve thymocytes is in accord with previous observations that IL-4 or T cell clones that posses the potential to help B cells produce antibody can support the differentiation of CD4+ and CD8+ T cells into competent alloreactive cells (Bagley, Sawada, Wu, & Iacomini, 2000; Pfeifer et al., 1987; Ramarli, Parodi, Fabbi, Corte, & Lanzavecchia, 1984), and leaves open the possibility that the effector CD4+ T cells present in the periphery of normal mice could be Th0, Th1, or Th2 in character.

The CD4+ T cell phenotype of the radiation-resistant effector helper cell has been described previously (Pilarski, 1977; Pilarski et al., 1980). Results obtained with bone marrow chimeras discussed previously show that this cell is a CD4+ T cell that displays antigen-specificity and self-tolerance (Pilarski, 1977). The radiation-resistant nature of "helper activity" of the effector CD4+ T cells indicates that these cells are activated T cells that provide helper signals and/or cytokines without prior proliferation; γ-irradiation induces lethal DNA damage that will result in cell death

upon engaging in the process of proliferation (Kettman & Dutton, 1971; Sprent et al., 1974).

Experiments were performed to further characterize the activation status of the radiation resistant effector CD4+ T helper cells. To expand upon the findings of L. Pilarski, the syngeneic irradiated CBA/J spleen cells were depleted of CD4+, CD8+, and Thy1.2+ positive cells by antibody dependent complement mediated lysis, and to further characterize the activation status, the syngeneic spleen cells were also depleted of cells expressing either T cell surface markers typically associated with naïve and resting memory T cells or surface markers associated with activated T cells before irradiation and assessment of their "helper" activity. The removal of cells expressing high levels of CD62L or CD45RB is expected to deplete naïve or resting memory cells, whereas, the removal of cells expressing high levels of CD44 is expected to result in the depletion of activated cells (Dutton et al., 1998). The number of irradiated spleen cells added to thymocytes was determined before depletion and cell numbers were not adjusted to compensate for cell loss. High expression of the latter T cell surface marker, CD44, has to date been the most reliable marker to characterize activated, or blasting, T cells.

The ability of the "helper" spleen cell populations, after depletion by antibody dependent complement mediated depletion, to provide "help" for thymocytes to produce CTLs and cytokine-producing cells was assessed. In agreement with the findings of L. Pilarski examining the generation by thymocytes of CTL responses (Pilarski, 1977), the removal of CD4+ T cells from the syngeneic spleen by antibody dependent complement mediated depletion significantly reduces

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the *in vitro* generation of CD8+ CTLs and IFN-γ producing cells, and CD4+ IL-2 producing cells from thymocytes upon stimulation with allo-MHC antigens, see Table 2 and Figure 4.4.4. a. When cells expressing high levels of the effector memory T cell/activation marker CD44, which is up modulated in the late stages of T cell activation (Swain, 1999), were removed by antibody dependent complement mediated depletion, the generation of CD8+ CTLs and IFN-γ producing cells was also markedly decreased, whereas the generation of CD4+ IL-2 producing cells was only partially affected. It is reasonable to speculate that some of the intermediate expressing CD44+ CD4+ T cells are in a transitional phase of CD44 expression. The presence of residual cells may be sufficient to result in the generation of IL-2 producing cells or the generation of some IL-2 producing cells may require these cells. It is less easy to explain in the latter case why the generation of IL-2 producing CD4+ T cells from thymocytes is dependent upon radiation resistant CD4+ Thy1.2+ cells in the spleen cell population.

By contrast, the removal of cells expressing high levels of markers characteristic of naïve and resting memory T cells, namely CD62L or CD45RB (Dutton et al., 1998), failed to decrease the ability of the irradiated syngeneic CBA/J spleen cells to provide thymocytes with "help" upon stimulation with alloantigen. Rather, the generation of CD8+ CTL and IFN-γ responses was enhanced by the removal of these cells. The enhancement may be the result of the removal of CD62L^{high} or CD45RB^{high} expressing NK cells, which could lead to the more prolonged presence of irradiated stimulators in culture (Moretta, 2002; Nikolic, Cooke, Zhao, & Sykes, 2001). Alternatively, the enhanced responses may be the

result of the removal of a subset of suppressor cells, which express surface markers either associated with naïve/resting memory cells or activated cells (Huehn et al., 2004; Szanya, Ermann, Taylor, Holness, & Fathman, 2002).

The heterogeneity of the expression profiles of the relevant surface markers on un-stimulated, normal, and for comparison purposes on un-stimulated DO11.10 TCR transgenic Thy1.2+ cells that should contain a higher proportion of naïve T cells, are presented in Figures 4.4.4. b and c. High, intermediate and low expression of CD44 is not as obvious on total Thy1.2 cells as it is for CD4+ T cells, see Figure 4.4.4.b. The expression profiles of the relevant markers on thymocytes presented in 4.4.4 c conform to the profiles already established in the literature (Lee et al., 2001). It has been suggested that the heterogeneous expression of such markers on T cells that emerge from the thymus, and potentially also activated T cells that emerge from tissues, is more related to a commitment to different migration patterns and/or reversible adaptation to anatomical microenvironments, respectively, than definitive markers of previous activation status (Kassiotis & Stockinger, 2004; Lee et al., 2001).

Table 2 Characterization of the CD4+ T cells present in irradiated CBA/J spleen that

helps thymocytes

Culture	Syngeneic Spleen Cell Treatment	Lytic Units*
T	None	0 ± 0
T + H	None	143 ± 15
T + H	Complement	129 ± 24
T + H	Anti-CD4 and C"	2.4 ± 1.2
T + H	Anti-CD8 and C"	120 ± 30
T + H	Anti-Thy1.2 and C"	0 ± 0
T + H	Anti-CD44 and C"	10 ± 1.0
T + H	Anti-CD45RB and C"	175 ± 8.0
T + H	Anti-CD62L and C"	180 ± 7.6
H	None	0 ± 0

* The errors represent the standard deviation of the LU values. Cultures were set up as described in section 3.4. Cell populations were depleted by antibody dependent complement mediated depletion as described in section 3.7.1. The generation of CTLs was assessed in the CTL assay as described in section 3.5.1. Similar findings were obtained in three separate experiments for all markers and two additional experiments for the removal of CD44 cells. In one replicate experiment, the trend of results was identical, but LU values were low due to a Lot of rabbit complement that was abnormally toxic for thymocytes. Values ranged from 0 ± 0 for T, to 47± 3.4 LU for anti-CD45RB and C" Rx T + H. In experiments where the removal of CD44 expressing cells was assessed as a function of hybridoma supernatant dilution, the percentage of **L**ow, **I**ntermediate, and **H**igh expressing CD44+ CD4+ cells as defined (Wang & Mosmann, 2001) was determined by flow cytometry as described in section 3.9.1. to be: C" treated (Rx), L:62.4%, I:27.7%, H:8.9; C" and undiluted anti-CD44 ab Rx, L:65.3%, I:25.5%, H:0.6%; C" and 1/2 diluted anti-CD44 ab Rx, L: 65.6%, I:24.9%, H:0.5%; and lastly C" and 1/4 diluted anti-CD44 ab Rx, L:65.5%, I:25.7%, H:0.9%.

Figure 4.4.4. a. *The Syngeneic Radiation Resistant Spleen Cells that Help Thymocytes Generate IFN-γ and IL-2 Cytokine Producing Cells upon Stimulation with Allo-MHC Stimulators are CD44^{hi}, CD45RB^{low} and CD62L^{low} CD4+ Thy1.2+ Cells.* Thymocytes, **T**, were stimulated with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells or **H**-help as described in section 3.4. Standard culture conditions were employed. Before irradiation and culture, T cell subsets were depleted from CBA/J spleen cells by C'-mediated depletion as described in section 3.7.1. Cells were untreated (1, 2, 10), treated (Rx) with C' alone (3), with C' and anti-CD4 (GK1.5, 4), anti-CD8 (TIB 211, 5), anti-Thy1.2 (TIB 99, 6), anti-CD44 (IM7.8.1,7), anti-CD62L (MEL14.D54, 8), or anti-CD45RB (MB23G2, 9) hybridoma supernatants. Analysis of the degree of depletion is described in section 3.9.1 and in the legend of Table 2 for the removal of CD44 expressing cells. Adjustments were not made to cell numbers to compensate for cell loss. The ability of the treated syngeneic spleen cells to help in the generation of IFN-γ and IL-2 producing cells was assessed on Day 6 with the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar results were obtained in three separate experiments for all markers, and two additional experiments where the removal of CD44 expressing cells was assessed independently.
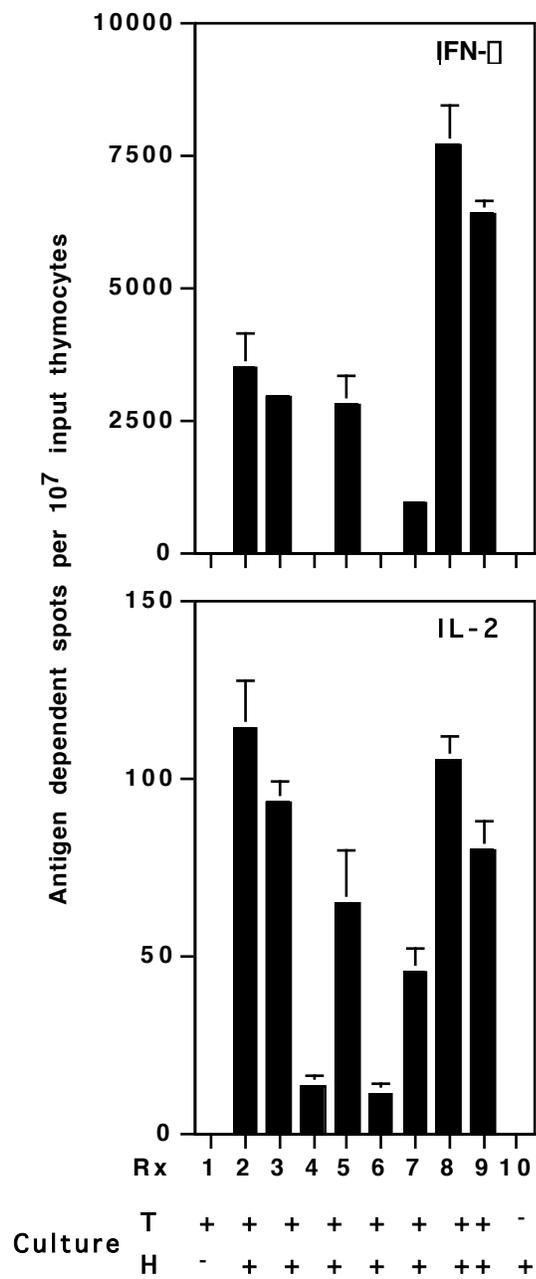
Figure 4.4.4. b. *The Expression Profile of T Cell Activation Markers on Normal and TCR Transgenic Splenocytes*. The expression profile of CD44, CD62L, and CD45RB on normal (upper row) and D011.10 TCR Transgenic (lower row) gated on Thy1.2+ splenocytes was assessed directly *ex vivo* by flow cytometry as described in section 3.9.1. The heterogeneous expression of CD44 is more evident on CD4+ gated cells. The approximate positions of **L**ow, **I**ntermediate, and **H**igh gates are presented.

CD44　　　**CD62L**　　　**CD45RB**

NORMAL

TCR TG

Events

FLORESCENCE INTENSITY ON THY1.2 + ⟶

Events

L　**I**　**H**

CD44 Expression on CD4+ T cells

129
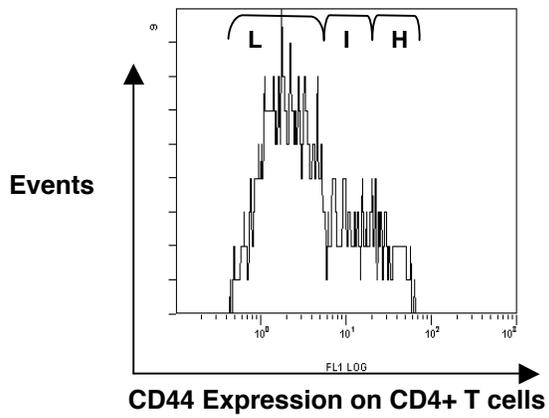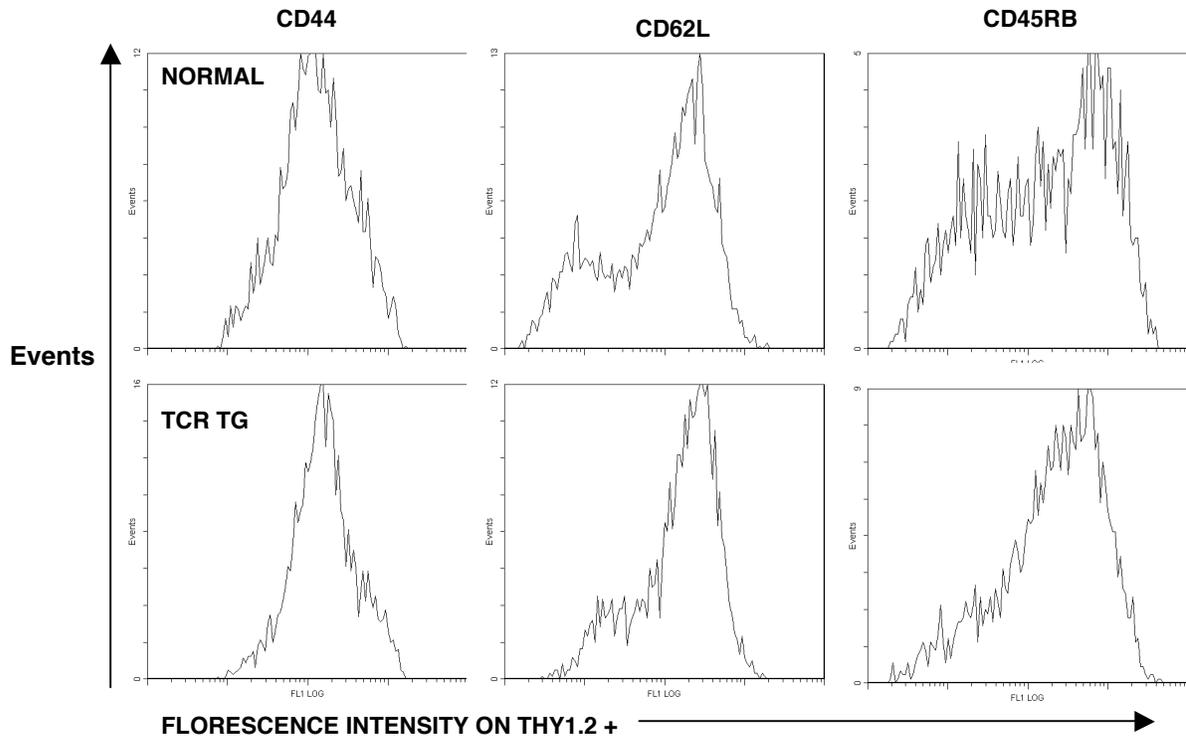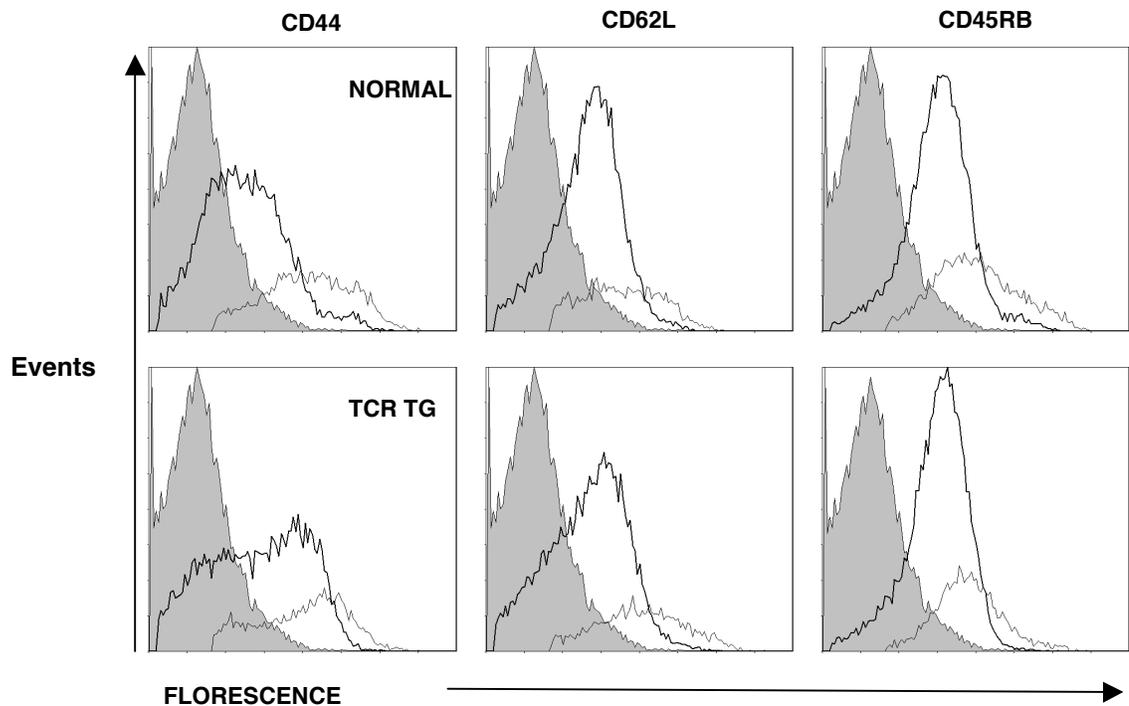
Figure 4.4.4. c. *The Expression of T Cell Activation Markers on Immature and Mature Normal and TCR Transgenic Thymocytes.* The expression profile of CD44, CD62L, and CD45RB on gated populations of immature Thy1.2^{high} (——) and mature Thy1.2^{low} (——) normal (upper row) and D011.10 TCR Transgenic (lower row) thymocytes was assessed by flow cytometry as described in section 3.9.1. Background staining is represented as a shaded histogram.

4.5. Nature of the Antigen Presenting Cell Involved in Primary T Cell Activation: Stimulatory Capacity of Dendritic Cells

Experimental evidence has been presented that shows conditions can be established under which the generation of allo-MHC specific CTLs and cytokine responses from thymocytes upon stimulation with allogeneic spleen cells depleted of T cells involves radiation resistant CD4+ T spleen cells syngeneic with the thymocytes. Precursor CD4+ thymocyte T helper cells can also participate in the generation of these responses. CD4+ T helper cells are restricted to MHC Class II antigens (Goldrath & Bevan, 1999). Their TCRs must therefore recognize antigenic peptides in the context of MHC Class II complexes in order to license/activate antigen-presenting cells that then can activate MHC Class I restricted, CD8+ T cells (Bennett et al., 1998; Bennett et al., 1997; Ridge et al., 1998). Competing models of primary T cell activation predict that different kinds of antigen-presenting cells are centrally involved in initiating the activation of naïve T cells (Bretscher, 1999; Janeway, 1989; Matzinger, 1994).

The proponents of the Infectious Non-Self and Danger models of naïve T cell activation propose that non-specific antigen-presenting cells, such as dendritic cells, under the correct circumstances, stimulate naïve CD4+ T cells to become activated cells that can help B cells produce antibody and/or help in CD8+ T cell activation (Janeway, 1989; Matzinger, 1994). The Two-step, Two-signal Model of primary CD4+ T cell activation states that non-specific antigen presenting cells, such as dendritic cells, are important for stimulating the proliferation of naïve CD4+ and

CD8+ T cells, but that such cells are unable to deliver the critical costimulatory signals and/or cytokines required for full naïve T cell activation (Bretscher, 1999). The latter model proposes that a critical signal needed for primary T cell activation, termed the second signal of step two, must be delivered in an antigen specific manner. The antigen-specific delivery of the second step second signal is postulated to occur upon recognition of cognate antigen presented on activated B cells, whose activation is dependent upon effector CD4+ T helper cells.

Experiments were performed to delineate which antigen-presenting cell(s) in the allogeneic BALB/c spleen cell population depleted of T cells are necessary for stimulating the generation of allo-MHC specific CTL and cytokine producing cells from thymocytes when irradiation resistant spleen cell "help" is provided. The allogeneic spleen cell populations depleted of T cells used to stimulate thymocytes were further depleted of different cell populations by antibody dependent complement mediated lysis and assessed for their ability to stimulate alloresponses. The stimulatory capacity of irradiated T cell depleted BALB/c spleen cells, T cell depleted BALB/c spleen also depleted of B220+ cells, and graded numbers of dendritic cells derived from the bone marrow of BALB/c mice were assessed. It is evident from the results presented in Table 3 that the removal of B220+ cells from the allogeneic stimulator spleen by antibody dependent complement mediated depletion decreased the generation of allo-MHC specific CTLs by three-fold. In comparison to the response generated upon stimulation with T cell depleted BALB/c spleen cells, a marked decrease in the generation of CD8+ CTL and IFN-γ producing cells, as well as CD4+ IL-2 producing cells, was evident when irradiated bone-

marrow derived dendritic cells were used to stimulate thymocytes, see Table 2 and Figure 4.5.1. When high numbers of bone marrow-derived dendritic cells were used to stimulate thymocytes, marginal CTL responses, in comparison to the control cultures, were evident. Possible reasons for the generation of the latter response are discussed below.

Low numbers of B cells that escape anti-B220 antibody dependent complement mediated depletion are inevitably present in the former treated allogeneic spleen population that was also depleted of T cells. The presence of residual B cells may explain the inability to dramatically affect the generation of responses from thymocytes if B cells are required to generate responses, or alternatively the generation of such responses may not require B cells. It is pertinent in assessing the plausibility of the two possibilities that some activated B cells have been reported to down regulate B220 expression (McHeyzer-Williams, Cool, & McHeyzer-Williams, 2000). The presence of B220- B cells in the treated allogeneic stimulator cells might also explain the inability to markedly affect the generation of responses from thymocytes by the depletion of B220+ cells from the BALB/c spleen cells. Low numbers of B cells may also be present in the bone marrow derived dendritic cell populations because B cells and dendritic cells can both develop from an early B-lineage precursor population found in adult mouse bone marrow (Coligan et al., 1998; Inaba et al., 1992; Izon et al., 2001). To minimize the presence of B cells in the bone marrow derived dendritic cells employed in all subsequent experiments, bone marrow cultures were extensively washed to remove contaminating cells. The efficiency of the removal of contaminating cells from bone

marrow derived dendritic cells was confirmed by flow cytometry and is discussed in the next section. The issue of whether B cells are required for the generation of immune responses from thymocytes, and whether the dendritic cells generated from BALB/c bone marrow are in fact conventional dendritic cells is not resolved and will be considered further in the next sections and as well as the discussion.

Table 3: The Efficiency of Different Allogeneic APC at Stimulating the Generation of CTLs from Thymocytes.

Culture	Stimulator	Number of Stimulator Cells per Culture	Lytic Units[1]
T	α Thy1.2 Rx Spleen	2×10^6	0 ± 0
T + H	α Thy1.2 Rx Spleen	2×10^6	320 ± 11
T + H	α Thy1.2 , α B220 and Ad- Rx spleen	2×10^6	110 ± 2
T + H	BMDC	1×10^2	0 ± 0
T + H	BMDC	1×10^3	0 ± 0
T + H	BMDC	1×10^4	1 ± 0
T + H	BMDC	1×10^5	35 ± 3
H	α Thy1.2 Rx Spleen	2×10^6	0 ± 0

[1] The errors represent the standard deviation of the LU values. Similar observations were obtained in three separate experiments. Cultures were set up employing standard culture conditions of 2×10^6 cells/well thymocytes and 1.0×10^7 cells/well irradiated spleen cells as described in section 3.4 and CTL assays were performed as described in section 3.5.1. Adherent cells (Ad) were removed by incubation of spleen cells in plastic Petri plates at 37 C for 2 hrs as described (Coligan et al., 1998). Bone marrow derived dendritic cells were prepared as described in section 3.8.1. and were sub cultured in the absence of exogenous activation stimuli.

Figure 4.5.1. *Efficiency of Different Allo-MHC Bearing APC in Stimulating the Generation of IFN-γ and IL-2 Producing Cells from Thymocytes in the presence of Irradiated Syngeneic Spleen.* CBA/J thymocytes, **T**, were stimulated with different irradiated T cell depleted BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells or **H**-Help employing standard conditions as described in section 3.4. The irradiated BALB/c spleen cells employed were Thy1.2 depleted spleen (Spl), spleen that was further depleted of adherent and B220+ cells, (Spl, *), or graded numbers of bone marrow derived dendritic cells (DC) that were generated from BALB/c bone marrow as described in section 3.8.1. Cell populations were depleted of cell populations by antibody dependent complement mediated depletion as described in section 3.7.1. Adjustments were not made to cell numbers after antibody dependent complement mediated depletion to compensate for cell loss. The number of IFN-γ and IL-2 producing cells generated by Day 6 was enumerated in the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen-dependent spots. Similar results were obtained in three independent experiments.

10^x DC	-	-	-	2	3	4	5	-
T	+	+	+	+	+	+	+	-
H	-	+	+	+	+	+	+	+
Spl	+	+	+*	-	-	-	-	+
DC	-	-	-	+	+	+	+	-

Culture/Stimulator
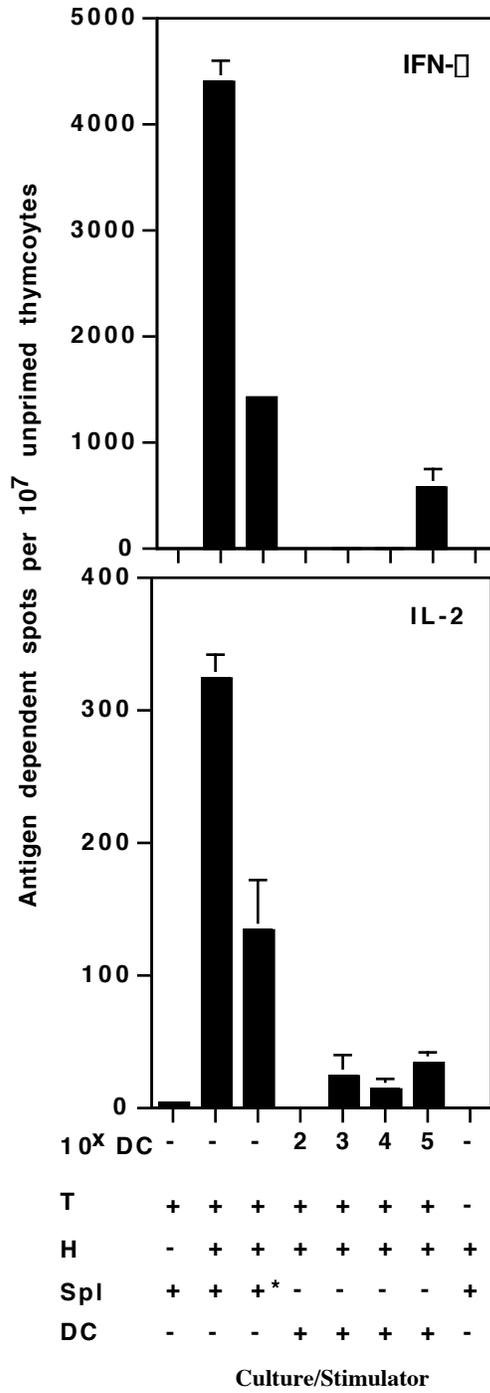
One could argue from the observations just described that the dendritic cells derived from murine proliferating bone marrow progenitor cells are inherently inefficient at stimulating the generation of CTL and cytokine producing cells from naïve thymocytes because they are functionally immature or alternatively are unconventional dendritic cells (Bonifaz et al., 2002; Girolomoni et al., 1990; Hawiger et al., 2001; Inaba et al., 1986; Jonuleit et al., 2000; Steinman et al., 2003). Experiments in which CBA thymocytes or splenocytes were stimulated with bone marrow derived dendritic cells matured/activated by exposure to different maturation stimuli for 24 hrs were performed to assess the stimulatory capacity of the *in vitro* generated dendritic cells. The bone marrow derived dendritic cells were sub-cultured in the presence of complete media, in the presence of media containing anti-CD40 mab, or complete media containing LPS. The latter two stimuli have been extensively documented to trigger the activation of dendritic cells (Banchereau & Steinman, 1998). The results presented in the right panels of Figures 4.5.2. a and b show that CBA splenocytes readily generate CTL responses upon stimulation with irradiated allogeneic bone marrow derived dendritic cells, with those matured in the absence of exogenous maturation stimuli and subsequently matured by the allogeneic T cells in culture and those matured in the presence of anti-CD40 ligation stimulating the generation of comparable responses. The bone marrow derived dendritic cells matured in the presence of LPS stimulated the generation of more vigorous CTL and IFN-γ responses. The ability of LPS-matured dendritic cells to stimulate more vigorous responses from thymocytes may be directly related to their increased

production of IL-12 upon such stimulation. In contrast to the responses generated from CBA splenocytes, CBA thymocytes failed to generate responses when stimulated with any of the matured dendritic cells, even though an adequate number of irradiated syngeneic spleen cells were present, see the left panels of Figure 4.5.2. a and Figure 4.5.2 b. The same thymocytes, however, readily generated CTL and cytokine producing cells upon stimulation with irradiated, T cell depleted, BALB/c splenocytes when irradiated syngeneic spleen is provided. The latter finding indicates that the inability to detect the generation of responses from thymocytes upon stimulation with allogeneic dendritic cells is unlikely to be due to an inherent inability of thymocytes to generate immune responses.

Figure 4.5.2. a. *Activated Bone Marrow Derived Dendritic Cells Stimulate the Generation of CTL Responses by Responding Splenocytes, but Fail to Stimulate Responses from Thymocytes in the Presence of Syngeneic Irradiated Spleen*. CBA/J thymocytes were stimulated with irradiated T cell depleted BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells (❏ or ■, respectively) employing standard conditions as described in section 3.4. Separate CBA/J thymocyte cultures that contained irradiated syngeneic spleen cells (left panel) or CBA/J splenocyte cultures (right panel) were stimulated with 1 x 10^4 cells/well irradiated DCs derived from the bone marrow of BALB/c mice as described in sections 3.4 and 3.8.1. Before the addition to cultures, bone marrow derived DCs were sub-cultured for 24 hours in the presence of media (●), anti-CD40 monoclonal antibody (▲), or 1ug/ml LPS (▼). The generation of CTL responses was assessed on Day 6 against labeled P815 (H-2^d) target cells or against labeled third party targets, EL-4 (H-2^b- inset) in the standard ^{51}Cr CTL assay as described in section 3.5.1. The percent specific lysis of thymocytes stimulated with all matured dendritic cells in the presence of irradiated syngeneic spleen cells, and irradiated spleen cells stimulated with T cell depleted BALB/c spleen cells was < 2%. Similar observations were obtained in three independent experiments.
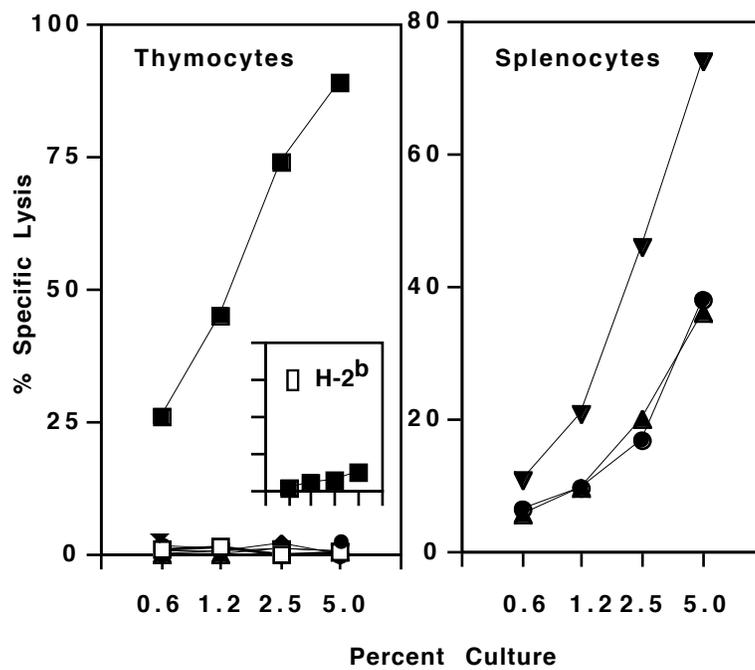
Figure 4.5.2. b. *Activated Bone Marrow Derived Dendritic Cells Stimulate the Generation of IFN-γ, and IL-2 Cytokine Producing Cells from Responding Splenocytes, but Fail to Stimulate Responses from Thymocytes in the Presence of Syngeneic Irradiated Spleen*. CBA/J thymocytes, **T**, were stimulated with irradiated, T cell depleted, BALB/c spleen (Spl) cells in the absence or presence of irradiated CBA/J spleen cells or **H**-Help. Standard culture conditions as described in section 3.4 were employed. Separate CBA/J thymocyte cultures that contained irradiated syngeneic spleen or CBA/J splenocyte cultures (insets) were stimulated with 1×10^4 cells/well irradiated DCs derived from the bone marrow of BALB/c mice that were generated as described in section 3.8.1. Standard culture conditions were also employed for CBA/J splenocyte cultures. Prior to the addition to cultures, bone marrow derived DCs were sub-cultured for 24 hours in the presence of the following maturation stimuli (MS): media (1), anti-CD40 monoclonal antibody (2), or 1μg/ml LPS (3). The number of IFN-γ and IL-2 producing cells generated by Day 6 was enumerated in the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar observations were obtained in three independent experiments.

Thymocytes

IFN-γ

Splenocytes

IL-2

Splenocytes
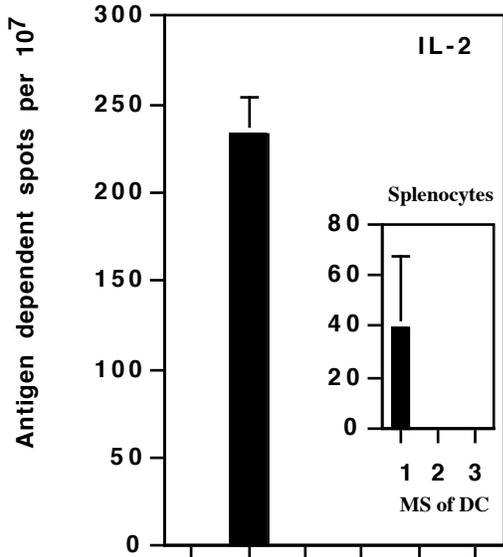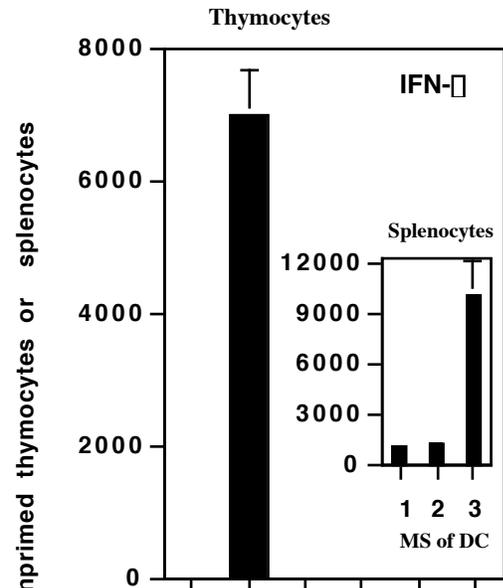
T	+	+	+	+	+	-
H	-	+	+	+	+	+
Spl	+	+	-	-	-	+
DC	-	-	+	+	+	-
MS	-	-	1	2	3	-

Culture/Stimulator

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The inability to detect the generation of CTL and cytokine responses from thymocytes upon stimulation with bone marrow derived cells could, in principle, be due to the generation of a cell other than a dendritic cell from the proliferating bone marrow progenitor cells that inhibits the generation of responses, though no precedence has been documented. To assess whether the cells generated from the proliferating bone marrow progenitors are in fact dendritic cells, the expression of known costimulatory and dendritic cell antigens, in addition to the antigen presenting capacity of the bone marrow derived cells (Inaba et al., 1992) was examined. The increased expression of the B7 and CD40 costimulatory molecules, and the stable expression of the dendritic cell marker DEC-205 (Bonifaz et al., 2002; Bonifaz et al., 2004; Coligan et al., 1998; Hawiger et al., 2001; Inaba et al., 1992), upon Toll-like receptor ligation support the suggestion that the bone marrow derived cells are in fact dendritic cells, see Figure 4.5.3.

The latter observations are further supported by the finding that only bone marrow derived cells sub-cultured in the absence of exogenous activation stimuli retain the capacity to efficiently endocytose and subsequently present antigen to BALB/c ovalbumin-specific CD4+ T cell clones, see Figure 4.5.4. This finding agrees with previous observations that matured and activated dendritic cells down modulate both macropinocytosis and their antigen processing machinery upon activation, which was discussed previously in the Introduction, see section 1.3.6. (Delamarre et al., 2003; Kampgen et al., 1991; Koch et al., 1995; Pure et al., 1990; Romani et al., 1989; Sallusto et al., 1995; Steinman, 1991; Wilson et al., 2003).

While it has been reported that LPS activated dendritic cells retain the capacity to endocytose antigens by receptor-mediated endocytosis, such as 0.5-μm latex beads or anti-adsorptive endocytosis receptor antibody complexes, in matured and activated dendritic cells these internalized complexes reportedly fail to reach lysosomal compartments (Engering et al., 2002; Kamath et al., 2000). Antigens internalized in such a manner have been reported to reside in small organelles called early endosomes that are located in proximity to the cell surface and have a neutral pH (Engering et al., 2002).
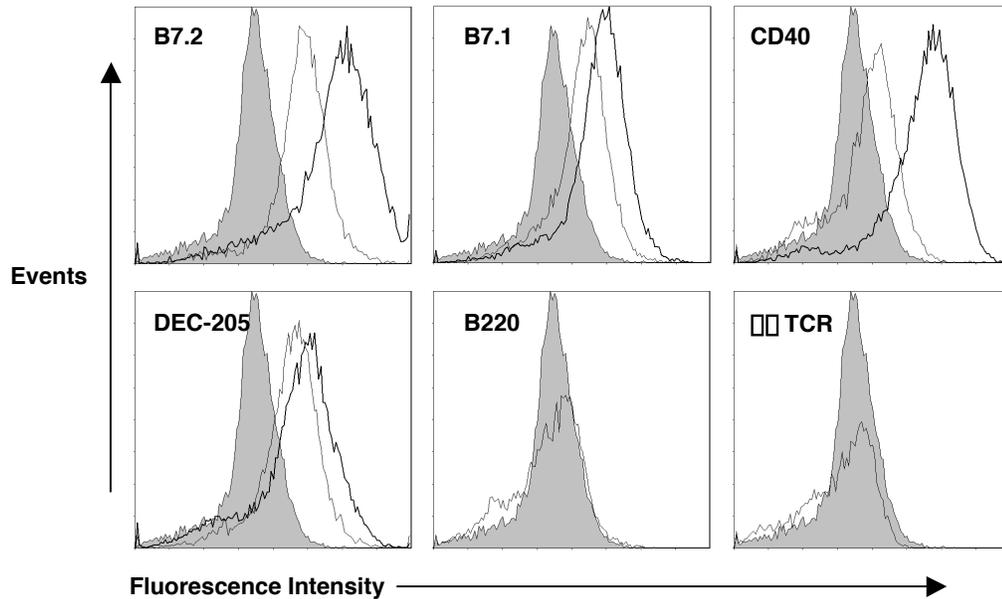
Figure 4.5.3. *Bone Marrow Derived Cells Up Regulate Costimulatory Molecules upon Stimulation with LPS and Express the DEC-205 Dendritic Cell Surface Marker.* The expression of B7.2, B7.1, CD40, DEC-205, B220, or αβ TCR on bone marrow derived cells that were generated as described in section 3.8.1 was assessed by flow cytometry that was performed as described in section 3.9.1. Expression profiles after 24 hr maturation with media (—) or with LPS (━) stimulation is shown. Background staining is represented as shaded histograms.

Figure 4.5.4. *Bone Marrow Derived Cells Present Antigen to BALB/c Ovalbumin (OVA) Specific CD4+ T cell Clones in a Manner Indicative of Dendritic Cells*. Bone marrow derived DC were prepared as described in section 3.8.1 and were sub-cultured for 24 hrs in the presence of media or media containing 1 μg/mL of LPS.   Sub-cultured DCs were subsequently used as APC in a standard proliferation assay and their ability to acquire and present antigen assessed. 1×10^4 BALB/c Th1, IFN-γ secreting OVA-Specific CD4+ T cell clones were stimulated with 1×10^3 DC in the presence of no antigen, 1.25 mg/mL OVA, or Hen Egg Lysozyme (HEL) in 96-well trays.   The ability of the DC to stimulate the proliferation of the BALB/c OVA-specific T cell clones was determined via the amount of ^{3}H uptake during the last 18hrs of a 3-day culture as described in section 3.8.2.
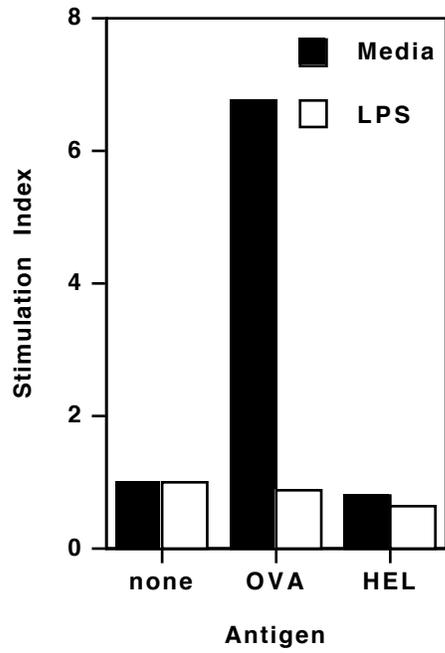
In light of these observations, we can suggest that the cells derived from BALB/c bone marrow progenitors are in fact conventional dendritic cells. The inability of thymocytes to generate significant CTL and T cells producing cytokines upon stimulation with bone marrow derived dendritic cells, because the dendritic cells are inefficient at up modulating costimulatory molecules, is unlikely because CBA/J spleen cells generate alloresponses upon stimulation with these cells, with LPS activated dendritic cells stimulating the most vigorous responses. The difference in the generation of CTL and cytokine responses from unseparated splenocytes upon stimulation with the differentially activated dendritic cells, specifically that between CD40 and LPS activated dendritic cells, is most likely the result of a combination of the higher levels of MHC class II expression and increased secretion of IL-12 from dendritic cells upon stimulation with LPS, (Edwards et al., 2002; Kelleher & Beverley, 2001; Maxwell, Ruby, Kerkvliet, & Vella, 2002; Reis e Sousa et al., 1997).

The requirement for additional antigen-presenting cell-naive T cell interactions to achieve full T cell activation is at odds with reports in the literature (Banchereau & Steinman, 1998). Dendritic cells are renowned for their ability to stimulate the proliferation and activation of T cells, especially those that have been activated by exposure to maturation stimuli (Banchereau & Steinman, 1998; Bonifaz et al., 2002; Bonifaz et al., 2004; Cella et al., 1996; Grohmann, Fallarino, Silla et al., 2001; Hawiger et al., 2001; Schuurhuis et al., 2000; Steinman & Witmer, 1978; Tough, Sun, & Sprent, 1997). Another group has demonstrated that separated normal CD8+ T cells isolated from the spleen fail to generate responses against

ovalbumin-pulsed, LPS activated bone marrow derived dendritic cells when CD4+ T cell signals such as CD40L-CD40 ligation are absent (Kelleher & Beverley, 2001). Ligation of CD40 restores the responsiveness of the normal CD8+ T cells. In our experimental system, naïve thymic CD4+ and CD8+ T cells fail to give rise to T cell responses upon stimulation with dendritic cells even though irradiated syngeneic splenic cells or CD40 ligation, both of which have been widely reported to activate dendritic cells, are present. In light of these observations, it may be reasonable to speculate that the potent capacity of dendritic cells to act as stimulators for T cells (Croft, Bradley, & Swain, 1994), is the result of the presence of previously activated T cells in the responding lymphocyte populations. The experimental data presented supports the possibility that additional cellular interactions, in addition to those with bone marrow derived dendritic cells, are necessary to stimulate the generation of CTL and cytokine responses from naïve CD4+ and CD8+ thymocytes.

In order to determine how CD4+ and CD8+ T cells respond to stimulation with allogeneic dendritic cells, experiments were performed to assess whether stimulation influences the expression of costimulatory ligands on T cells (Banchereau et al., 1994), and whether stimulation triggers the initiation of T cell proliferation. The expression of CD40L and stimulation of proliferation at the single cell level were assessed by flow cytometry.

CD40L expression is known to peak at very early time points after T cell stimulation, and is maintained at low levels on Th1 CD4+ T cells thereafter (Jaiswal et al., 1996; Lee et al., 2002). The results presented in Figure 4.5.5 show that on Day 5 of culture, when responses have been established, thymocytes stimulated with

dendritic cells in the absence or presence of irradiated syngeneic CBA/J spleen cells express CD40L. Expression is at a high level for thymocytes stimulated in the absence of help, and is down-modulated to a low level for thymocytes stimulated in the presence of help. Thymocytes stimulated with allogeneic spleen cells express only low levels of CD40L, as is expected for Th1 responses (Lee et al., 2002). We are currently exploring the kinetics of peak CD40L expression in the differentially stimulated thymocyte populations.

The proliferation of CBA/J thymocytes was also assessed upon stimulation with either irradiated allogeneic, bone marrow derived dendritic cells or T cell depleted spleen cells. The results presented in Figure 4.5.6 show that thymocytes stimulated with BALB/c dendritic cells, matured by the presence of irradiated syngeneic CBA/J spleen cells, divide. Division of thymocytes is evident from the loss of CFSE intensity over time (Lyons & Parish, 1994). The proliferation of thymocytes stimulated by dendritic cells in the presence of irradiated CBA/J spleen cells is comparable to that seen when thymocytes are stimulated with irradiated BALB/c, T cell depleted, spleen cells in the presence of irradiated CBA/J spleen cells. As discussed previously, only the latter cultures of thymocytes stimulated in the presence of "help", generate allo-MHC specific CD8+ CTL and IFN-γ producing cells, and CD4+ IL-2 or IL-4 producing cells.

In summary, the results presented indicate that dendritic cells derived from proliferating bone marrow progenitor cells are conventional dendritic cells that possess the capacity to stimulate the naïve T cells present among thymocytes to increase expression of CD40L, and progress into cell cycle. However, in the absence

of further cellular interactions, the proliferating CBA/J thymocytes fail to differentiate into functional CTLs and T cells producing cytokines. In marked contrast, such stimulation of CBA/J splenocytes, which contain both naïve and previously activated T cells (Dutton et al., 1998), results in the generation of CTL and cytokine responses.
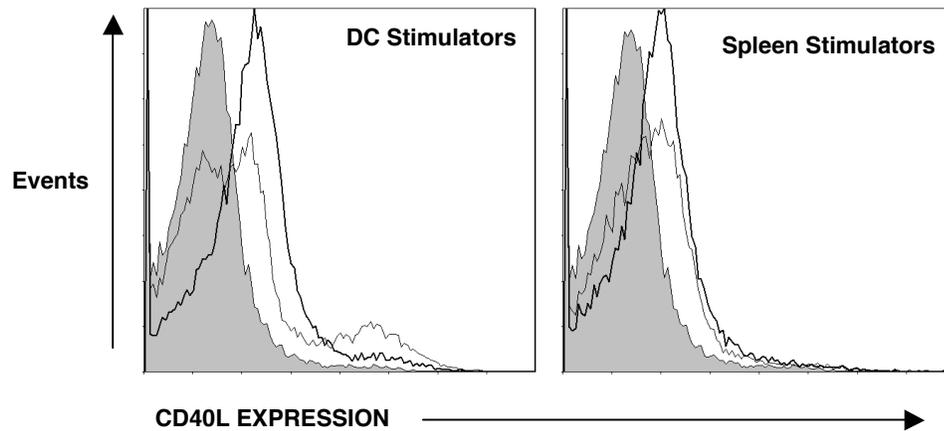
Figure 4.5.5. *Thymocytes Stimulated with Allogeneic Bone Marrow Derived Dendritic Cells or Allogeneic Spleen Cells Up-Regulate the Costimulatory Molecule CD40L (CD154).* CBA/J thymocytes were stimulated with 1 x 10^4 cells/well irradiated DC derived from the bone marrow of BALB/c mice (left panel) or with irradiated, T cell depleted, BALB/c spleen cells (right panel) in the absence (—) or presence (—) of irradiated CBA/J spleen cells. Standard culture conditions as described in section 3.4 were employed. The expression of the costimulatory ligand CD40L (CD154) on stimulated thymocytes was performed by flow cytometry as described in section 3.9.1 on day 5. Background staining is indicated by the shaded histogram. Similar results were obtained in two independent experiments.

Figure 4.5.6. *Thymocytes Stimulated with Allogeneic Bone Marrow Derived Dendritic Cells in the Presence of Syngeneic Irradiated Spleen are Stimulated to Divide*. CBA/J thymocytes, T, were stimulated with 2 x 10^4 cells/well irradiated DCs derived from the bone marrow of BALB/c mice or with irradiated, T cell depleted, BALB/c spleen cells in the absence or presence of irradiated CBA/J spleen cells or H-Help. Standard culture conditions as described in section 3.4 were employed. Before the addition to culture, responding CBA/J thymocytes were labeled with CFSE as described in section 3.9.2. Single cell division was assessed at 24 hr intervals on Days 2 (—), 3 (---), and 4 (—) by the loss of CFSE fluorescence intensity after the removal of dead cells by Ficoll density centrifugation. Background fluorescence of unlabelled and stimulated thymocytes is indicated by the shaded histogram. Similar observations were obtained in three independent experiments.

Thymocytes w/o Help　　　　**Thymocytes w/ Help**

DC
Stimulators

Events

Spleen
Stimulators

CFSE Intensity ⟶

155
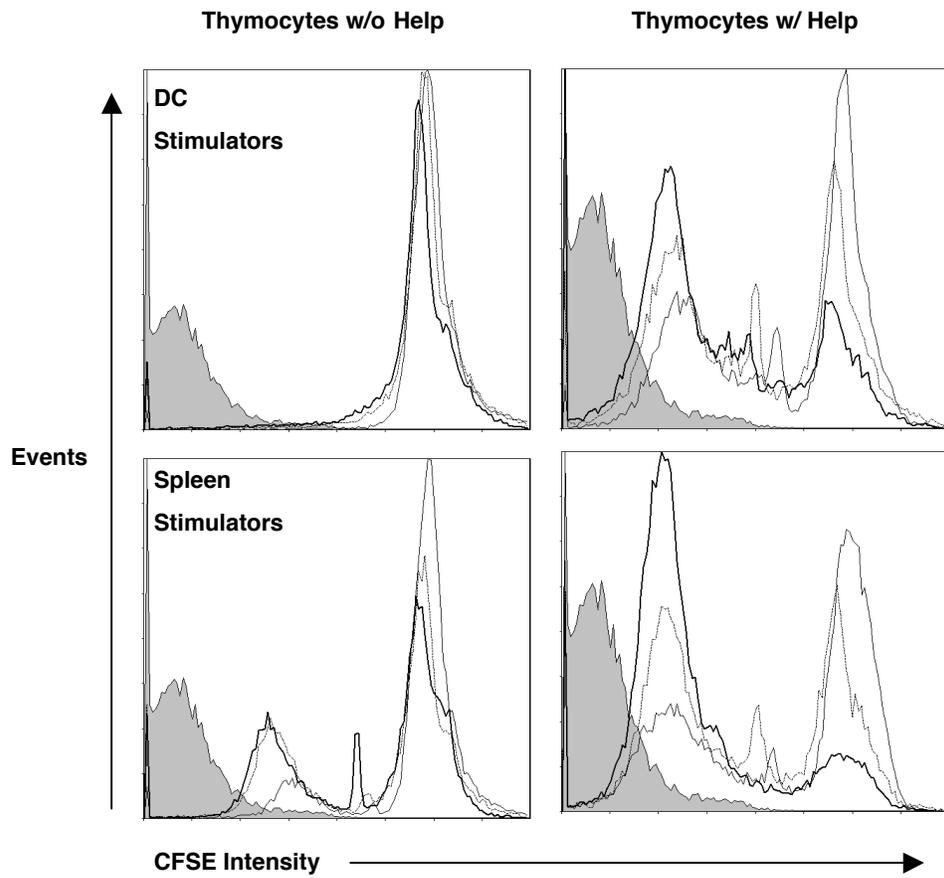
4.6. Nature of the Antigen Presenting Cell Involved in Primary T Cell Activation: Stimulatory Capacity of B cells

We have shown that allogeneic bone marrow derived dendritic cells fail to stimulate thymocytes to generate CTLs and cytokine producing cells, even after they have been exposed to maturation signals such as CD40 ligation or exposure to LPS, and when radiation-resistant effector CD4+ T helper cells are provided. Thymocytes stimulated with allogeneic dendritic cells up-regulate CD40L and proliferate, but fail to differentiate into competent effector cells. The two-step, two-signal model predicts that the inability of thymocytes to generate significant CTL and cytokine producing cells upon dendritic cell stimulation in the presence of "help" is due to the absence of antigen-specific B cells that act as antigen-presenting cells (Bretscher, 1999).

Indirect experimental evidence, such as the ability of the BALB/c T cell depleted spleen to stimulate thymocytes to generate immune responses in the presence of irradiated syngeneic CBA/J help, and the reduction in the stimulatory capacity of the BALB/c splenocytes after the removal of both Thy1.2 and B220+ cells, support the possibility that B cells may participate in the delivery of activation signals to naïve T cells that have initiated clonal expansion.

Experiments were performed to obtain more conclusive evidence that B cells are capable of stimulating the generation of CTL and cytokine producing cells from naïve cells obtained from the thymus. B cells, as operationally defined as CD45RA (B220+) cells (Coffman & Weissman, 1981), were purified from BALB/c

splenocytes using MACs beads. The ability of the purified B220+ cells, which are predominately B cells, to stimulate the generation of responses from thymocytes was assessed in either the absence or presence of irradiated, syngeneic CBA/J spleen cells. The results presented in Figure 4.6.1 a and b show that thymocytes readily generate allo-MHC specific CTL and cytokine responses against irradiated B220+ cells when irradiated syngeneic spleen cells are provided.

We next examined the capacity of combined bone marrow derived dendritic cells and MACs sorted B220+ cells, which are predominately B cells, to stimulate the generation of allogeneic responses from thymocytes. Responses similar to that generated upon stimulation with B220+ cells alone were generated when both B220+ cells and bone marrow derived dendritic cells were present in culture, which indicates that the cells in the bone marrow derived dendritic population or any cytokine they may produce, are not inhibiting the generation of responses from thymocytes. However, when the B220+ cells in the latter stimulators were depleted of immunoglobulin-bearing cells (Ig), thymocytes failed to produce CTL and T cells producing cytokines upon stimulation with allo-MHC bearing dendritic cells, see the left panels of Figure 4.6.2. a and b, as well as Figure 4.6.2. c, even though irradiated syngeneic spleen cell "help" was provided. This result appears to be very "clean", and provides strong support for the possibility that allogeneic B220+ Ig+ B cells are required in this system to generate responses.

Both allogeneic antigen presenting cell populations, the purified B220+ cells and the bone marrow derived dendritic cells, stimulate CBA/J spleen cells to generate CTL and cytokine producing cells, see the left panels of Figure 4.6.2 a and b, with

B220+ B cells stimulating more optimal CTL and cytokine responses when both stimulator cell populations are employed at physiological cell numbers.

Figure 4.6.1.a. *MACs Sorted CD45R (B220+) Allo-MHC Bearing Spleen Cells Stimulate the Generation of CTLs from Thymocytes in the Presence of Syngeneic Irradiated Spleen.* CBA/J thymocytes were stimulated with irradiated B220+ cells that were isolated from the spleens of BALB/c mice as described in 3.7.2. The number of T cell depleted, BALB/c spleen cells was determined before sorting. In order to accommodate for cell loss during the sorting procedure, a standard number of thymocytes were stimulated with 2 x 10^6 cells/well (◆), 4 x 10^6 cells/well (●), or 8 x 10^6 cells/well (■) stimulators as enumerated before sorting. Stimulated thymocytes were cultured either in the absence (left panel) or presence (right panel) of a standard number of irradiated CBA/J spleen cells as described in section 3.4. The response of irradiated CBA/J spleen cells stimulated with sorted B220+ cells in the absence of responding thymocytes is represented by the open triangle. The generation of CTL responses was assessed on Day 6 using a standard ^{51}Cr CTL assay against labeled P815 (H-2^d) target cells or against labeled third party EL-4 (H-2^b- inset) targets as described in section 3.5.1. Similar results were obtained in two independent experiments.

Thymocytes without Help

Thymocytes with Help

% Specific Lysis

80

60

40

20

0

80
60
40
20
0

α H-2^b

0.6   1.2   2.5   5

0.6   1.2   2.5   5

Percent Culture

160
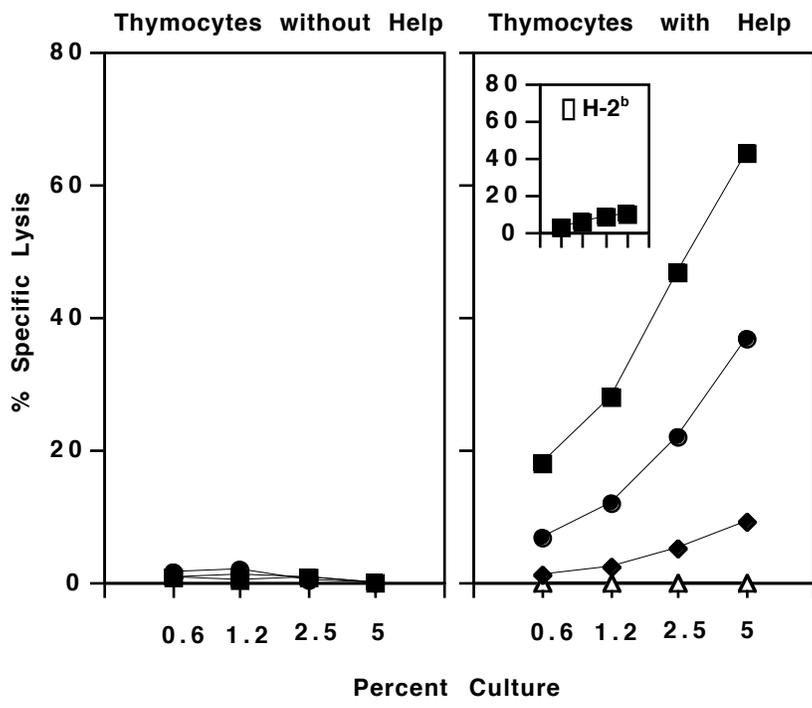
Figure 4.6.1. b. *MACs Sorted CD45R (B220+) Allo-MHC Bearing Spleen Cells Stimulate the Generation of IFN-γ, IL-2, and IL-4 Producing Cells from Thymocytes in the Presence of Syngeneic Irradiated Spleen.* CBA/J thymocytes, T, were stimulated with irradiated B220+ cells that were isolated from the T cell depleted spleens of BALB/c mice as described in section 3.7.2. The number of stimulator cells was determined before sorting. In order to accommodate for cell loss during the sorting procedure, thymocytes were stimulated with 2 x 10^6 cells/well (1), 4 x 10^6 cells/well (2), or 8 x 10^6 cells/well (3) as enumerated before sorting. Stimulated thymocytes were cultured employing standard conditions as described in section 3.4 in either the absence or presence of irradiated CBA/J spleen cells or H-Help. The number of IFN-γ, IL-2, and IL-4 producing cells generated by day 6 was enumerated in the ELISPOT Assay as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar results were obtained in two independent experiments.

T	+	+	+	+	+	+	-
H	-	-	-	+	+	+	+
B220+	1	2	3	1	2	3	3

Culture/Stimulator

162
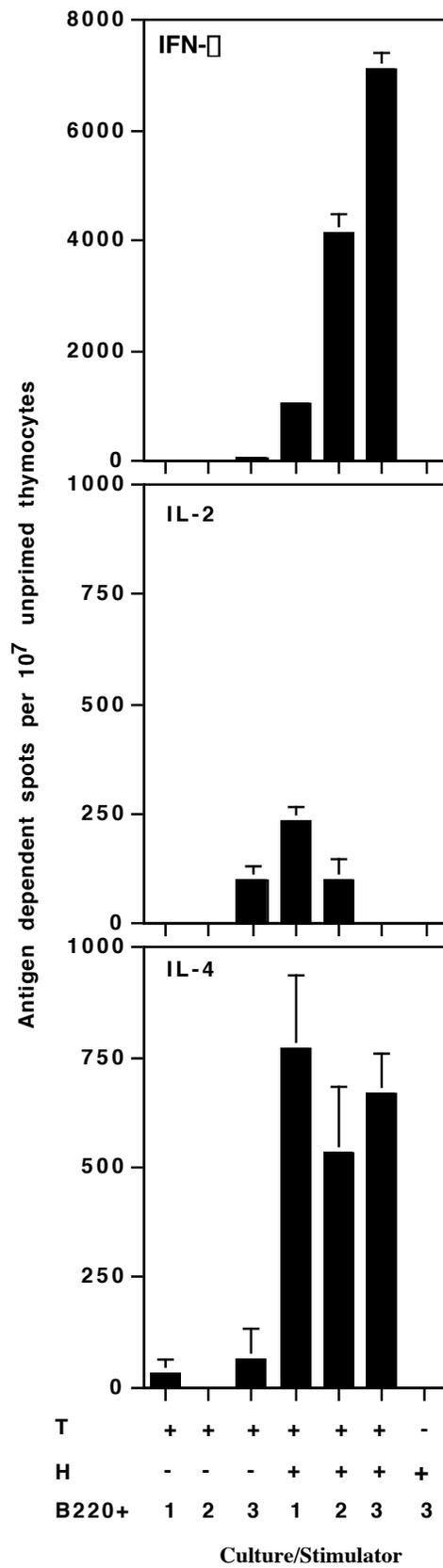
Figure 4.6.2.a. *The MACs Sorted CD45R (B220+) Allo-MHC Bearing Spleen Cell that Stimulates the Generation of CTLs from Thymocytes in the Presence of Syngeneic Irradiated Spleen also Bears Surface Immunoglobulin.* CBA/J thymocytes were stimulated with combinations of irradiated T cell depleted BALB/c cells in the absence or presence of irradiated CBA/J spleen cells (open and filled symbols, respectively) employing standard conditions as described in section 3.4. DCs were derived from bone marrow, and B220+ cells were isolated from the spleens of BALB/c mice using MACs beads as described in sections 3.8.1 and 3.7.2, respectively. Immunoglobulin bearing cells were removed from the sorted B220+ cells by panning on Ig coated plates as described in section 3.7.3. Thymocytes (left panel) were stimulated with 1 x 10^4 DC/well alone (■), DC plus 1 x 10^6 Ig+ B220+ cells/well (●), DC plus 0.5 x 10^6 Ig+ B220+ cells/well (▲), DC plus 1 x 10^6 B220+ cells/well depleted of Ig+ cells (✚), or with 1 x 10^6 Ig+ B220+ cells/well alone (◆). CBA/J thymocytes stimulated in the absence of irradiated spleen cells were stimulated with DC plus 1 x 10^6 Ig+ B220+ cells/well (□) and the hatched circle, which is hidden under the square, represents similarly stimulated irradiated CBA/J spleen cells without thymocytes. CBA/J spleen cells (right panel) were also stimulated with either 1 x 10^4 DC/well (■) or 1 x 10^6 Ig+ B220+ cells/well (◆). The ability of the different APCs to support the generation of CTLs was determined on day 7 against labeled P815 (H-2^d) in the standard ^{51}Cr CTL assay as described in section 3.5.1. Similar observations were obtained in three separate experiments.
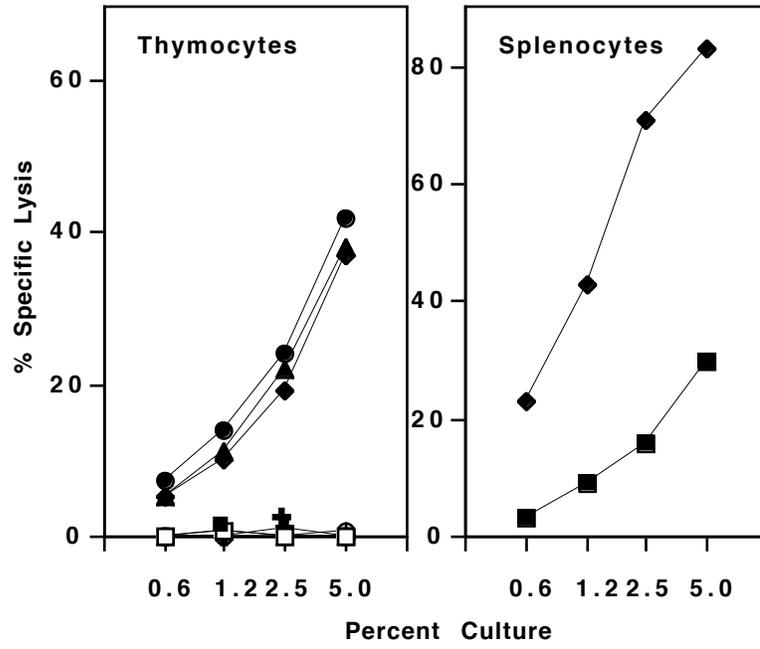
Figure 4.6.2.b. *The MACs Sorted CD45R (B220+) Allo-MHC Bearing Spleen Cell that Stimulates the Generation of IFN-γ, IL-2, and IL-4 Producing Cells from Thymocytes in the Presence of Syngeneic Irradiated Spleen also Bears Surface Immunoglobulin*. CBA/J thymocytes, T, were stimulated with combinations of irradiated BALB/c APC in the absence or presence of irradiated CBA/J spleen cells or H –help employing standard conditions as described in section 3.4. DCs were derived from bone marrow, and B220+ cells were isolated from the spleens of BALB/c mice using MACs beads as described in sections 3.8.1 and 3.7.2, respectively.   Thymocytes were stimulated with 1 x 10^4 DC/well alone, DC plus 1 x 10^6 Ig+ B220+ cells/well, DC plus 0.5 x 10^6 Ig+ B220+ cells/well, DC plus 1 x 10^6 B220+ cells/well depleted of Ig+ cells, or with 1 x 10^6 Ig+ B220+ cells/well alone. The +* in the B220 row represents 0.5 x 10^6 Ig+ B220+ cells/well, and +/- represents 1 x 10^6 B220+ cells/well depleted of Ig+ cells. CBA/J spleen cells (S) were also stimulated with either 1 x 10^4 DC/well, or 1 x 10^6 Ig+ B220+ cells/well. The ability of the different APCs to support the generation of cytokine producing cells was determined on day 7 in the ELISPOT assay in the absence or presence of BALB/c re-stimulation as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar observations were obtained in three independent experiments.
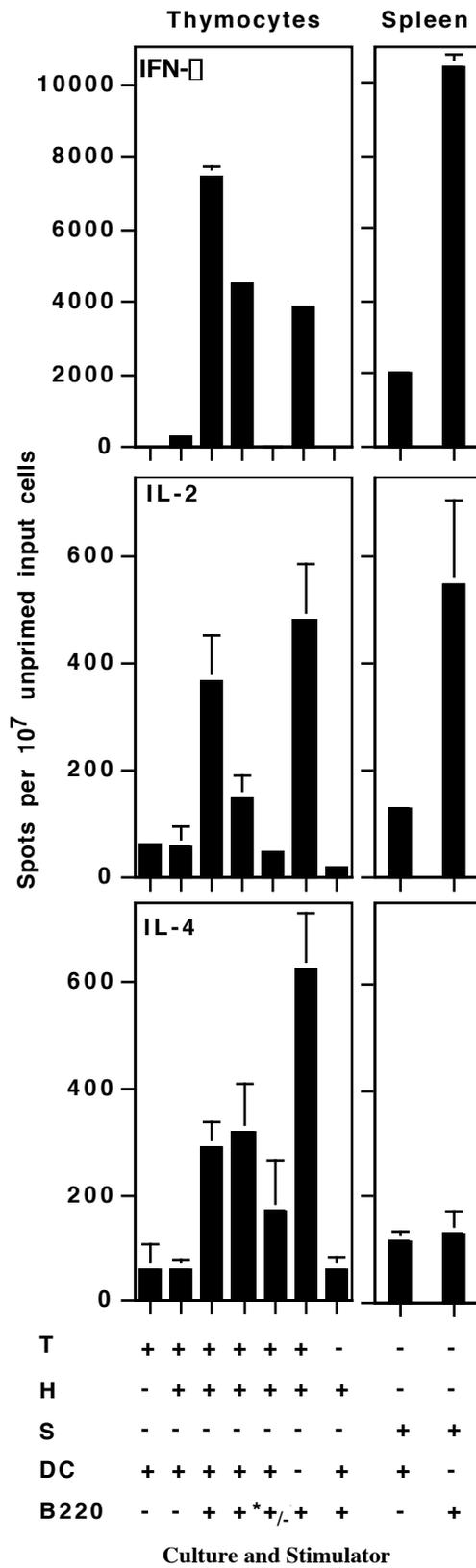
Figure 4.6.2. c. *The MACs Sorted CD45R (B220+) Allo-MHC Bearing Spleen Cell that Stimulates the Generation of IFN-γ Producing Cells from Thymocytes in the Presence of Syngeneic Spleen also Bears Surface Immunoglobulin*. CBA/J thymocytes, T, were stimulated with combinations of irradiated BALB/c APCs in the absence or presence of irradiated CBA/J spleen cells or H –help employing standard conditions as described in section 3.4. DCs were derived from bone marrow, and B220+ cells were isolated from the spleens of BALB/c mice using MACs beads as described in sections 3.8.1 and 3.7.2, respectively. Thymocytes were stimulated with 1×10^4 DC/well alone, DC plus 1×10^6 Ig+ B220+ cells/well, DC plus 0.5×10^6 Ig+ B220+ cells/well, DC plus 1×10^6 B220+ cells/well depleted of Ig+ cells, or with 1×10^6 Ig+ B220+ cells/well alone. The +* in the B220 row represents 0.5×10^6 Ig+ B220+ cells/well, and +/- represents 1×10^6 B220+ cells/well depleted of Ig+ cells. The ability of the different APCs to support the generation of IFN-γ producing cells was determined on day 7 in the ELISPOT assay in the absence or presence of BALB/c re-stimulation as described in section 3.5.2. Effector phase cells were seeded in the ELISPOT at a concentration of 1×10^4 effectors/well with or without 2.5×10^5 irradiated BALB/c spleen cells/well. Similar observations were obtained in three independent experiments.

Culture/ Stimulator				BALB/c Stimulation in ELISPOT	
T	H	DC	B220	-	+
1. +	-	+	+		
2. +	+	+	-		
3. +	+	+	+		
4. +	+	+	+*		
5. +	+	+	+/-		
6. +	+	-	+		
7. -	+	+	+		

In order to assess in another context a requirement for Ig+ B220+ B cells in primary thymic T cell activation, and ensure that the inability of the bone marrow derived dendritic cells to stimulate the generation of CTL and cytokine responses is not a unique property of the dendritic cells generated from proliferating bone marrow progenitor cells, B220+ cells and B220- cells were isolated from normal BALB/c splenocytes depleted of T cells by MACs sorting. The sorted B220+ and B220- cells were used to stimulate CBA/J thymocytes and spleen cells. Thymocytes were stimulated in either the absence or presence of irradiated syngeneic CBA/J spleen cells. The results presented in Figure 4.6.3. a and b show that unsorted BALB/c spleen depleted of T cells and sorted B220+ spleen cells readily stimulate thymocytes to generate CTL and cytokine producing cells when "help" is provided, whereas sorted B220- cells, which contain macrophages and the majority of the different dendritic cells subsets found in the spleen, fail to optimally stimulate the generation of allo-MHC specific CTL and IFN-γ cytokine producing cells, but do stimulate the generation of IL-2 and IL-4 producing cells. The control CBA/J spleen cell cultures readily generate potent CTL and cytokine responses against both the B220+ and B220- cells sorted from BALB/c spleen, see the left panels of Figure 4.6.3. a and b.

FACs analysis on the sorted B220+ and B220- spleen cells is shown in Figure 4.6.3.c. The B220+ cells sorted using MACs beads were 98% pure B220+ CD19+ B cells. The B220- fraction, was less pure, and was found to still contain a small population of cells expressing a low level of B220. The presence of this small

population in addition to any memory B cells that have completely down regulated B220 (McHeyzer-Williams et al., 2000) may explain the residual generation of IL-2 and IL-4 cytokine producing cells from thymocytes stimulated with Thy1.2- B220- allogeneic spleen cells. In preliminary experiments where residual immunoglobulin cells were removed via MACs sorting, the residual generation of IL-2 cytokine producing cells from thymocytes was abrogated (data not shown).

Figure 4.6.3. a. *MACs Sorted CD45R (B220-) Allo-MHC Bearing Spleen Cells Stimulate the Generation of CTLs from CBA/J Splenocytes, but Fail to Optimally Stimulate the Generation of CTL Responses from CBA/J Thymocytes Stimulated in the Presence of Syngeneic Irradiated Spleen.* CBA/J thymocytes were stimulated with combinations of irradiated BALB/c APCs in the absence or presence of irradiated CBA/J spleen cells (open and filled symbols, respectively) employing standard conditions as described in section 3.4. B220+ and B220- cells were isolated from T cell depleted spleens of BALB/c mice using MACs beads as described in section 3.7.2 and 3.8.3. Thymocytes (left panel) were stimulated with 2 x 10^6 unsorted anti-Thy1.2 treated spleen cells/well (■), 1 x 10^6 B220+ spleen cells/well (▲), or 1 x 10^6 anti Thy1.2 treated B220- spleen cells/well (●). CBA/J spleen cells (right panel) were also stimulated with 2 x 10^6 anti-Thy1.2 treated spleen cells/well (■), 1 x 10^6 B220+ cells/well (▲), or 1 x 10^6 anti Thy1.2 treated B220- spleen cells/well (●). The ability of the different APCs to support the generation of CTLs was determined on day 7 against labeled P815 (H-2^d) and EL-4 (H-2^b) target cells as described in section 3.5.1. Specific lysis of third party EL-4 target cells by effector cells generated by stimulation with anti-Thy1.2 treated spleen stimulators is shown by the hatched square. The specific lysis of irradiated CBA/J spleen cells stimulated with T cell depleted BALB/c spleen cells was < 2% and is hidden by the other symbols in the left panel. Similar observations were obtained in three independent experiments.
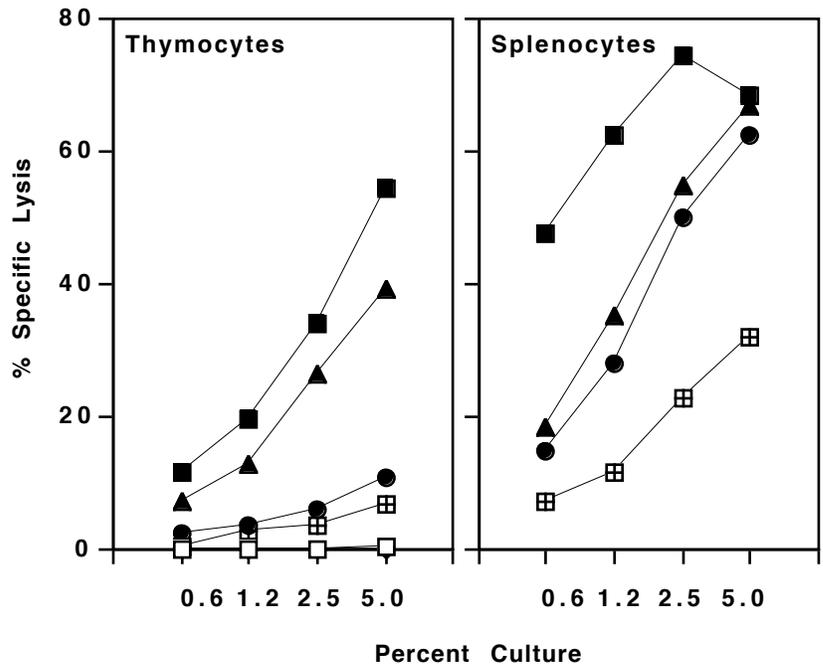
**Figure 4.6.3. b**. *MACs Sorted CD45R (B220-) Allo-MHC Bearing Spleen Cells Stimulate the Generation of IFN-γ, IL-2, and IL-4 Producing Cells from Responding Splenocytes, but Fail to Optimally Stimulate the Generation of IFN-γ Responses from Thymocytes Stimulated in the Presence of Syngeneic Irradiated Spleen.* CBA/J thymocytes, T, were stimulated with combinations of irradiated BALB/c APCs in the absence or presence of irradiated CBA/J spleen cells or H-Help employing standard conditions as described in section 3.4. B220+ and B220- cells were isolated from the spleens of T cell depleted BALB/c spleen cells using MACs beads as described in section 3.7.2. Thymocytes (left panels) were stimulated with 2 x 10^6 unsorted anti-Thy1.2 treated (Rx) spleen cells/well (anti-Thy1.2 Rx Ag), 1 x 10^6 B220+ spleen cells/well, or 1 x 10^6 anti-Thy1.2 treated B220- spleen cells/well. CBA/J spleen (S) cells (right panels) were also stimulated with 2 x 10^6 anti-Thy1.2 treated B220+ spleen cells/well, or 1 x 10^6 anti Thy1.2 treated B220- spleen cells/well. The ability of the different APCs to support the generation of cytokine producing cells was determined on day 7 in the ELISPOT assay in the absence or presence of BALB/c re-stimulation as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar results were obtained in three independent experiments.

Thymocytes   **Spleen**

IFN-γ

IL-2

IL-4

Antigen dependent spots per 10^7 unprimed thymocytes or 10^6 splenocytes

T	+	+	+	+	-	-	-	-
H	-	+	+	+	+	-	-	-
S	-	-	-	-	-	+	+	+
αThy1 Rx Ag	+	+	-	-	+	+	-	-
αThy1 Rx B220-	-	-	+	-	-	-	+	-
B220+	-	-	-	+	-	-	-	+

Culture/Stimulator

174
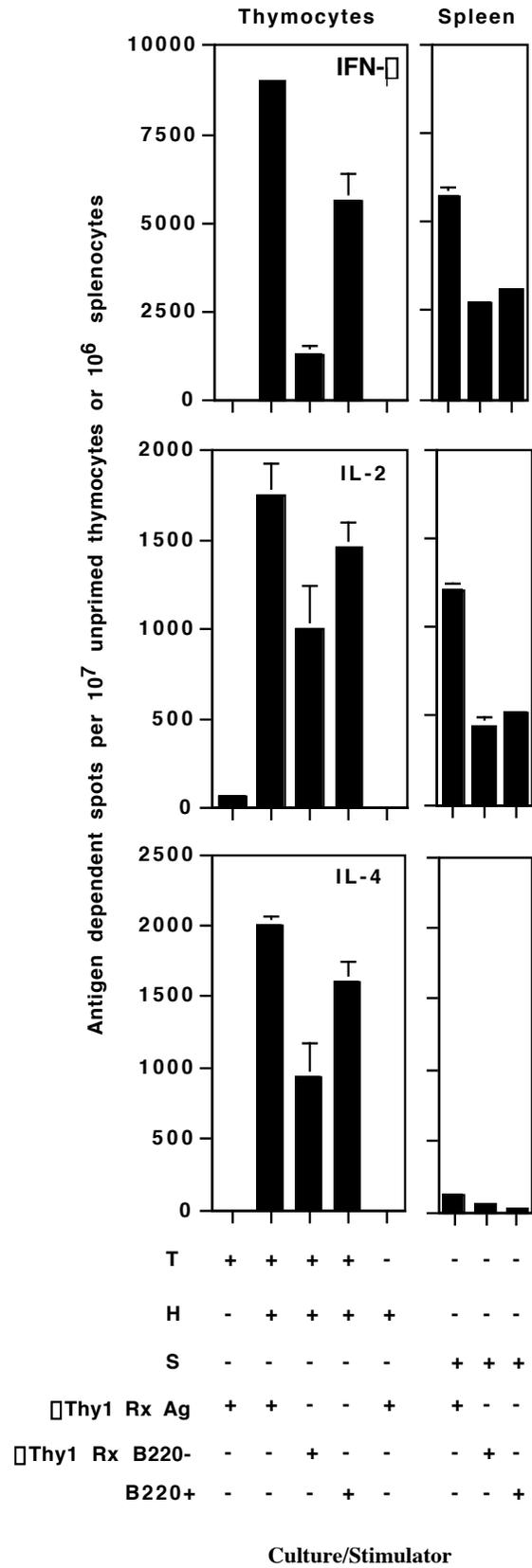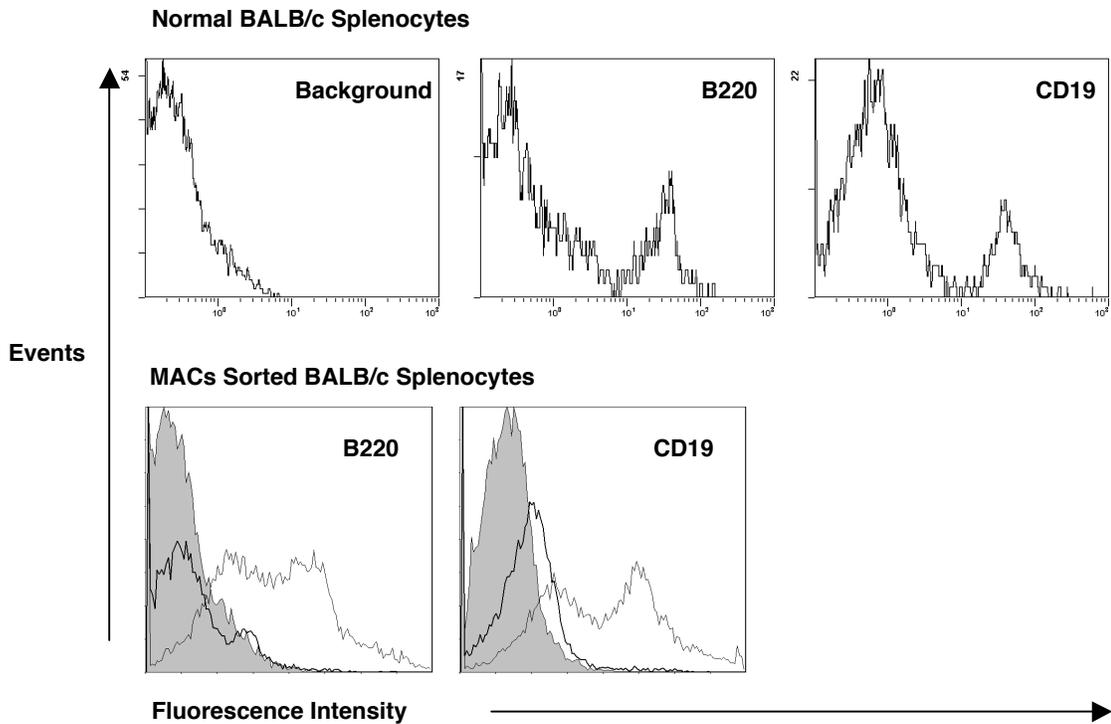
Figure 4.6.3. c. *CD45R (B220) and CD19 Expression on Normal Spleen and on CD45R (B220+) and CD45R (B220-) MACs Sorted Cells*. The expression profiles of B220 and CD19 on normal (upper row) and MACs sorted BALB/c spleen cells (lower row) were assessed by flow cytometry as described in section 3.9.1. Expression profiles of MACs sorted B220+ (—), or B220- (▬) cells are shown. Background staining is represented as shaded histograms.

4.7. Stimulation of Naïve Thymic T cells with Dendritic Cells in the Absence of B cells Appears to result in a State of Antigen-Specific CTL Unresponsiveness

Experimental evidence has been presented that shows that thymocytes, the naïve precursors of the peripheral T cell pool, fail to generate CTL and cytokine-producing T cells upon stimulation with allogeneic bone marrow derived dendritic cells matured by maturation stimuli, such as CD40 ligation or exposure to LPS, even when irradiated syngeneic spleen cell "help" is provided. To determine if a state of allo-MHC specific tolerance follows the cellular proliferation initiated by stimulation with activated, allogeneic dendritic cells as has been documented for fetal thymic cells (Matzinger & Guerder, 1989), stimulated thymocytes were re-stimulated after 5 days with either T cell depleted allogeneic spleen cells of the same MHC haplotype of the primary LPS activated DC stimulators, or with those of an alternate MHC haplotype, in either the absence or presence of irradiated syngeneic spleen cell "help". To avoid disturbing the initial cultures, irradiated syngeneic spleen cells and T cell depleted allogeneic spleen cells were added to thymocyte cultures after the careful removal of half of the culture media (1mL for a 2 mL culture). The ability of the re-stimulated thymocytes to generate CTL against both allo-MHC antigens was assessed after 6 days.

The findings presented in Figure 4.6.1 show that the ability of thymocytes to generate allo-MHC specific CTL responses against cells bearing the same allo-MHC antigens as dendritic cells used as stimulators in primary culture, is significantly

reduced, even though irradiated syngeneic spleen cell "help" was provided with the T cell depleted allogeneneic spleen cell stimulators. By contrast, the generation of alternate allo-MHC specific CTLs in restimulated cultures is minimally affected by previous exposure to dendritic cells of a different MHC haplotype. A reduction in the response generated against stimulators that share MHC antigens with the primary stimulating dendritic cells is more dramatic for the response against BALB/c MHC-antigens than for C57BL/6. It is possible that the kinetics of tolerance induction against the different allogeneic cells varies, for example thymocytes stimulated with C57BL/6 dendritic cells may still be "rescue-able" on Day 5 but not on Day 6. Future experiments will thoroughly analyze the kinetics of the proliferative potential of the C57BL/6 allogeneic dendritic cell stimulated thymocytes as well as the kinetics of tolerance induction and rescue of differentially stimulated CBA/J thymocytes.

The corresponding cytokine data (data not shown) reveals some interesting differences in the ability of thymocytes to generate cytokine-producing cells upon secondary stimulation. Thymocytes stimulated with allogeneic spleen cell stimulators in the presence of irradiated syngeneic spleen cell "help" after a pre-exposure to allogeneic bone marrow derived dendritic cells appear to only generate cytokine producing cells against non-dendritic cells alloantigens present on non-Thy1.2 + allogeneic spleen cells as revealed by a comparison of spleen-dependent and dendritic cell dependent development of spots in the ELISPOT assay. These findings are preliminary and warrant further examination.

A number of reports in the literature argue that the provision of the growth factor IL-2, which is normally produced by CD4+ T helper cells, can rescue CD8+ T cells from activation induced cell death (Smith, 1984; Teh & Teh, 1980). Experiments were performed to determine if the addition of this cytokine prevents the induction of allo-MHC specific CTL and cytokine unresponsiveness, and thereby helps thymocytes generate allo-MHC specific CTLs and cytokine producing cells. The results presented in Figure 4.6.2 a and b show that thymocytes do generate allo-MHC specific CTL and IFN-γ producing cells, which in this experimental system derive from CD8+ thymic precursors, upon stimulation with irradiated, LPS-activated, allogeneic dendritic cells when exogenous IL-2 is provided and irradiated syngeneic spleen cells are absent. Provision of IL-2, which is normally produced in large amounts by CD4+ T cells (Harris et al., 2000; Swain, Weinberg, & English, 1990), can therefore bypass the cellular requirements normally involved in the primary activation of CD8+ T cells. It is interesting to note that B cells have been demonstrated to stimulate CD4+ T cells to produce more IL-2 than other antigen-presenting cells (Harris et al., 2000).

In summary, the observations presented provide supportive experimental evidence that naive thymic T cell activation upon stimulation with conventional allogeneic bone marrow derived dendritic cells can be dependent upon costimulatory signals and/or cytokines provided by activated, cognate, allogeneic B cells. In the absence of such cells, thymic naïve T cells appear to fail to become fully activated upon stimulation with bone marrow derived dendritic cells. At least for CD8+ CTLs,

the induction of antigen-specific tolerance is preventable by the presence of high levels of the CD4+ T helper cell derived cytokine, IL-2.

Figure 4.7.1. *Exposure of Thymocytes to Activated Dendritic Cells in the Absence of Effector CD4+ T Helper Cells and B220+ B cells Results in Antigen Specific Immune Unresponsiveness.* CBA/J thymocytes, 2 x 10^6 cells/well, were stimulated for 5 days with either 2 x 10^4 cells/well irradiated BALB/c (left panel) or C57BL/6 (right panel) LPS-activated, bone marrow derived DCs as described in section 3.4 and 3.8.1, respectively. On Day 5, separate DC-pretreated cultures were stimulated with irradiated anti-Thy1.2 and C" treated (Rx) BALB/c (■) or C57BL/6 (●) spleen cells in absence or presence of irradiated CBA/J spleen cells (open and filled symbols, respectively). The latter cells were added to cultures in situ at standard numbers as described in section 3.4. The ability of the different DC pretreated thymocytes to generate CTL responses was assessed six days after re-stimulation using the standard ^{51}Cr CTL assay against labeled P815 (H-2^d) target cells or against labeled third party targets, EL-4 (H-2^b) as described in section 3.5.1. The specific lysis of pre-treated thymocytes stimulated with T cell depleted BALB/c or C57BL/6 spleen cells without "help", and the specific lysis of irradiated CBA/J spleen cells stimulated with T cell depleted BALB/c or C57BL/6 spleen cells in the absence of thymocytes was < 2% and are hidden by the other symbols in the appropriate panels. Similar observations were obtained in three independent experiments. Similar observations were obtained in two independent experiments.

BALB/c BMDC
Pretreatment

C57BL/6 BMDC
Pretreatment

% Specific Lysis

α EL-4

α P815

α P815

α EL-4

0.6 1.2 2.5 5   0.6 1.2 2.5 5

Percent Culture

181
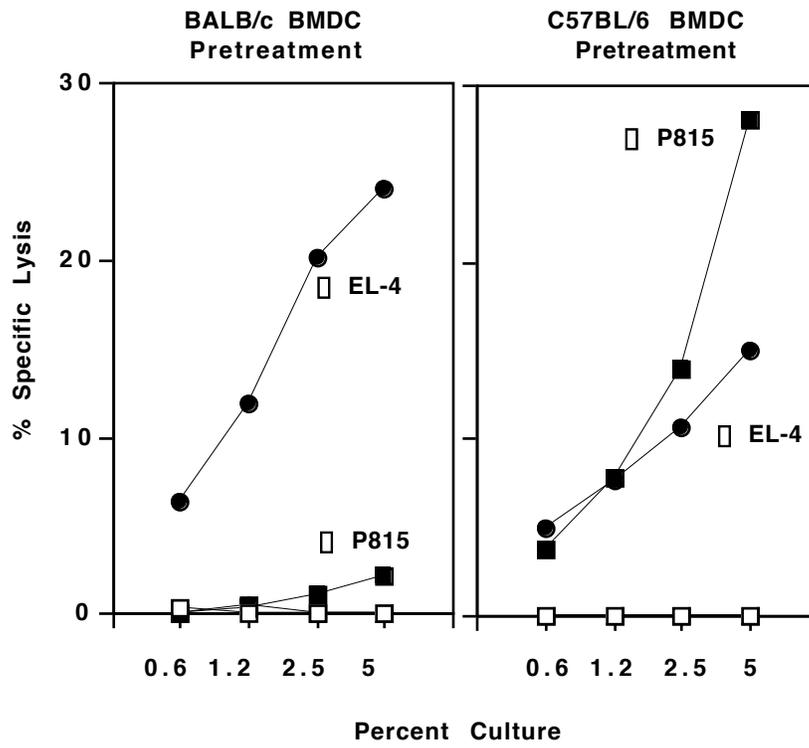
Figure 4.7.2. a. *High Levels of IL-2 Prevent the Antigen-Specific CTL Unresponsiveness Generated by Exposure of Thymocytes to Dendritic Cells.* Varied numbers of CBA/J thymocytes were stimulated for 6 days with 1 x 10^4 cells/well LPS-activated, irradiated BALB/c bone marrow derived DCs in the absence or presence of 10 Units of recombinant IL-2. Thymocyte cultures and bone marrow derived dendritic cells were prepared as described in sections 3.4 and 3.8.1. Irradiated, syngeneic CBA/J spleen cells were not added to any cultures. The numbers of CBA/J thymocytes seeded in culture were as follows: 1 x 10^6 (❏), 2 x 10^6 (✧), 4 x 10^6 (❍), and 8 x 10^6 (∇) cells/well. The ability of the DCs to stimulate the generation of CTL responses was assessed on Day 6 using the standard ^{51}Cr CTL assay against labeled P815 (H-2^d) target cells as described in section 3.5.1. The specific lysis of the varied numbers of thymocytes represented by the above symbols in the absence of IL-2 was < 2% lysis and the corresponding symbols are hidden under the ❏. Similar results were obtained in two independent experiments.

Stimulation minus IL-2   **Stimulation plus IL-2**

% Specific Lysis

Percent Culture
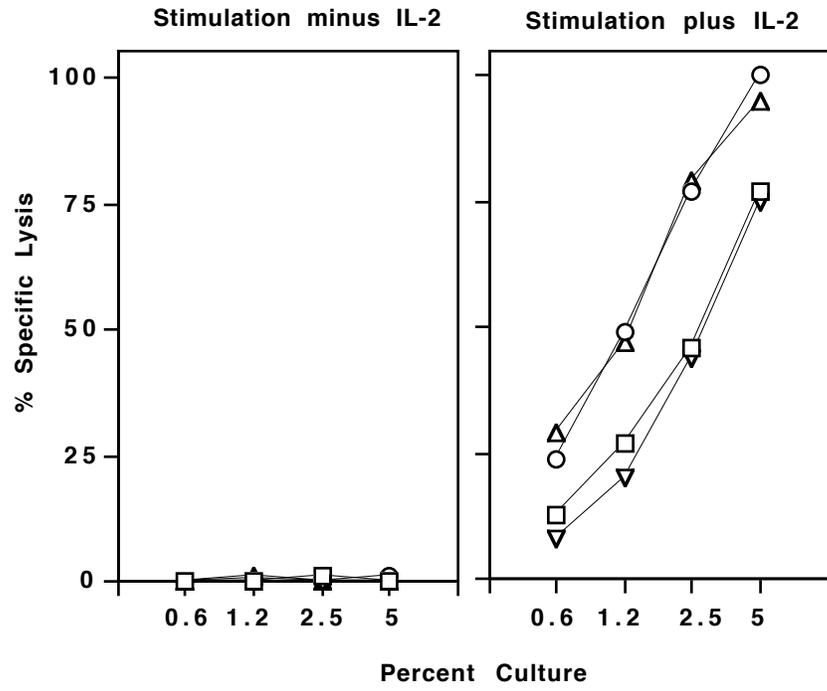
Figure 4.7.2. b. *High Levels of IL-2 "Help" Thymocytes Generate the Antigen-Specific IFN-γ Producing cells upon Stimulation with Bone Marrow Derived Dendritic Cells*. Varied numbers of CBA/J thymocytes were stimulated with 1 x 10^4 cells/well, LPS-activated, irradiated BALB/c bone marrow derived DCs in the absence or presence of 10 Units of recombinant IL-2. Thymocyte cultures and bone marrow derived dendritic cells were prepared as described in sections 3.4 and 3.8.1. Irradiated, syngeneic CBA/J spleen cells were not added to any cultures. The numbers of CBA/J thymocytes seeded in culture were as follows 1 x 10^6 (1), 2 x 10^6 (2), 4 x 10^6 (3), and 8 x 10^6 (4) cells/well. The ability of DCs to support the generation of IFN-γ producing cells was determined on Day 7 in the ELISPOT assay in the absence or presence of BALB/c re-stimulation as described in section 3.5.2. The error bars represent the standard deviation of the number of antigen dependent spots. Similar results were obtained in two independent experiments.

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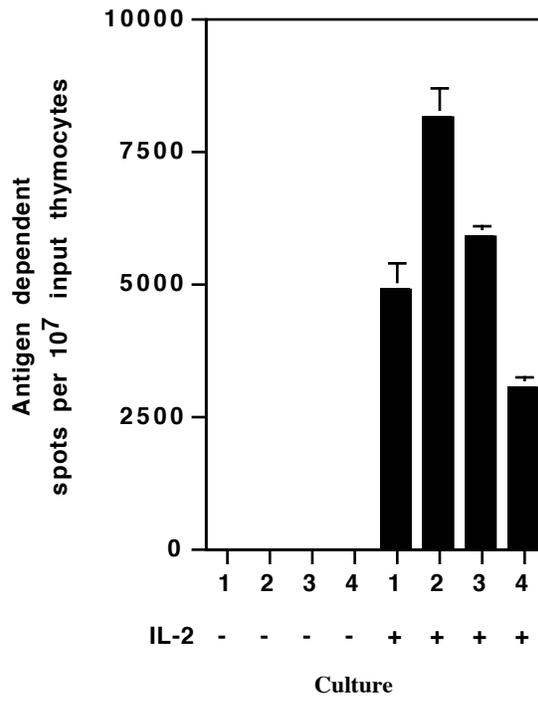
5. GENERAL DISCUSSION

5.1. Prologue

CD4+ T cells play an essential role in the body's defense against infectious pathogens. They orchestrate the antigen specific immune responses that provide both primary and secondary immunological protection against infection (Abbas et al., 2000). In order to ensure that the appropriate type of immune response is generated upon antigenic challenge, and to ensure that immune responses against self are subverted, the activation of naïve CD4+ T helper cells must be regulated.

The need to regulate the activation of immune cells to prevent the generation of autoimmune responses or "horror autotoxis" has been evident since the days of Ehrlich (Ehrlich, 1900). As discussed in the Introduction, an immunological mechanism to account for the regulation of peripheral self-reactive T cells, which escape central tolerance in the thymus, during the primary activation of naïve, foreign antigen-specific T cells remains to be established. Several models of naive T cell activation that attempt to describe how tolerance towards peripheral self occurs during the activation of foreign antigen specific cells have been discussed in the Introduction. These include the Infectious Non-Self, the Danger, the Two-Step, Two-Signal, and the Steady State Models, which may or may not also involve CD25+ CD4+ T regulatory cells (Bretscher, 1999; Janeway, 1989, 2001; Matzinger, 1994; Sakaguchi, 2004; Steinman & Nussenzweig, 2002). The major goal of this

thesis was to establish an experimental system that would allow one to begin to delineate which model most validly describes the cellular interactions required for generation of primary immune responses from naïve T cells.

5.2. The Generation of Primary Immune Responses from the Naïve T Cells present among Thymocytes Requires the Presence of Effector CD4+ T helper Cells

In order to delineate the cellular interactions involved in naive T cell activation, we chose to use an *in vitro* culture system in which thymocytes are the responding naïve lymphocytes (Pilarski, 1977). The use of thymocytes as responding naïve T cells has enabled us to study the cellular interactions required for naive T cell activation in a manner uncomplicated by the presence of any partially or previously activated T cells. A number of studies have demonstrated that mature thymocytes harvested from mice at time periods 24 hours after birth, or thymic emigrants that have recently exited the thymus are immunocompetent cells (Bennink & Doherty, 1981; Claman et al., 1968; Claman et al., 1966a; Fink et al., 1985; Pilarski, 1977; Ramsdell et al., 1991. In fact, thymocytes were the lymphocyte population employed by Claman in the landmark experiments that suggested that antibody-producing cells derived from bone marrow and thymus dependent lymphocytes interact with one another for the efficient generation of antibody responses (Claman, 1966 #1492; Scollay et al., 1984; Wagner et al., 1980; Widmer et al., 1981). Of particular interest to this work is the demonstration that the

frequency of CTL precursors reactive against either alloantigens or viruses amongst thymocytes does not vary dramatically from frequency of CTL precursors isolated from the secondary lymphoid organs (Bennink & Doherty, 1981; Fink et al., 1985; Widmer et al., 1981). However, it must be acknowledged that some propose that thymocytes receive additional maturation signals in the periphery, and that thymocytes, particularly CD4+ T cells, are therefore functionally different from naïve T cells isolated from the periphery (Boursalian et al., 2004; Lutz, 1981). At the present time, it is difficult to distinguish whether differences in the activation requirements of thymic T cells in comparison to peripheral naïve T cells reflects inherent differences in the functional capacity of the two T cell populations or whether the cellular requirements for their activation are truly different, as will be discussed later in this discussion.

Employing an *in vitro* system similar to the one established by L. Pilarski in the 1970's (Pilarski, 1977), we were able to reliably stimulate the generation of primary antigen-specific IL-2 CD4+ and IFN-γ CD8+ cytokine producing cells and CD8+ CTL immune responses from the naïve, competent T cells present among thymocytes. We have confirmed and extended previous observations in that we have found that not only the generation of CTL responses but cytokine producing cells from thymocytes upon stimulation with allogeneic spleen cells is dependent upon the presence of a sufficient number of irradiated spleen cells syngeneic to the responding thymocytes (Pilarski, 1977). The cells in the irradiated syngeneic spleen that are required to "help" in the activation of naïve T cells present among thymocytes have previously been shown to be CD4+ T cells (Pilarski, 1977; Pilarski et al., 1980). We

have found that these radiation resistant CD4+ T cells can act in conjunction with thymic CD4+ T cells to activate naïve CD8+ effector cells.

Our findings show that effector CD4+ T helper cells are required to activate naïve, thymic, precursor CD4+ T helper cells. However, the requirement for effector CD4+ T helper cells for the activation of thymocytes could conceivably be due to thymic CD4+ T cell functional immaturity (Boursalian et al., 2004; Lutz, 1981). Conclusive experimental evidence that thymocytes behave the same as naïve T cells isolated from the periphery remains a topic for future investigation that will be addressed in the Future experiments section.

5.3. The Surface Marker Phenotype and Antigen Specificity of the Radiation resistant, Effector CD4+ T helper Cells

A number of studies have lead to the estimate that 5-10% of peripheral CD4+ T cells survive *in vivo* after lethal irradiation (Cederbom et al., 1998; Pereira et al., 1985; Williams et al., 1994). The ability of a fraction of splenic CD4+ T cells to provide "help" for thymocytes after exposure to gamma irradiation indicates that these surviving cells are activated cells that can provide "help" independently of having to go through some divisions before being able to provide such help. In agreement with previous findings characterizing the surface marker phenotype of the CD4+ T cells that survive lethal doses of irradiation (Cederbom et al., 1998; Pereira et al., 1985), our results, employing a functional assay, indicate that the radiation resistant cell that "helps" thymocytes generate CTL and cytokine responses is a

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CD4+ T cell that expresses surface markers normally associated with activated T cells (Dutton et al., 1998). The removal of cells expressing high levels of a surface molecule normally associated with recently activated cells, namely CD44 (Budd et al., 1987), abrogates the ability of the irradiated syngeneic spleen to provide "help" for the *in vitro* generation of CTL and cytokine producing cells from thymocytes. By contrast, the removal of cells bearing high levels of surface markers normally associated with naïve T cells or resting memory cells, for example CD62L or CD45RB (Birkeland et al., 1989; Gallatin et al., 1983), fails to abrogate the ability of the irradiated syngeneic spleen to provide help for thymocytes. The radiation resistant effector CD4+ T helper cell is thus an activated CD44hi, CD62Llo, and CD45RBlo T cell.

Previous experiments, in which the spleen cells from CBA/J into CBA/J X BALB/c bone marrow chimeric mice were employed as a source of "help" for thymocytes, have demonstrated that the radiation resistant "helper" cells display tolerance towards self-antigens and specificity towards foreign antigens (Pilarski, 1977). Spleen cells from such chimeric mice fail to provide CBA/J thymocytes with radiation resistant "help" for the generation of allospecific CTL responses against BALB/c MHC antigens, but do provide help for the generation of alloresponses against third party allogeneic spleen cells. Effector CD4+ T helper cells expanded *in vitro* also display specificity. Using spleen cells from normal un-manipulated F1 mice, L. Baum was able to expand up a population of radiation resistant effector CD4+ T cells that fail to provide "help" for the generation of responses against both parent MHC antigens, but do provide "help" for the generation of responses against

the third party allogeneic spleen cells (Baum & Pilarski, 1978). Using effector CD4+ T cells clones derived from responsive thymocyte cultures as "helper cells", we have shown that thymocytes are capable of generating cells that can function as radiation resistant "helper" cells for other thymocytes. The thymocyte-derived clones are able to "help" thymocytes generate significantly greater responses against the allogeneic spleen cells for which they are specific than for third party allogeneic spleen cells. In conjunction with previously published findings (Baum & Pilarski, 1978; Pilarski, 1977), these observations indirectly support the suggestion that the irradiation resistant, effector CD4+ T cells that "help" thymocytes generate primary responses are thymus dependent lymphocytes, which bear antigen-specific $\alpha\beta$ T cell receptors.

If the requirement for effector CD4+ T helper cells is general, their presence could determine whether the generation of immune responses occurs. The Two-step, Two-signal Model hypothesizes that the effector CD4+ T helper cells that are present in the periphery of normal, un-manipulated mice, primarily bear T cell receptors that recognize foreign antigens, and that those with self-specificity do not exist in the periphery (Bretscher, 1999). This model hypothesizes that self-specific CD4+ T helper cells are deleted as they are generated, and that such deletion results in the peripheral population of effector CD4+ T helper cells with specificity for foreign antigens. According to this model, it is the presence of effector CD4+ T cells that determines, in part, the ability to generate responses against foreign or self-antigens, and not the "stranger" or "danger" signals the antigens may or may not induce (Bretscher, 1999; Janeway, 1989; Matzinger, 1994).

Findings obtained in an unrelated experimental system also provide support for the postulate that effector CD4+ T cells with self-specificity are lacking in the periphery of normal mice. These results come from studies analyzing the T cell receptor specificity of peripheral T cells that have undergone homeostatic driven proliferation upon transfer into lymphopenic mice (de Souza et al., 2002). After T cell hybridomas were generated from such cells, it was determined that the small minority of CD4+ T cells, which proliferated, and acquired an activated surface marker phenotype, primarily bear TCR with specificity for foreign antigens as assessed by the lack of responsiveness towards autologous cells (de Souza et al., 2002). While the ontogeny of effector CD4+ T helper cells requires further and direct examination, the possibility that the lymphopenic environment of the developing immune system supports the initial activation of foreign antigen-specific, effector CD4+ T helper cells is potentially supported by the previous study (Cho et al., 2000; Min et al., 2003; Murali-Krishna & Ahmed, 2000; Schuler et al., 2004; Tanchot et al., 2001).

5.4. The Nature and Role of the Antigen Presenting Cells Involved in the Primary Activation of Naïve Thymic T Cells *in Vitro*

The discussed requirement for effector CD4+ T helper cells is a plausible mechanism whereby the immune system decides to preferentially generate immune responses against foreign antigens, whilst maintaining a state of unresponsiveness towards peripheral self. The Two-step, Two-signal Model postulates that effector

CD4+ T cells are required to activate cognate antigen-specific B cells to up modulate the expression of the costimulatory ligands that collectively lead to the generation and delivery of signal two to naïve CD4+ T helper cells (Bretscher, 1999). The other models of primary T cell activation discussed propose that non-specific antigen presenting cells, such as dendritic cells that express pattern recognition receptors for "dangerous" or "stranger" signals, are the cells that deliver signal two to naïve T cells during T cell activation (Janeway, 1989; Matzinger, 1994; Steinman & Nussenzweig, 2002). While the requirement for effector CD4+ T cells for the activation of naïve thymic T cells supports certain predictions of the Two-step, Two-signal Model, the determination of whether such a requirement also applies to peripheral naïve T cells remains a topic for investigation. In light of this uncertainty, other issues must be resolved to determine which model of primary T cell activation, if any, most validly describes the activation of naïve CD4+ T cells. A major prediction that distinguishes between the competing models is that dendritic cells can activate naïve T cells.

We designed a number of *in vitro* experiments to address whether dendritic cells are capable of stimulating the generation of immune responses from the competent naïve T cells present among thymocytes, realizing that separation of different antigen-presenting cells into separate populations may never be absolute. Our results show that when bone marrow derived dendritic cells (Inaba et al., 1992), which were thoroughly washed to remove the majority of contaminating B cells, are employed as allogeneic stimulator cells the generation of CTL and cytokine responses from naïve thymic T cells occurs minimally if at all, even when radiation

resistant effector CD4+ T cell "help" is present. The same bone marrow derived dendritic cells that fail to stimulate the generation of responses from thymocytes readily stimulate the generation of allo-MHC specific cytokine and CTL responses from splenic T cells. The latter observation leads us to suggest that the inability of dendritic cells to stimulate the generation of responses from thymocytes is unlikely to be due to the release of inhibitory cytokines and/or by the expression of inhibitory ligands, which are down-modulated upon CD40 ligation (Cella, Dohring et al., 1997; Grohmann, Fallarino, Bianchi et al., 2001; Grohmann, Fallarino, Silla et al., 2001; Powell, Jenkins, Hattori, & MacPherson, 2003). The possibility that thymocytes fail to respond because they are lacking competent CD4+ T helper cells is also unlikely because functional "help", in the form of irradiated syngeneic spleen cells, was present (Boursalian et al., 2004; Lutz, 1981).

Under the appropriate circumstances, other allogeneic spleen cells, in contrast to purified dendritic cells, readily stimulate the generation of alloresponses from thymocytes when radiation resistant CD4+ effector T helper cells are present. This discrepancy in the ability of allogeneic bone marrow derived dendritic cells, in comparison to allogeneic spleen cells depleted of T cells, to stimulate the generation of competent CD4+ and CD8+ effector cells from thymocytes prompted us to examine the fate of the differentially stimulated thymocytes. We therefore assessed whether stimulation with allogeneic bone marrow derived dendritic cells results in thymocyte proliferation, and whether T cell costimulatory molecule receptors, such as CD40L, are up modulated (Banchereau et al., 1994; Croft & Dubey, 1997). Both allogeneic spleen and dendritic cell stimulator populations stimulate thymocytes to

cycle and up-regulate the expression of CD40L. However, only thymocytes stimulated with allogeneic spleen cells depleted of T cells appear to differentiate into competent effector cytokine producing cells and CTLs. It is reasonable to speculate, given our observations, that stimulation of competent thymocytes with allogeneic bone marrow derived dendritic cells in the absence of allogeneic B cells and/or effector CD4+ T helper cells induces activation induced cell death (Schwartz, 2003; Wells et al., 1999).

According to the tenets of the Two-step, Two-signal Model, the discrepancy in the ability of the two allogeneic populations to stimulate the generation of responses from thymocytes is most likely a consequence of the absence of B cells in the former, and their presence in the latter (Bretscher, 1999). Our initial findings with T cell depleted allogeneic spleen cell stimulators, further depleted of B220+ cells, which are predominately B cells, by antibody dependent complement mediated lysis indicate that the generation of responses can be reduced when such cells are removed. In order to more conclusively examine whether B cells are necessary to stimulate the generation of responses from naïve T cells, we sorted B220+ and B220- cells from the T cell depleted allogeneic spleen by magnetic cell sorting, and used the separate fractions as allogeneic stimulators for CBA/J thymocytes and responding splenocytes. The T cell depleted, BALB/c spleen cell fraction devoid of B220 expressing cells, which harbors macrophages and the majority of splenic dendritic cell subsets, failed to optimally stimulate the generation of significant CTL and IFN-γ cytokine producing cells from competent thymocytes given irradiated "help", but did stimulate the generation of such responses from spleen cells. By

contrast, the B220+ cells stimulated the generation of responses from both thymocytes and responding splenocytes in a manner very similar to that generated upon stimulation with irradiated, T cell-depleted spleen.

To determine if the presence of B220+ cells can restore the responsiveness of thymocytes stimulated with bone marrow derived dendritic cells, we again sorted B220+ cells from the T cell depleted BALB/c spleen by magnetic cell sorting and assessed the ability of combined allogeneic populations to stimulate the generation of responses when CD4+ T cell "help" in the form of irradiated syngeneic spleen was present. We also removed immunoglobulin bearing cells (Ig) from the sorted B220+ cell fraction via panning on immunoglobulin coated plates to ensure that the B220+ cell necessary for stimulating the generation of responses is in fact a B cell. Stimulation of thymocytes with combined bone marrow derived dendritic cells, and Ig+ B220 cells restored the generation of cytokine producing cells and CTLs from thymocytes when irradiated syngeneic spleen cell "help" was present. The removal of Ig+ cells abrogated the ability of the combined allogeneic cells to stimulate the generation of alloresponses from thymocytes. Thus, in regards to the question of whether allogeneic, bone marrow derived dendritic cells can activate naïve thymic T cells in the absence of B cells, we conclude that the *in vitro* generation of alloresponses from the competent naïve T cells present among thymocytes requires the presence of an additional cell, which is an immunoglobulin bearing allogeneic B cell. According to the postulates of the Two-step, Two-signal Model, the potential requirement for a B cell for the generation of immune responses, which possesses the potential to specifically acquire and present protein antigens to naïve T cells

(Hodgkin & Basten, 1995; Lanzavecchia, 1990; Rush & Hodgkin, 2001), adds a level of control over the generation of immune responses that is lacking if the delivery of signal two occurs from a non-antigen specific antigen-presenting cell. The specific acquisition and presentation of antigens to T cells by B cells is not easily addressed in *in vitro* systems. However, in our experimental *in vitro* system, which relies on the use of alloantigens, cognate allo-MHC bearing B cells can be required for the generation of responses from naïve thymic T cells.

5.5. *In vivo* evidence to support the postulate that B cells are required for the activation of naïve antigen-specific CD4+ T cells

Literature to support the suggestion that B cells are required for the *in vivo* generation of primary Th1- and Th2-type immune responses dates back to the days of Burnet (N. L. Warner, A. Szenberg, & F. M. Burnet, 1962). At that time, it was commonplace to generate B cell deficient chickens via the early administration of testosterone (Szenberg, 1970). Such hormonal treatment blocks the development of the Bursa, and subsequently blocks B cell development (Glick et al., 1956). In his experiments, Burnet found B cell deficient chickens to lack both cell-mediated, DTH and anti-viral responses, as well as antibody responses (N. L. Warner et al., 1962).

Before the appearance of genetically modified mice, the generation of B cell deficient mice required the continuous administration of anti-IgM immunoglobulin (Gordon, 1979). This treatment results in the depletion of all B cells. In some

instances, this regime was commenced immediately after birth. Findings obtained with mice treated in this manner largely support the position that B cells play an essential role in the generation of primary immune response against protein and infections antigens (Hayglass et al., 1986; Janeway et al., 1987; Kurt-Jones et al., 1988; Ron & Sprent, 1987; Schultz et al., 1990; Schultz et al., 1995). However, the demonstration that uMT genetically modified B cell deficient mice generate primary Th1 and Th2 responses, *in vitro* and *in vivo*, against a broad array of antigens, cast considerable doubt on the proposition that B cells participate in the primary activation of naïve T cells (Epstein et al., 1995; Leef et al., 2000). The influence of the continuous administration of anti-IgM immunoglobulin on the ability to generate functional T cell responses in the previous studies became subject to question, and Burnet's findings appear to have been forgotten (Epstein et al., 1995; N. L. Warner et al., 1962).

While the uMT model of B cell deficiency appears ideal, considerable controversy developed over the use of such mice. This is primarily due to the variable ability of these mice to generate T cell responses (Epstein et al., 1995; Greeley et al., 2001; Leef et al., 2000). The finding that uMT B cell deficient mice, on backgrounds other than the original C57BL/6 strain in which they were developed, possess serum immunoglobulin of isotypes other than IgM led to the realization that genetic recombination events can occur that allow for the survival of a small number of B cells (Hasan et al., 2002; Melamed et al., 2000; Rivera et al., 2001). The ability to generate responses in such mice against some antigens and not others may therefore be the result of a randomly altered B cell repertoire.

A more complete model of B cell deficiency is the JhD B cell deficient mouse (Liu et al., 1995). Immunoglobulin recombination is not possible in JhD mice because a gene segment necessary for genetic recombination, the joining region, is missing. The generation of Th1 and Th2 immune responses, and the maintenance of memory CD4+ T helper cells, is severely impaired in these mice (Chan, Madaio, & Shlomchik, 1999; Leef et al., 2000; Linton, Harbertson, & Bradley, 2000; Liu et al., 1995; Macaulay et al., 1998). Repopulation of the B cell compartment in JhD B cell deficient mice with B cells that are either capable or incapable of secreting immunoglobulin restores immune responsiveness (Chan, Hannum et al., 1999; Chan, Madaio et al., 1999), which supports the suggestion that there is a role for B cells in primary T cell activation. The inability of passively administered immunoglobulin from responsive mice to restore the ability to generate T cell responses in JhD mice supports the latter argument (Chan, Hannum et al., 1999; Leef et al., 2000).

However, the JhD *in vivo* model of B cell deficiency may also have limitations. According to Cyster, the requirement for B cells in the generation of Th1 and Th2 immune responses in B cell deficient mice, is indirect, and is unrelated to B cell antigen presenting functions (Golovkina et al., 1999; Ngo et al., 2001). He suggests that B cells are necessary *in vivo* for the development of proper lymphoid architecture (Cyster, 2003). While B cells may be essential for proper lymphoid development *in vivo*, our *in vitro* findings support the position that B cells also play an essential antigen-presenting role in the primary activation of naïve T cells.

The cellular requirements for primary T cell activation *in vitro* may not be directly applicable to the cellular requirements *in vivo*. However, studies analyzing

the *in vivo* traffic of T cells undergoing T cell activation support the possibility that the two rare antigen specific cells the Two-step, Two-signal Model predicts to be involved in the generation of signal two in the second step, effector CD4+ T cells and cognate antigen-specific B cells (Bretscher, 1999), interact during the generation of cellular and humoral responses (Attanavanich & Kearney, 2004; Garside et al., 1998; Moser, Schaerli, & Loetscher, 2002; Schaerli, Loetscher, & Moser, 2001). For example, B cells that encounter their specific antigen migrate from the B cell areas of lymphoid organs to the marginal zones to come into close contact with T cells (Attanavanich & Kearney, 2004; Garside et al., 1998). Conversely, uncommitted CD4+ T cells producing IL-2, which express the chemokine receptor CCRX5, that have encountered antigen on antigen presenting cells such as dendritic cells, migrate from the T cell zones of the lymphoid organs into the B cell areas (Moser et al., 2002; Schaerli et al., 2001). These CD4+ T cells subsequently differentiate into either Th1 or Th2 effector T helper cells.

Collectively, *in vivo* observations support the possibility that B cells are centrally involved in the activation of naïve T cells. Conclusive experimental observations that B cells are directly required for the *in vivo* activation of naïve CD4+ T cells rather than for the development of the architecture of lymphoid organs necessary to support immune responses, is not yet available. We are currently in the process of establishing a system to address the *in vivo* requirement for B cells in primary CD4+ T cell activation.

5.6. Is there evidence that dendritic cells stimulate the activation of naïve antigen-specific CD4+ T cells?

Presently, the arguments that B cells are essential antigen presenting cells that participate in primary T cell activation, *in vitro* and *in vivo*, are controversial, and most believe that dendritic cells are the most potent activators of naïve T cells (Banchereau & Steinman, 1998; Steinman, 1991). To understand how support for the role of dendritic cells in primary T cell activation arose, one must review the discovery of dendritic cells and consider the experimental techniques employed to study their stimulatory capacity.

The belief that dendritic cells are, on a per cell basis, the most potent activators of naïve T cells emanated from a number of *in vitro* studies published shortly after the discovery that antigen-presentation by dendritic cells is maturation dependent (Cella, Sallusto et al., 1997; Girolomoni et al., 1990; Kampgen et al., 1991; Kelly et al., 1978; Klinkert et al., 1982; Koch et al., 1995; Pure et al., 1990; Romani et al., 1989; Sallusto et al., 1995; Schuler & Steinman, 1985; Steinman et al., 1975; Steinman & Cohn, 1973; Steinman & Witmer, 1978; Wilson et al., 2003; Wolff & Schreiner, 1970). It was demonstrated that allogeneic dendritic cells are 100 times more efficient at clustering and initiating the proliferation of splenic T cells than allogeneic B cells or macrophages (Steinman & Witmer, 1978).

At this time, the separation of naïve T cells from those previously activated by antigen encounter was uncommon, and the surface markers discriminating between naïve/resting memory cells and activated T cells were unknown (Dutton et

al., 1998). The procedures to purify the desired lymphoid populations, in particular, dendritic cells, were also somewhat elemental (Steinman, Kaplan, Witmer, & Cohn, 1979). Even current protocols to purify dendritic cells state that 65% purity may be acceptable for most assays (Coligan et al., 1998). Most importantly, at the time the stimulatory capacity of dendritic cells was first being analyzed, it is not clear whether a disassociation was being made between stimulation of T cell proliferation and full activation as it is today (Cunningham & Lafferty, 1974; Hawiger et al., 2001; Heckford et al., 1986; Hernandez, Aung, Marquardt, & Sherman, 2002; Kurts et al., 2001; Kurts, Kosaka et al., 1997; Townsend & Goodnow, 1998).

The advance of experimental techniques, and the realization that fractionated lymphocyte populations are not as pure as was once hoped, has led to a re-examination of a number of experimental findings regarding primary T cell activation. In most current studies, these experiments rely on the use of genetically modified mice. The use of TCR transgenic mice, knockout mice deficient in genes of immunological interest, or knockout mice lacking whole lymphocyte subsets, has become standard because of the ease by which desired lymphoid populations can be studied. Genetically modified mice are extremely valuable experimental tools. However, one must keep in mind a number of confounding issues that may influence the experimental outcome obtained when using such animals. These include a marked increase in the precursor frequency of mono-specific cells, or endogenous TCRα chain rearrangement in TCR transgenic mice that harbor wild-type Rag1 and Rag2 recombination genes, and, as is evident from the models of B cell deficiently, the inadvertent alteration of developmental process in gene knockout mice

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(Golovkina et al., 1999; Legrand & Freitas, 2001; Radu, Noben-Trauth, Hu-Li, Paul, & Bona, 2000; Smiley & Grusby, 1997; Zhou et al., 2004). Generally, also, most molecules of immunological interest that we know of have multiple functions, and so the latter gene knockout mice are intrinsically complicated.

The general consensus resulting from studies employing such mice as experimental tools appears to be that dendritic cells are the most potent stimulators of naïve T cell proliferation and activation (Croft et al., 1992; Langenkamp et al., 2002; Levin, Constant, Pasqualini, Flavell, & Bottomly, 1993; Liu, Kanzler, Soumelis, & Gilliet, 2001). The most convincing experimental finding to support the argument that dendritic cells are capable of stimulating the generation of primary immune responses from naïve T cells comes from a study in which both TCR transgenic and knockout mice were employed (Lemos, Fan, Lo, & Laufer, 2003). In this study, the generation of primary immune responses from peripheral TCR transgenic CD4+ T cells was examined in an adoptive transfer system in which mice believed to be deficient in Class II expression on all antigen-presenting cells except dendritic cells were employed as host animals. Upon antigenic challenge, the responding TCR transgenic CD4+ T cells generate cytokine and DTH responses efficiently, even though the B cells present in the host mice lack MHC Class II expression and, in theory, cannot interact with the TCR transgenic CD4+ T cells (Lemos et al., 2003). These observations at face value appear to demonstrate that presentation by dendritic cells alone can result in T cell activation and thus appear to invalidate the postulates of the Two-step, Two-signal Model and the potential

significance of the findings described in this thesis. However, two confounding issues brought about by the use of genetically modified mice require consideration.

First, similar to the majority of studies using TCR transgenic T cells, the naivety of the responding T cells in the latter study is uncertain (Smiley & Grusby, 1997). The TCR transgenic mice from which the responding CD4+ T cells were isolated harbor the Rag recombination genes. Thus, endogenous TCRα chain rearrangement is possible, and leads to the possibility that the transgenic CD4+ T cells express the desired transgenic receptor, and a hybrid receptor containing an endogenously rearranged TCRα chain and the transgene encoded β chain. Hence, the idea that the TCR transgenic CD4+ T cells are naïve because they have not been deliberately exposed to antigen can in theory be questioned because the potential exists that the hybrid T cell receptor has encountered environmental antigens (Croft et al., 1992; Zhou et al., 2004). The generation of T cells producing cytokines and DTH responses in the experimental mice may, therefore, have been obtained by activating T cells primed in the donor, which has B cells and activated T cells (Lemos et al., 2003).

Second, knowledge gained from studies of T cell proliferation under lymphopenic conditions leads one to question the completeness of the Class II deficiency in the *in vivo* model employed (Martin, Bourgeois, Dautigny, & Lucas, 2003). Using similar, I-A_βb -/- Class II deficient mice on the C57BL/6 background, which are naturally deficient in I-E MHC class II expression because of the lack of a functional E_α chain, it has been shown that homeostatic driven proliferation of adoptively transferred CD4+ T cells still occurs, albeit less efficiently (Martin et al.,

2003). Homeostatic driven proliferation of adoptively transferred CD4+ T cells can be prevented by backcrossing the I-A$_\beta$b -/- host mice to mice engineered to also be deficient in the I-E$_\beta$ chain. While the chains encoded by the I-A or the I-E regions of the MHC complex normally associate with their respective β chains, it has been suggested that a normally rare association of I-A$_\alpha$ and I-E$_\beta$ chains to form a functional class II MHC antigen occurs at a higher frequency in the I-A$_\beta$b -/- mice (de Souza et al., 2002). Expression of a hybrid I-A$_\alpha$ and I-E$_\beta$ MHC class II molecule on B cells in I-A$_\beta$b -/- mice is thus possible. Hence, whether the delivery of signal two occurs after interaction with dendritic cells or whether it occurs after encounter with antigen-specific B cells is uncertain.

The possibility that the naïve CD4+ TCR transgenic cells contain previously activated cells, in addition to the possibility that the B cells of the I-A$_\beta$b -/- mice may express a hybrid class II MHC molecule, opens up the possibility that more than recognition of "danger" or "stranger" signals by non-antigen-specific dendritic cells is required for the primary activation of naïve, precursor CD4+ T helper cells (Lemos et al., 2003). While modern experimental tools have broadened our understanding of the cells that mediate immune responses, the delineation of the cellular requirements necessary for primary T cell activation *in vivo* remain enigmatic.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The major goal of this thesis was to establish and utilize an experimental system that would allow one to begin to delineate which model of immune T cell activation most validly describes the cellular interactions required for generation of primary immune responses from naïve CD4+ T cells, whilst the generation of immune responses towards self antigens is avoided. We have obtained experimental evidence that additional cellular interactions involving B220+ Ig+ B cells can be required for full activation of naïve thymic T cells *in vitro* after encounter with antigen presented by non-specific antigen-presenting cells, as represented by dendritic cells derived from bone marrow progenitors. While the latter antigen presenting cells are efficient at stimulating the proliferation of naïve T cells, our findings show that such stimulation fails to result in the acquisition of full effector functions. We have also obtained some experimental evidence, which warrants further examination, that suggests that naïve thymic T cells, more specifically CD8+ T cells, can be rendered tolerant in an antigen specific manner by exposure to MHC-alloantigens presented by non-specific antigen presenting cells, such as bone marrow derived dendritic cells when effector CD4+ T helper cells and cognate B cells are absent.

Our results thus favor the predictions of the Two-step, Two-signal Model of T cell activation, which postulates that T cell-T cell cooperation and antigen-specific B cells are involved in the generation and delivery of the critical activation signals to precursor CD4+ T helper cells (Bretscher, 1999).

While our *in vitro* findings are supportive of this model, *in vivo* confirmation of these cellular requirements with peripheral naïve T cells and sorted antigen presenting cell populations is required before one can definitively state that the Two-step, Two-signal model most validly describes the cellular events involved in primary T cell activation, and the means by which immune responses towards peripheral self are avoided.

We are currently in the process of establishing an *in vivo* system to test whether the cellular requirements predicted by this model apply to the *in vivo* activation of naïve T cells. We plan to use a murine adoptive transfer system with lethally irradiated host animals to address whether the same antigen presenting and effector CD4+ T helper cell requirements apply to the *in vivo* activation of transferred naïve thymic T cells. Preliminary observations obtained from collaborative experiments with K.K. McKinstry are supportive of the *tro* observations presented in this thesis that B220+ cells, which are predominately B cells, are necessary for the generation of IFN-γ and IL-4 producing cells from thymocytes when mice are challenged with sheep red blood cells (SRBCs). We plan to employ this system, rather than the *in vitro* system that relies on the use of allo-MHC antigens, to assess whether antigen-specific B cells are in fact required to initiate the generation of immune responses (Rivera et al., 2001).

Confirmation of a requirement for B cells in primary T cell activation in such an *in vivo* system will provide support for the re-examination of the generation of primary immune responses from TCR transgenic CD4+ T cells in an *in vivo* system where MHC class II expression is restricted to defined subsets of antigen-presenting cells (Lemos et al., 2003). The use of TCR transgenic mice on a Rag-deficient background as naïve CD4+ T cell donors, and C57BL/6 mice as host animals that are I-A$_\beta$b -/- and I-E$_\beta$ -/- deficient on all antigen presenting cells except dendritic cells (Martin et al., 2003), will allow for a valid assessment of whether B cells are in fact required for primary T cell activation *in vivo*. Such a system will also allow for a distinction between whether the requirement for B cells *in vivo* is directly related to the delivery of the critical signals needed for the generation of immune responses or whether their role is indirectly related to the development of lymphoid architecture (Bretscher, 1999; Ngo et al., 2001).

In closing, such experiments may allow for a determination of whether the Two-step, Two-signal Model, does in fact, validly describe the cellular requirements for naïve T cell activation.

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