Chapter 1: General Introduction and Specific Aims

Introduction:

The role of the immune system is to protect the host against foreign pathogens. The foreign substances that induce an immune response are referred to as antigens. The first line of defense involves the innate immune system. One component involves phagocytic cells attacking bacterial pathogens. In addition, the skin and mucosal surfaces serve as a physical barrier to protect the host. Another defense mechanism, the acquired (specific) immunity, characteristic of the vertebrate immune system, is stimulated by exposure to foreign substances, which are distinct and specific as well as increasing in intensity upon reexposure. The immune system remembers exposure to the antigens and mounts an enhanced immune response upon the second exposure to an antigen. This allows for protective vaccination against diseases. Immunity has been seen in the ancient Chinese customs where children inhaled powders made from skin lesions of patients recovering from smallpox (Abbas et al., 1994).

Many people have contributed to forming the basis for understanding immunology. The studies by Edward Jenner showed that milkmaids who contracted cowpox were immune to smallpox. In his classic experiment he inoculated a boy with fluid from a cowpox pustule and then infected him with smallpox. Luckily for Jenner, the boy did not develop smallpox. This technique was referred to as variolation (Kuby, 1997). Louis Pasteur was another great scientist whose work with bacterium led to the discovery of vaccines using attenuated strains to protect against the disease. The mechanism behind the protection was not revealed until Emil Von Behring and Shibasaburo showed that the serum from animals previously immunized to diphtheria could be transferred to unimmunized animals and protect them from the disease (Kuby,

1997). In the 1930ís, the protective component characterized as an antitoxin, precipitin, bacteriolysin and agglutinin was termed antibody.

The immune responses can be categorized into two types, humoral immunity which is mediated by antibodies and cell-mediated immunity which is mediated by T lymphocytes. The primary defense mechanism against extracellular microbes is the humoral immunity because the antibodies can bind to them and help eliminate them. The intracellular microbes including viruses and some bacteria are not accessible by the circulating antibodies and are eliminated by the cellular immune response. Studies by Elie Metchnikoff in 1893 demonstrating the presence of phagocytes around lesions on starfish that had been stuck with thorns showed that cells responded to foreign substances (Abbas et al, 1994). Studies by Sir Almroth Wrightís in the 1900ís demonstrated a process known as opsonization where substances in the immune serum enhanced the phagocytosis of bacteria. Clear evidence for a cell-mediated immunity came in the 1950ís with studies by Mackaness showed that resistance to an intracellular bacteria can be transferred with cells but not with serum. Also in the 1950ís, the lymphocyte was identified as the cell responsible for both cellular and humoral immunity (Kuby, 1997).

Two types of cells involved in the immune response of vertebrates include the B lymphocytes, which develop into antibody-producing cells, and the T lymphocytes, which mediate the cellular immune response. The immune response can be divided into three phases, the cognitive phase, activation phase and the effector phase. In the cognitive phase, the foreign antigen binds specific receptors on mature lymphocytes. In the activation phase, the lymphocytes undergo proliferation and differentiation and in the effector phase the antigen is eliminated (Abbas et al, 1994).

Antigens are substances that bind specifically to an antibody or a T-cell receptor (Kuby, 1997). The T and B lymphocytes recognize specific sites on the antigen called epitopes. Although the B lymphocytes can recognize the epitope

by itself, the T lymphocyte needs to recognize a major histocompatibility complex (MHC) molecule on the surface of an antigen presenting cell or altered self-cell, in order to recognize an antigen (Kuby, 1997). Thus, the T and B lymphocytes recognize different components of the immune response. The two major classes of membrane molecules encoded by MHC loci code are MHC Class I and MHC Class II. Class I MHC molecules are present on all nucleated cells. Class II MHC molecules are present on antigen-presenting cells. T helper cells ($CD4^+$) recognize antigen associated with Class II MHC molecules and T cytotoxic cells ($CD8^+$) recognize antigen associated with Class I MHC molecules.

The primary lymphoid organs, the thymus and bone marrow, provide the appropriate environment for lymphocyte maturation. The thymus is the site for T cell maturation. About 95% of all thymocyte progeny undergo apoptosis (programmed cell death) in the thymus. The cells that can not recognize foreign antigen displayed by self-MHC molecules and the cells that recognize self antigens displayed by self-MHC molecules are deleted from the thymus. This cell death prevents an autoimmune reaction. Immature B cells proliferate and differentiate in the bone marrow. The secondary lymphoid organs, the spleen and lymph nodes, trap antigen and provide a place for lymphocytes to interact with antigen.

Specific Aims

It is well established that activation of T or B lymphocytes through their antigen-specific receptors leads to their differentiation and growth. In addition to the antigen-specific receptors, the T and B cells also express a variety of adhesion molecules, which are known to participate in cell-cell interaction, migration, homing and signal transduction. Also, the immune system is taught to react against foreign, "nonself" molecules. However, sometimes cells capable of responding to "self" molecules escape the screening process and generate an autoimmune response. Various effector mechanisms such as circulating autoantibodies and autoreactive T lymphocytes are responsible for tissue injury in different autoimmune diseases. There are murine models of human systemic lupus erythematosus (SLE) that provide excellent experimental models for analyzing the pathogenesis of autoimmune disease. In the current study, we wished to test the hypothesis that lymphocytes can not only be triggered through their antigen-specific receptors but also through certain adhesion molecules such as CD44. Inasmuch as, hyaluronate (HA) constitutes an important component of the extracellular matrix, our studies can provide useful information on how CD44-hyaluronate interactions can regulate the functions of lymphocytes at sites of inflammation in a variety of diseases. In addition, we wished to test the hypothesis that CD44 and hyaluronate interactions may play an important role in the development of vascular disease. The current study should provide novel information on the role of CD44 and its involvement in the development of autoimmune disease as well as possible approaches to treat human SLE. The specific aims are as follows:

1. **Role of CD44-hyaluronate interactions in the activation of lymphocytes:**

   To test the hypothesis that lymphocytes that express CD44, can undergo growth and differentiation when stimulated with

hyaluronate. We will test the mechanisms involved in CD44-induced lymphocyte activation. Lastly, we will investigate whether T and B lymphocytes respond differentially to stimulation with HA.

2. Mechanism of induction of vascular leak syndrome (VLS) following interleukin-2 administration:

To test the hypothesis that IL-2 induced VLS results from the activation of cytotoxic lymphocytes which mediate lysis of endothelial cells, using perforin and Fas Ligand..

3. Role of CD44-HA in endothelial cell lysis and vascular leak syndrome (VLS):

To investigate whether the damage to the endothelial cells seen in a variety of diseases results from CD44-hyaluronate interactions between lymphocytes and endothelial cells and devise possible approaches to prevent endothelial cell damage and induction of VLS *in vivo*.

4. Characterization of CD44-knockout mice:

To investigate whether the CD44 deficiency causes any abnormalities in the immune response, particularly the functions of cytotoxic lymphocytes.

Chapter 2: Review of Literature

I. Adhesion Molecules

Adhesion molecules play an important role in cell-cell and cell-extracellular matrix interactions (Grossi et al., 1992). Such interactions are crucial to all developmental processes (Wegner et al., 1993). Adhesion receptors of the immune system are important in regulating the mechanism of cell adhesion. In vivo, adhesion molecules guide cell interactions. The size and shape of adhesion molecules involved with the immune system have been determined by electron microscopy or X-ray crystallography. The distance between two cell membranes or a cell membrane and the extracellular matrix can be determined because the binding sites for several adhesion receptors and their counter-receptors are known (Springer, 1990). Lymphocyte adhesion receptors regulate lymphocyte antigen-specific interactions and also transmit information that affects cellular differentiation and responsiveness and interactions with the environment. There are important interactions between adhesion molecules and antigen receptors involving signaling and induction of gene expression. Adhesive interactions take place when lymphocytes have been activated by foreign antigen. These interactions will direct their localization and migration before receptors determine lymphocyte homing to different lymphoid organs and neutrophil localization in inflammation. Three families of adhesion receptors control these interactions: the immunoglobulin superfamily (includes the antigen-specific receptors of T and B lymphocytes), the integrin family (is important in dynamic regulation of adhesion and migration), and the selectins (are prominent in lymphocyte and neutrophil interaction with vascular endothelium) (Springer, 1990).

II. Significance of CD44

CD44 (also known as Pgp-1, Ly-24, extracellular matrix receptor III and Hermes) is synthesized as a 37 kDa molecule (Stamenkovic et al., 1989). CD44 is an acidic, sulfated integral membrane glycoprotein ranging in molecular weight from 80 kD up to 200 kD (Haynes et al., 1989). The gene for CD44 has recently been cloned. The sequence has revealed that the CD44 protein backbone is a 37 kDa molecule that is extensively glycosylated via N- and O-linkages, and is rich in serine and threonine residues (22%) (Stamenkovic et al., 1989). Both physically and functionally, the CD44 molecule can be separated into three main regions: the cytoplasmic domain (mediates the interaction with the cytoskeleton), the middle domain (responsible for the lymphocyte homing) and the amino-terminal domain (which binds to HA) (Underhill, 1992). The amino terminal portions of CD44 are homologous to cartilage link proteins, which promote proteoglycan- and collagen-dependent extracellular matrix adhesion. CD44 has also been shown to bind to extracellular matrix components such as hyaluronate, collagen, and fibronectin (Messadi and Bertolami, 1993). Hyaluronate (HA) is a common component of the extracellular matrix and extracellular fluid (Lesley et al., 1993). The CD44 family belongs to a larger group of HA-binding proteins, called the hyaladherins. CD44 is thought to function by mediating cell-cell or cell-substrate interactions through recognition of components of the ECM, intercellular milieu, and/or pericellular layer (Lesley et al, 1993). CD44 ligand-binding function on lymphocytes is strictly regulated, such that most CD44-expressing cells do not constitutively bind ligand (Lesley et al., 1993). There is not a one-to-one correspondence between the expression of CD44 on the cell surface and the ability to bind HA. CD44 ligand-binding functions may be activated due to differentiation, inside-out signaling, and/or extracellular stimuli (Lesley et al., 1993). The affinity for hyaluronate can be experimentally controlled and depends on the cytoplasmic domain of CD44 (Kincade, 1992).

CD44 is a diverse family of molecules produced by alternate splicing of multiple exons of a single gene and by different posttranslational modifications in different cell types (Lesley et al., 1993). The influence of these modifications on ligand-binding are not fully understood and are still being studied. In mature lymphocytes, CD44 is upregulated in response to antigenic stimuli and may participate in the effector stage of immunological responses (Lesley et al., 1993). Examination of the cDNA sequence of CD44 which showed homology between the amino-terminal portion of CD44 to chick and rat cartilage link proteins has provided evidence that CD44 has an ECM ligand. Subsequent studies have shown that HA is a ligand for CD44 (Aruffo et al., 1990). It has been reported that lymphoid cell lines (Lesley et al., 1993), B cell hybridomas (Miyake et al., 1990), and IL 5-activated B cells (Murakami et al., 1990) have all been shown to bind to purified HA and the binding can be specifically inhibited by anti-CD44 mAbs. More importantly it has been shown that the CD44 expressed on the B cell hybridoma is involved in binding to HA present on the surface of the stromal cells in vitro (Miyake et al., 1990). These results along with earlier data showing inhibition by anti-CD44 mAb's of B cell lymphopoiesis in long-term bone marrow cultures (Miyake et al., 1990) suggests that the CD44-HA interaction may be important in B cell differentiation.

CD44 is expressed by various lymphoid and nonlymphoid tissues (Haynes et al., 1989; Flanagan et al., 1989) and has been demonstrated to participate in lymphocyte adhesion to the matrix, lymph node homing and lymphopoeisis (Haynes et al., 1989; Miyake et al., 1990). Recent studies have demonstrated that the CD44 molecule may also participate in lymphocyte activation. Studies from our lab demonstrated that antibodies against CD44 can trigger the lytic activity of the cytotoxic T lymphocytes (CTL) as well as the double-negative T cells that accumulate in the MRL lpr/lpr mice (Hammond et al., 1993; Seth et al., 1991). We have also shown that the cytotoxicity induced by T cells can be inhibited in the presence of soluble HA thereby suggesting

that HA may serve as an important molecule involved in the target cell recognition by the cytotoxic T lymphocytes (unpublished observation). Similarly, monoclonal antibodies (mAbs) directed against CD44 molecules have been shown to either upregulate (Huet et al., 1989; Shimizu et al., 1989) or downregulate (Rothman et al., 1991) anti-CD3 and anti-CD2 mAb induced proliferation of T cells. Furthermore, certain anti-CD44 mAbs have also been shown to induce proliferation of resting human T cells in the absence of costimulation with anti-CD3 or anti-CD2 mAbs (Galandrini et al., 1993; Pierees et al., 1992; Dennin et al., 1990). All of these data together demonstrate that activation via CD44 can trigger effector functions in human T lymphocytes. In addition, antibodies against CD44 have also been shown to activate human monocytes and enhance the natural killer (NK) cell mediated cytotoxicity (Tan et al., 1993; Webb et al., 1990).

III. Significance of *lpr* and *gld* mutations

The murine *lpr* gene encodes for an aberrant form of Fas (CD95) a molecule involved in apoptosis. Also, the *gld* gene leads to a nonfunctional expression of Fas ligand (FasL). Mice homozygous for *lpr* and *gld* mutations develop severe lymphoproliferative and autoimmune disease similar to human systemic lupus erythematosus (SLE) characterized by the appearance of unique $CD4^{-}CD8^{-}$ (double negative,DN) T cells. The nature and functional significance of these DN T cells is not clear. These cells may represent autoreactive T cells which proliferate in response to stimulation with self antigens and are unable to undergo apoptosis and deletion *in vivo* due to lack of expression of Fas or FasL, necessary for the cells to undergo apoptosis (Hammond-McKibben et al., 1996). These cells have an unusual phenotype, particularly expression of high levels of CD44.

IV. Are cytotoxic lymphocytes involved in nonspecific vascular tissue injury seen in vivo?

Cytotoxic lymphocytes play an important role in killing virally infected cells and cancer cells and thereby provides protection to the host. Such cells include the cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Recent studies have characterized two distinct mechanisms of cytotoxicity, Fas-based and perforin-based, which appear to be independent based on the fact that cytotoxic cells from perforin knockout (ko) mice can lyse target cells using Fas-dependent pathway and *gld* cytotoxic cells can lyse by perforin pathway (Nagata and Goldstein, 1995). Perforin is a molecule similar to complement component C9, and is secreted by activated cytotoxic T cells and natural killer (NK) cells (Lee et al, 1996).

There are many disease models in which factors other than cytolytic lymphocytes have been shown to participate in endothelial cell injury, such as, neutrophils, complement components, etc. However, there is growing evidence for the involvement of cytolytic lymphocytes in endothelial cell injury. For example, IL-2 activated T cells and other leukocytes have been shown kill endothelial cells ex vivo and in vivo (Damle et al., 1987; Hammond-McKibben et al., 1995; Damle et al., 1989; Bechard et al., 1989; Fujita et al., 1991; Hammond-McKibben et al., 1995). There are several unexplained disease models in which endothelial cell damage has been reported and it is tempting to speculate that such instances, damage can result from promiscuous killing exhibited by cytotoxic cells, using CD44 receptor.

Endothelial cell injury is one of the most widely noted phenomenons in a variety of clinical diseases. Murine lymphocytic choriomeningitis (LCM) viral infection represents a well-characterized experimental infection where massive delayed-type hypersensitivity (DTH) reaction occurs in the CSF, caused by $CD8^+$ T cells (Doherty et al., 1990). The induction of inflammation and clinical symptoms of the disease can be prevented by immunosuppression, specifically inhibition of T cell responsiveness. It has been speculated that virally activated $CD8^+$ T cell which express high levels of CD44, kill endothelial cells leading to

massive extravasation of monocytes and $CD4^+$ T cells in the subarachnoid space. In experimental allergic encephalomyelitis, a model for human multiple sclerosis, damage to the blood-brain barrier has been reported to occur, following injury to the endothelial cells, the exact mechanism of which is not known. This facilitates infiltration of $CD4^+$ T cells into the CNS. CD44-HA interactions have been shown to mediate lymphocyte adhesion to the white matter thereby contributing towards the pathogenesis of inflammation of CNS (Aho et al., 1994). Moreover, in autoimmune disease models involving vasculitides, the lesions have been associated with infiltration of lymphocytes and macrophages at the vascular wall structure (Moyer et al., 1984). Such types of vasculitis have been described in human autoimmune disease (McCluskey and Feinberg, 1983) as well as in the mouse models (Hewicker and Trautwein, 1987). Also, lymphocytes activated with cultured endothelial cells have been shown to induce experimental autoimmune type of vasculitis (Hart, 1983). The T cell involvement in the endothelial cell damage leading to vascular disease in scleroderma has also been described (Kahaleh, 1990). The peripheral blood lymphocytes from some patients with rheumatoid arthritis and giant cell arthritidis have been shown to be cytotoxic to endothelial cells but not to fibroblasts (Blann and Scott, 1991). Similarly, in arteriosclerosis, endothelial cell damage and inflammatory cell activation have been shown to contribute to the further development of cardiovascular disease (Van Hinsbergh, 1992; vanderWal et al., 1992). The endothelial cells lining the inflamed vessels have been shown to play an important role in initiating chronic rejection of the graft, by the NK cells (Inverardi and Pardi, 1994). Accelerated arteriosclerosis is a major complication in cardiac transplantation, caused in part by the rejection related cell-mediated immune response. Furthermore, examination of coronary arteries has revealed the presence of CTL expressing perforin (Fox et al., 1993). Donor heart endothelial cells have been shown to act as targets for infiltrating lymphocytes of the host after clinical cardiac

transplantation (Inverardi and Pardi, 1994; Jutte et al., 1993). Acute lethal GVH reaction can be induced using the T cells that can trigger VLS causing death of the recipient (Lehmann et al., 1990). All such examples stress the possible participation of cytolytic lymphocytes in endothelial cell damage.

V. Is CD44 Involved in the Induction of Vascular Leak Syndrome (VLS)?

At sites of chronic inflammation as seen in certain infections, autoimmune diseases, allograft refection, GVH disease and treatment of cancer patients with high doses of IL-2, significant damage to the endothelial cells has been noted, although the mechanism of such endothelial cell damage is not understood. The destruction of endothelial cells leads to loss of vascular fluid, edema in various organs and often death. This pathological condition is called vascular leak syndrome (VLS). VLS is seen only in immunocompetent but not in nude or immunodeficient mice receiving IL-2 (Rosenstein et al., 1986; Ettinghausen et al., 1988). Also, the toxicity associated with IL-2 therapy has been shown to decrease after depletion of NK/LAK cells *in vivo* (Peace and Cheever, 1989).

The role of CTL in VLS induction was demonstrated in earlier studies from our lab in which it was noted that administration of a CTL clone and IL-2 into irradiated syngeneic mice but not the CTL clone or IL-2 alone, triggered VLS (Hammond-McKibben et al., 1995). Immunotherapy using such cells caused damage to the vascular endothelial cells and led to pulmonary edema and respiratory problems. Also, IL-2 activated CTL clone could mediate efficient lysis of an endothelial cell line but not a fibroblast cell line, in a MHC-unrestricted fashion (Hammond-McKibben et al., 1995). Such studies together suggest that IL-2 induced VLS may result from the direct cytotoxicity of endothelial cells by LAK cells. We and others have shown earlier that CTL, double-negative T cells and NK cells upon activation express high levels of CD44 and mediate efficient MHC-unrestricted TCR-independent lysis following

ligation of CD44 (Seth et al., 1991; Galandrini et al., 1993; Tan et al., 1993; Hammond-McKibben et al., 1995; Hammond-McKibben et al., 1996; Sconocchia et al., 1994). We have also demonstrated that the lysis of endothelial cells *ex vivo* by cytolytic lymphocytes can be blocked by soluble CD44 fusion protein, anti-CD44 Fab fragments or soluble hyaluronate (unpublished data). These data suggested that CD44-hyaluronate interactions may play an important role in the migration and homing of lymphocytes as well as sometimes lysis of endothelial cells. The interaction between lymphocytes and endothelial cells plays a crucial role in the extravasation of lymphocytes to sites of infection or tumor growth. This interaction is carefully regulated. In the current study, we wish to test the hypothesis that dysregulation in CD44-hyaluronate interactions can lead to nonspecific killing of endothelial cells by cytotoxic lymphocytes.

We have proposed that VLS results from the dysregulated interaction between CD44 expressed on cytotoxic T cells and LAK cells and hyaluronate expressed on endothelial cells leading to endothelial cell injury. Future studies will investigate whether preventing the interaction between CD44 and its ligand on the endothelial cells will inhibit the damage to these endothelial cells. The results of these studies may offer new avenues in the treatment of a range of diseases where endothelial cell damage occurs.