The Role of CD44 in Concanavalin A-Induced Hepatitis

By

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(Abstract)

Administration of Concanavalin-A (Con A) induces severe injury to the hepatocytes in mice and is considered to be a model for human hepatitis. In the current study, we investigated the role of CD44 in Con A induced hepatitis. Although immune cells have been identified as the causative agent of Con A-induced hepatitis, the exact mechanism of pathogenesis remains unclear. When Con A was injected into CD44 wild type (WT) mice, it induced hepatitis as evident from increased plasma aspartate aminotransferase (AST) levels accompanied by active infiltration of mononuclear cells in the liver and significant induction of apoptosis. Interestingly, Con A injected C57BL/6 CD44-knockout (KO) mice exhibited increased hepatitis with higher levels of apoptosis in the liver and increased plasma AST levels when compared to the CD44 WT mice. Also, transfer of T cells from Con A injected CD44-KO mice into CD44 WT mice induced higher levels of hepatitis when compared to transfer of similar cells from CD44 WT mice into CD44 WT mice. The increased hepatitis seen in CD44-KO mice was partially due to increased production of cytokines such as TNF-α, IL-2 and IFN-γ, but not Fas or FasL. Also, it was not caused by altered presence of T cell subsets. The increased susceptibility of CD44 KO mice to hepatitis correlated with increased resistance of T cells from CD44 KO mice to undergo

apoptosis when compared to the CD44 WT mice. Together, these data demonstrate that activated T cells use CD44 to undergo apoptosis, and dysregulation in this pathway could lead to increased pathogenesis in a number of diseases, including hepatitis.

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List of Abbreviations

Fas	Fas receptor
FasL	Fas Ligand
PKO	Perforin knock-out
CTL	Cytotoxic T lymphocyte
APC	Antigen presenting cells
bp	Base pair
CD	Cluster of differentiation
Con A	Concanavalin A
FITC	Fluorescein isothiocyanate
IL-2	Interleukin-2
LAK	Lymphokine activated cells
NK	Natural killer cells
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
RT-PCR	Reverse trancribed PCR
TCR	T cell receptor
TUNEL	Terminal transferase mediated dUTP nick end labeling
sFasL	Soluble form of Fas ligand
mFasL	Membrane bound Fas ligand
H&E	Hemotoxylin and eosin
DN	Double negative

Chapter 1: Introduction and Specific Aim

Introduction:

Immunology is a science that involves study of the immune system. The main role of the immune system is to protect the host from infections and development of cancer. The immune system consists of organs, cells, and molecules. Immunity is divided into two groups: innate and acquired. Innate immunity includes the anatomical and physiological barriers, and phagocytic cells, including macrophages, monocytes, neutrophils, basophils, and eosinophils. Even though phagocytic cells do not have memory, they become more active after subsequent exposure to the same pathogen. In contrast to the innate immunity, acquired immunity has specificity, diversity, memory, and self/non-self recognition. The immune system is so specific that it is capable of distinguishing a single mutation, or minor differences between two molecules. Because of this specificity, the immune system can also generate enormous diversity to respond to any molecule found in nature or artificially synthesized. Cells involved in the specific immune system are educated in such a way that they can distinguish self molecules from non-self or foreign molecules. Therefore, immune responses are only generated against non-self molecules, and after secondary exposure to the same molecules, a much stronger reaction is produced in a short time (Kuby, 1994; Abbas et al., 1994; Austyn Wood, 1994).

B and T lymphocytes are the predominant cells in the acquired immune system and originate from stem cells found in the bone morrow. B cells stay in bone morrow until they become mature. When mature B cells leave the bone morrow, they express a unique antigen specific receptor, a membrane bound antibody. These receptors include IgD and IgM molecules.

B cell receptors only recognize soluble antigens preserved in their native form. Therefore, degraded proteins are not recognized by B cells in conventional ways. However, the production of antibodies against double stranded DNA indicated that these cells can respond to many molecules, including lipid and carbohydrates. Following exposure to antigens, B cells undergo immunoglobulin class-switching and differentiate into memory B and plasma cells. Plasma cells actively secrete one of five classes of antibodies. Plasma cells in their short life span produce a large number of antibodies, which have a single antigenic specificity. IgG is the most common antibody found in the serum. IgA is involved in mucosal immunity, and therefore when IgAs are passing through the epithelial cells, they are further modified to become a dimer. Thus, secreted IgA molecules exist as dimers. IgE binds to the Fc receptor on mast cells and is associated with allergies (Charles et al. 1997).

In contrast to the B cells, some stem cells migrate to the thymus in response to the chemokines secreted by stromal cells, which are located in the thymus. As they interact with cells and molecules in the thymus, T cells differentiate by expressing unique receptors, T-cell receptor (TCR), and CD3, a co-receptor. The TCR is a heterodimer, consisting of either $\alpha\beta$ or $\gamma\delta$ polypeptide chains. The majority of T cells express $\alpha\beta$-TCR and only 3-5 % of T cells carry the $\gamma\delta$ TCR. Following the expression of TCR and CD3 receptor, the T cells differentiate into two types. Those T cells, which express CD8 molecules, are known as cytotoxic T lymphocytes (CTLs), and those T cells that express CD4 molecules are designated as T helper cells. During the subsequent maturation stages in thymus, most self antigens are processed and presented in association of Major Histocompatability Complex (MHC) to the T cells. T cells, which can not

recognize MHC and self-molecules or respond strongly to the self-molecules, are eliminated through apoptosis. Mature self-restricted $CD4^+$ or $CD8^+$ T cells are released into the circulation.

The sources of the antigen determine their processing and presention pathways. Proteins expressed in the nucleus, or viral antigens, are endogenous antigens. These molecules are processed by antigen presenting cells (APC) and presented by MHC class I molecules. Such complexes are recognized by $CD8^+$ T cells. Other kinds of antigens enter the cells from outside. Such antigens are known as exogenous antigens. The exogenous antigens are presented in association with MHC class II molecules and activate the $CD4^+$ T helper cells.

The expression and distribution of Class I and Class II MHC encoded molecules are different. Class I MHC molecules are expressed on nucleated cells so that $CD8^+$ T cells can perform immunosurveillance. However, class II MHC molecules are more restricted to the professional antigen presenting cells (APC) including, dendritic cells, macrophages, monocytes, and B cells.

In response to an antigen, both CD4 and CD8 T cells are activated, but outcomes of the activation are different. CD4 T cells start secreting a variety of cytokines that stimulate cells involved in innate and acquired immunity, whereas activated CD8 T cells instead upregulate the expression of cytolytic genes used for killing infected cells or cancer cells.

Immune responses generated by B cells or antibodies are known as the humoral immune response, whereas cell-mediated immunity results from activation of T cells. To generate

efficient immunity to infections or cancer, the cells and molecules involved in innate, humoral, and cell-mediated immunity have to closely work.

Significance of Con A-induced Mice Hepatitis

Chronic hepatic diseases such as chronic hepatitis and liver cirrhosis are major causes of morbidity and mortality. It is known that hepatitis B virus (HBV) and hepatitis C virus infection may favor the development of both chronic hepatitis and hepatocellular carcinoma (HCC) (Beasley RP. Et al., 1981). There is evidence that HCC occurs in cirrhotic liver, and that chronic hepatitis is a prerequisite for HCC development. There is also evidence that cell-mediated immune phenomena directed against viral determinants on hepatocytes (Mondelli M, et al., 1982), and probably driven by cytotoxic T lymphocytes (Chisari FV. Et al., 1989), may be implicated in HBV-induced hapatocellular injury.

Animal models of human diseases provide suitable tools for the better understanding of pathogenic mechanisms and may lead to insights into novel therapeutic approaches for the clinical setting. Recently, a new experimental model of hepatitis has been described which can be induced in mice by a single i.v. injection of Concanavalin A (Tiegs G. et al., 1992). Unlike other commonly used models of immunoinflammarory liver injuries such as lipopolysaccharide (LPS)-induced hepatitis and the autoimmune hepatitis provoked by immunization with syngeneic liver antigens (Lohsi AW. Et al. 1994) both of which require the use of hepatotoxic agents (D-galactosamine and Freund's complete adjuvant, respectively) for disease induction (Decker K. et al., 1974), the sole injection of Con A is sufficient for liver lesions to develop. Within 8-24 h,

clinical and histological evidence of hepatitis occurs with elevation of transaminase activities (AST) in the plasma and hepatic lesions characterized by massive granulocyte accumulation and hepatic necrosis (Tiegs G. et al., 1992). Con A-induced hepatitis is both T cell- and macrophage-dependent; it can not be induced in nude athymic mice lacking immunocompetent T cells, and it is prevented by anti- T cell immunosuppresants such as cyclosporin A (CsA) and FK506, or by blockade of macrophages functions with silica particles (Tiegs G. et al., 1992).

The precise mechanism(s) by which T cells and macrophages exert their hepatogenic potential is not know. Because a massive release of macrophage and T cell-derived cytokines IL-2, IL-1, IL-6, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) occurs with different kinetics in response to Con A, a role has been envisaged for these cytokines in the development of the hepatic lesions (Mizuhara et al. 1994). Nonetheless, the role of cytokines in the pathogenesis of this immunoinflammatory condition remains to be defined.

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 mAbs 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). Recently, some studies in our lab have also shown that CD44 deficient T cells are resistant to induction of apoptosis. All of these data together demonstrate that activation via CD44 can trigger effector functions in human T lymphocytes and also help activated T cells undergo apoptosis to maintain homeostasis.

Significance of Cell Death

Cell death is a natural and necessary process observed in all living organisms and plays an important role in development, defense, aging, and homeostasis (Vaux and Strasser 1996). Cell death in bacteria occurs as suicide when they are infected by phages so that phages are not spread to the other bacteria. For the same reason, virus infected T cells also kill themselves, but the mechanisms used for suicide in bacteria and in T or vertebrate cells are totally different (Shub, 1994).

Cell death in vertebrates is developmentally regulated. For example, during the development of the nervous system in the fetus as many as 85 % of the developing neurons, 95% of T cells in thymus (Horvitz et al., 1994), and 80% of B cells in bone morrow, die even without leaving the primary site. The webbed structure of duck foot is due to the lack of cell death (Gilbert, 1988). Mammary development seen in females is not common in male because testosterone secretion in male ceased the mammary development by upregulating apoptosis at

this site (Gilbert, 1988). Also, steady state levels of cells is so tightly regulated that the number of the cells stays constant. For example, every day at a given time a person carries $50x10^9$ neutrophils in the circulation and at the end of the average one day life span, they are eliminated by apoptosis (Cohen et al., 1992). During infection, an individual may need large numbers of immune cells. Following the clearing of the infection, those activated cells are killed, otherwise they may pose a danger to self tissues.

There are two kinds of cell death seen in mammals: Necrosis and Apoptosis. Necrosis is initiated by overwhelming cellular injury resulting in swelling, bursting of cells, and finally releasing of cytolytic granules. These cytotoxic molecules cause inflammation, redness and pain (Steller, 1995; Kuby, 1995). In contrast, apoptosis is induced by gene products and characterized by relatively intact membrane, cell shrinkage, chromosomal condensation, cleavage of DNA into oligonucleosmal fragments, and packaging of the cytoplasmic contents into small granules. After undergoing apoptosis, cells are immediately phagocytosed by macrophages and thus, intracellular molecules are not released, and no inflammation occurs.

Apoptosis or programmed cell dead is programmed in each individual cell and encoded by genes or gene families of the host, including hormones, receptors, ligands, and cytolytic molecules. The apoptosis program can be triggered by many intrinsic or extrinsic signals, such as starvation, infection by virus, radiation, aging, hormones, drugs, stress, alcohol etc., leading to activation of internal death signals.

Many life threatening diseases today are associated with either inhibited or excessive apoptosis. Cancer, including breast, prostate and ovarian cancer, follicular lymphoma, hormone-driven tumor, autoimmune diseases, and viral infection are characterised by accumulation of cells resulting from either increased proliferation or failure of cells to undergo apoptosis in response to appropriate stimuli (Thomson, 1995; Hsu et al., 1994). Diseases resulting from increased apoptosis include AIDS, neuron damage after stroke and other ischemic injury, Alzheimer's disease, Parkinson's disease, cerebellar degeneration, toxin induced liver disease and alcohol mediated liver destruction (Thomson, 1995; Barinaga, 1998).

Programmed cell death is also important for establishing and maintaining a healthy immune system. Premature T cells migrate to the thymus and begin to rearrange T-cell receptor (TCR) receptor genes. $CD4^+CD8^+$ T cells that carry a productive TCR gene are further selected. Those T cells that have high affinity to self MHC and self antigens associated with MHC are eliminated by negative selection. Mature single positive T cells ($CD4^+$ or $CD8^+$) are released into the circulation where T cells are further selected in the periphery based on the recognition of the self-antigens. During the immune response, the number of $CD4^+$ and $CD8^+$ T lymphocytes increase so that they can attack the foreign invader efficiently. Some of the activated T cells become memory cells and rest of them are removed from the immune system, otherwise, they keep secreting cytokines that could be toxic to the host. Those T cells that do not have a productive TCR or fail to recognize self MHC, or carry TCR receptors that show high affinity to self antigens in association with MHC molecules are removed by apoptosis.

B cells are also selected under the same pressure and those B cells that fail one of these steps are removed by programmed cell death. The consequence of failing to undergo apoptosis in one of these steps is the development of the autoimmune diseases. Rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, autoimmune diabetes, and other autoimmune diseases are associated with inhibition of apoptosis (Kuby, 1995; Thompson, 1995). *Lpr* and *gld* mice are good examples to demonstrate the failing of apoptosis and the development of autoimmune diseases. The defect in Fas or Fas ligand in *lpr* and gld mice (Nagata, 1995), respectively, results in accumulation of double negative T cells in secondary lymphoid organs and in developing a disease similar to human lupus. The detection of higher levels of circulating auto-reactive antibodies in *lpr* and *gld* mice indicates the existence of large number of autoreactive B cells. The impairing of Fas-mediated apoptosis by deletion in the cytoplasmic domain of Fas (Rieux-Laucat et al., 1995) or by soluble Fas (Cheng et al., 1994) was associated with human lymphoproliferative syndrome and systemic lupus erythematosus. These data suggest that apoptosis, particularly Fas-mediated, is important for development and maintaining immune system homeostasis. The inhibition or downregulation of cell death leads to deleterious autoimmune diseases and cancer.

To prevent the propagation of virus, infected cells commit suicide by apoptosis or are killed by cytotoxic T cells following the presentation of viral proteins on host MHC class I molecules. Viruses depend on host cells to produce their viral proteins, and therefore they develop a mechanism to interfere with apoptotic pathway in infected cells. The viral gene products E1B or BHRF1 from adenovirus and Epstein-Barr virus, respectively were shown to block apoptosis using similar mechanisms as used by bcl-2 (Thompson, 1994). The CrmA, a

protease inhibitor from cowpox virus, disrupts apoptosis by inhibiting ICE, interleukin-1β-converting enzymes or cysteine proteases (Thompson, 1994). Some other viral products from Estein-Bar virus or Chronic Sindbis virus promote the survival of cells by upregulating bcl-2 expression (Thompson, 1994). These data demonstrated that inhibition of apoptosis in infected cells causes the establishment of viral colonies or latencies in the host. In contrast to other viruses, HIV infects and causes depletion of the CD4+ T cells by inducing apoptosis.

It is believed that several neurological diseases including Alzheimer's diseases, Parkinson's diseases, spinal mascular atrophy, and cerebellar degeneration are caused by apoptosis of neurons (Thompson, 1994). Furthermore, the morphologic characteristics of dying cells after stroke were found to be similar to apoptosis observed in normal cells. These data suggest that excessive cell death can be reduced with caspase inhibitors following the stroke.

Programmed cell death can be divided into two phases: transferring extracullular signals into the cells and activation of caspases in mammals or ced genes in *C. elegans*. Activation of caspases is sufficient for a cell to commit suicide. However, for delivering the cell mediated apoptosis, which is important for the immunotherapy of cancer, cell to cell contact provided by receptors and ligands, is necessary. Cytotoxic T cells use two major pathways to kill targets cells: Perforin and Fas ligand.

Significance:

This study will identify the role played by CD44 in the induction of hepatitis. Therefore it will provide useful information on the role of CD44 in hepatitis and may provide appropriate treatment modalities to prevent the development of hepatitis.

Specific Aim:

The exact mechanism by which Con A triggers hepatitis is not clear. In the current study, we investigated the role of CD44 in hepatitis induction. Since CD44 is involved in lymphocyte migration and activation as well as to mediate cytotoxicity, one can expect that CD44 KO mice may exhibit decreased hepatitis following Con A injection when compared to the WT mice. On the other hand, CD44 has also been shown to play a role in activation-inducted apoptosis. Thus, $CD44^+$ cells upon activation with Con A may undergo increased apoptosis when compared to the CD44-deficient cells. In such an instance, we may find that CD44 KO mice may exhibit increased hepatitis when compared to the CD44 WT mice. In this case, CD44 will provide protection in Con A-induced hepatitis by inducing activation-induced cell death in lymphocytes. These studies therefore provide useful information on the role of CD44 in hepatitis and may provide appropriate treatment modalities to clinically prevent the development of hepatitis.

Aim:

1. Identify the role played by CD44 in the Con A-induced hepatitis model.

2. To investigate whether Con A-induced hepatitis is triggered by T cells inducing apoptosis in hepatocytes.

3. To test whether Con A induced hepatitis can be blocked by mAbs against CD44.

Chapter 2: The Role of CD44 in Concanavalin A-Induced Hepatitis

Abstract:

Administration of Con A induces severe injury to the hepatocytes in mice and is considered to be a model for human hepatitis. In the current study we investigated the role of CD44 in Concanavalin-A (Con A) induced hepatitis. Although immune cells have been identified as the causative agent of Con A-induced hepatitis, the exact mechanism of pathogenesis remains unclear. When Con A was injected into CD44 wild type (WT) mice, it induced hepatitis as evident from increased plasma aspartate aminotransferase activity (AST) levels accompanied by active infiltration of mononuclear cells in the liver and significant induction of apoptosis. Interestingly, Con A injected C57BL/6 CD44-knockout (KO) mice exhibited increased hepatitis with higher levels of apoptosis in the liver and increased plasma AST levels when compared to the CD44 WT mice. Also, transfer of T cells from Con A injected CD44-KO mice into CD44 WT mice induced higher levels of hepatitis when compared to transfer of similar cells from CD44 WT mice into CD44 WT mice. The increased hepatitis seen in CD44-KO mice was partially due to increased production of cytokines such as TNF-α, IL-2 and IFN-γ, but not Fas or FasL. Also, it was not caused by altered presence of T cell subsets. The increased susceptibility of CD44 KO mice to hepatitis correlated with increased resistance of T cells from CD44 KO mice to undergo apoptosis when compared to the CD44 WT mice. Together, these data demonstrate that activated T cells use CD44 to undergo apoptosis, and dysregulation in this pathway could lead to increased pathogenesis in a number of diseases, including hepatitis.

Introduction

Recently, a new experimental model of hepatitis has been described which can be induced in mice by a single *i.v.* injection of Concanavalin A (Con A) (1). Unlike other commonly used models of immunoinflammatory liver injury such as lipopolysaccharide (LPS)-induced hepatitis and the autoimmune hepatitis provoked by immunization with syngeneic liver antigens (2), which require the use of hepatotoxic agents (D-galactosamine and Freund's complete adjuvant, respectively) for disease induction (3), the sole injection of Con A is sufficient for liver lesions to develop. Within 8-24h, clinical and histological evidence of hepatitis occurs with elevation of transaminase activities in the plasma and hepatic lesions characterized by massive granulocyte accumulation and hepatic necrosis (1). Thus, Con A-induced hepatitis is considered to be an experimental model for human autoimmune hepatitis (4). Con A-induced hepatitis is both T cell- and macrophage-dependent inasmuch as it can not be induced in nude athymic mice lacking immunocompetent T cells, and it is prevented by immunosuppressants such as cyclosporin A (CsA) and FK506, or by blockade of macrophage functions with silica particles (1).

The precise mechanism(s) by which T cells and macrophages induce hepatitis is not known. Because a massive release of macrophage and T cell-derived cytokines (IL-2, IL-1, IL-6, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) occurs with different kinetics in response to Con A, a role has been envisaged for these cytokines in the development of the hepatic lesions (5).

Nonetheless, the precise role of cytokines in the pathogenesis of this immunoinflammatory condition remains to be defined.

CD44 is an acidic, sulfated integral membrane glycoprotein ranging in molecular weight from 80 kD up to 200 kD (7). CD44 is expressed by various lymphoid and nonlymphoid tissues (7,8). The CD44 molecule has been demonstrated to participate in lymphocyte adhesion to the matrix, lymph node homing and lymphopoeisis (7,9). Studies from our laboratory have shown that T cells upon activation express increased levels of CD44, which can act as a signaling molecule in effector functions such as induction of cytotoxicity. Similarly, mAbs against CD44 were shown to induce B cell growth and differentiation (10) Other studies have shown that monoclonal antibodies (mAbs) directed against CD44 molecules can either upregulate (11,13) or down regulate (12) anti-CD3 and anti-CD2 mAb induced proliferation of T cells. Recent studies from our laboratory also demonstrated that interleukin-2 (IL-2) induced vascular leak and damage to the endothelial cells is mediated by CD44 (10) Together, the above studies demonstrate that CD44 expressed on activated lymphocytes participate in cell injury leading to autoimmune reactions. In the current study, we wished to test this hypothesis in Con A-induced hepatitis model. Interestingly, using CD44 KO mice we observed that CD44 expression on the lymphocytes was responsible for enhanced pathogenesis in Con A-induced hepatitis. The enhanced hepatitis resulted from inability of Con A-activated CD44-defcient T cells to undergo apoptosis.

Materials and Methods

Mice

Adult female C57BL/6 (CD44 wild type) mice were purchased from the National Institutes of Health (Bethesda, MD). CD44-knockout mice on C57BL/6 background were obtained from AMGEN Institute (Toronto, Ontario) and bred in our animal facilities and screened for the CD44 mutation. The phenotype of these mice has been described elsewhere (10,13).

Con-A induced hepatitis and its evaluation

To induce hepatitis, female CD44 wild type (WT) and CD44 knock out (KO) mice weighing 20-23 g were challenged with Con A (12mg/kg body weight, *i.v.* in 100 µl of saline). Control mice received 100µl saline(*i.v.*). Plasma from individual mice was separated from blood obtained through the sinus orbital under anesthesia with IsoFlo (Abbott Laboratories, North Chicago, IL) at various time intervals after Con A injection. Plasma aspartate aminotransferase activity (AST) levels in the plasma were measured using a commercial kit (Sigma Chemical, St. Louis, MO).

For histopathological studies, the harvested livers were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections were affixed to slides, deparaffinized and stained with hematoxylin and eosin to assess morphologic changes, as described (10)

Adoptive cell transfer

Groups of 5 female CD44 WT and CD44 KO mice were challenged with Con A (12mg/kg body weight, *i.v.* in 100 μl of saline). Control mice received 100μl saline. Twenty four hours later, the spleen cells were harvested and washed with PBS. The red blood cells were lysed and the spleen cells were washed three times using PBS before adoptive transfer. Next, spleen cells (1×10^8 cells by i.v. route) from CD44 WT or CD44 KO mice were adoptively transferred into CD44 WT or CD44 KO mice. Plasma from individual mice was obtained through the sinus orbitalis under anesthesia 24 hours after the adoptive transfer. Plasma AST levels were measured using a commercial kit as described above.

T cells

At different time points after Con A injection, spleen cells were harvested. T cells from spleen were purified using nylon wool. Next, T cells were studied for apoptosis using TUNEL assay as described (14). The cells were fixed with 4% p-formaldehyde for 30 min at room temperature. In some assays, T cells purified from normal CD44 WT mice were cultured with Con A (5μg/ml) *in vitro* for 24 hours. Then T cells were harvest and fixed with 4% p-formaldehyde for TUNEL assay. Briefly, the T cells were washed with PBS, permeabilized on ice for 2 min, and incubated with FITC-dUTP for 1 hour at 37^{o}C. Fluorescence of the cells was measured by flow cytometry as previously described (14). The analysis was performed by a Counter Epics V flow cytometer (Miami, FL). Five thousand cells were analyzed per sample.

Isolation of lymphocyte infiltrating the liver

The isolation of liver infiltrating lymphocytes was carried out as described by Hisami Watanabe et al. (1991). Briefly, the liver obtained 16 hour after Con A injection was pressed through a 200-gauge stainless steel mesh (17) and suspended in RPMI medium supplemented with 5% fetal calf serum (FCS). After one washing with the medium, the cells were resuspended in 30 ml of medium and infiltrating lymphocytes were separated form parenchymal hepatocytes and kupffer cells by ficoll-isopaque density (1.09) gradient centrifugation. The cell suspension (35ml) was overlaid on 15 ml of the ficoll-isopaque in a 50 ml conical plastic tube. Centrifugation was performed at 2500 rpm for 30 min at room temperature. After centrifugation, 10 ml of the interface was aspirated, mixed with 20 ml of the medium in a 50 ml conical tube and then washed twice.

Flow cytometry to detect cell surface markers

CD44 WT and CD44 KO mice were injected with Con A (12mg/kg body weight, *i.v.* in 100 μl of saline). Twenty four hours later, spleen cells were harvested and the lymphocyte population was screened for various cell surface markers using flow cytometry. Briefly, 1×10^6 spleen cells were incubated with Fc block (Pharmingen, San Diego, CA) follow by culture with PE-conjugated anti-mouse CD3, anti-mouse CD8, anti-mouse CD4, anti-mouse Mac3, or anti-CD45/B220 mAbs (Pharmingen, San Diego, CA) on ice for 30 minutes. The cells were washed with PBS three times and analyzed for fluorescence using Counter Epics V flow cytometer (16).

Detection of Fas, Fas Ligand, TNF-α, IFN-γ, and IL-2 expression using semi-automatic quantitative RT-PCR

RT-PCR was carried out to detect Fas, FasL, TNF-α, IFN-γ, and IL-2 gene expression in a similar way as previously described (19). The primers used were as follows: Fas primer, 5'-GCACAGAAGGGAAGGAGTAC-3' and 5'GTCTTCAGCAATTCTCGGGA-3'; FasL primer, 5'-GAGAAGGAAACCCTTTCCTG-3' and 5'- ATATTCCTGGTGCCCATGAT-3'; IFN-γ primer, 5'-TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC-3' and 5'-TGGACCTGTGGG TTGTTGACCTCAAACTTGGC-3'; TNF-α primer, 5'TTCTGTCTACTGAACTTCGGGGT GATCGGTCC-3' and 5'-GTATGAGATAGCAAATCTGGCTGACGGTGTGGG-3'; IL-2 primer, 5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG-3' and 5'-GACAGAAGGCTATCCAT CTCCTCAGAAAGTCC-3'; Mouse β-actin primer, 5'-ATCCTGACCCTGAACTACCCCATT-3'; and, 5' GCACTGTAGTTTCTCTTCGACACGA-3'. PCR amplification products were visualized in 1.5% agarose gels after staining with ethidium bromide. The density of the PCR products for various cytokines was compared to β-actin used as an internal control. Briefly, the spot density of each band in the gel was measured using AlphaImager™2000 digital imaging system. Then, the percentage(density of cytokine products divided by density of β-actin) was calculated.

Detection of apoptosis using TUNEL method

At different time points following Con A injection, one million purified freshly isolated T cells or T cells from normal mice cultured with Con A as described earlier were harvested and washed with PBS twice. Next, the cells were fixed with 4% p-formaldehyde for 30 min at room temperature. DNA

strand breaks were detected by TdT-mediated nick end-labeling, referred to as TUNEL assay (Boehringer Mannnheim, Indianapolis, IN), as described (14). Briefly, the T cells were washed with PBS , permeabilized on ice for 2 min, and incubated with FITC-dUTP for 1 hour at 37^{o}C. Fluorescence of the cells was measured by flow cytometry as previously described (14). The analysis was performed by a Counter Epics V flow cytometer (Miami, FL). Five thousand cells were analyzed per sample.

For *in situ* apoptosis detection, liver from control and Con A-injected mice were aseptically removed and fixed in 10% neutral formalin solution. Five μm paraffin-embedded sections were adhered to slides. Deparafinization was done by heating the sections at 60^{o}C for 25 min. Rehydration was carried out by transferring the slides through the following solutions: twice in xylene for 5 min, twice in 95% ethanol for 5 min, twice in 70% ethanol for 5 min, and 10 min in distilled water. The tissues were treated with 20 μg/ml proteinase K (Sigma, St Louis, MO) in 10 mmol Tris-HCL, pH 8 for 30 min at 37 ^{o}C and then washed with PBS twice for 10 min. Endogenous alkaline-phosphatase were inactivated by treating the slides with 10 mMol levamisole (Sigma, St Louis, MO) at room temperature for 1 hour. The sections were washed with PBS for 10 min and covered with TdT-FITC-dUTP enzyme-labeling solution and then incubated at 37 ^{o}C in a humidified incubator for 1.5 hours. The slides were rinsed for 10 min in PBS and covered with alkaline phosphatase converter solution. Following 1hour incubation, the slides were washed twice with PBS for 10 min and BM purple substrate (BCIP/NBT) was added. Dark-purple color was visible in 15-25 min. The slides were washed, counter-stained with eosin and cover-slip was placed on mounting media. The nuclear staining was evaluated under a light microscope. Identical slides were also stained with hematoxylin-eosin (H&E) to detect lymphocyte infiltration and to evaluate tissue structure.

Statistical Analysis

The statistical comparison between experimental and control groups was carried out using Students t-test and $p < 0.05$ was considered to be significant.

Result:

Nature of hapatocellular cell damage in Con A-induced hepatitis

To investigate the nature of hepatocellular cell damage in Con A-induced hepatitis, female CD44 WT and C57BL/6 CD44 KO mice were injected with Con A (12 mg/kg body weight, *i.v.* in 100 µl of saline). Control animals received 100µl saline (*i.v.*). Plasma AST levels were measured at different time point after Con A injection. As shown in Fig. 1, in both CD44 WT and CD44 KO mice, there was a significant increase of plasma AST level thereby indicating that hepatitis was induced after Con A injection. Increased AST levels were seen as early as 6 hours after Con A injection, reaching a peak at 12 hours and declining thereafter. At 48 hours the plasma AST reached normal levels. Although, CD44 WT and CD44 KO mice exhibited similar kinetics, CD44 KO mice exhibited higher plasma AST levels particularly at 12 and 24 hours after Con A injection. These data indicated that CD44 KO mice may develop more severe hepatitis than the CD44 WT mice (Fig. 1). Similar results were also obtained using histological studies and *in situ* apoptosis staining (Fig. 2). Hematoxylin and eosin staining of liver section was conducted 48 h after Con A challenge, because of significant liver damage seen at this time point. Damage to hepatocytes and presence of inflammatory cells was observed in both CD44 WT and CD44 KO mice. However, in CD44 KO mice, the liver damage was more severe than WT mice because of the larger areas of liver destruction. Because apoptosis occurs at earlier time points, it was studied at 16 hours after Con A injection. Liver sections were examined for apoptosis using TUNEL assay. The dark purple area demonstrates nuclei positive for DNA fragmentation (Fig. 2). It was noted that CD44 KO mice had higher number of nuclei positive for

DNA fragmentation when compared to CD44 WT mice (Fig.2). Together the above data demonstrated that CD44 KO mice exhibited increased susceptibility to hepatitis when compared to the CD44 WT mice

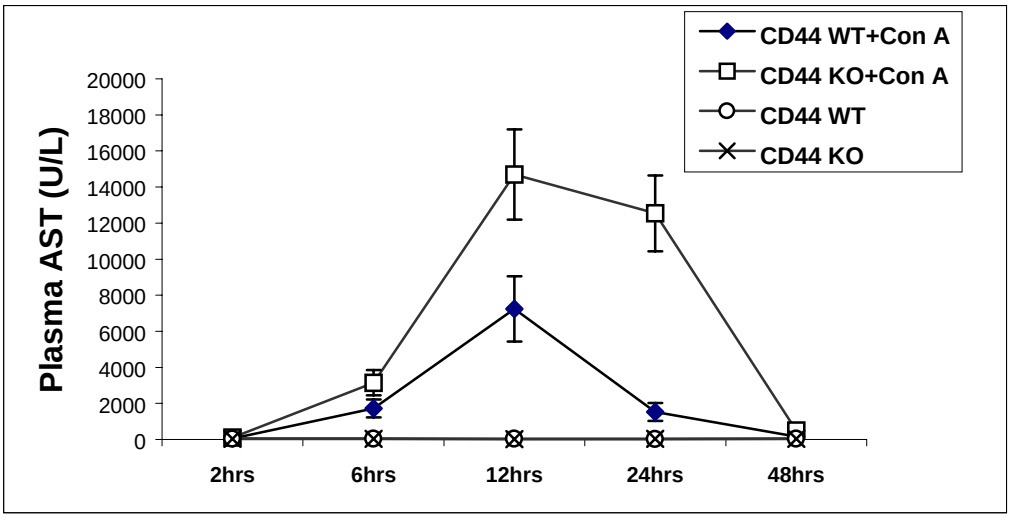

FIGURE 2.1: Plasma AST levels following Con A treatment. CD44 wild type (WT) and CD44 Knock out (KO) mice weighing 20-23 g were challenged with Con A (12mg/kg body weight, *i.v.* in 100 µl of saline). Control mice received 100µl saline (*i.v.*). Plasma AST levels were measured at 2, 6, 12, 24 and 48 hour respectively after Con A injection.

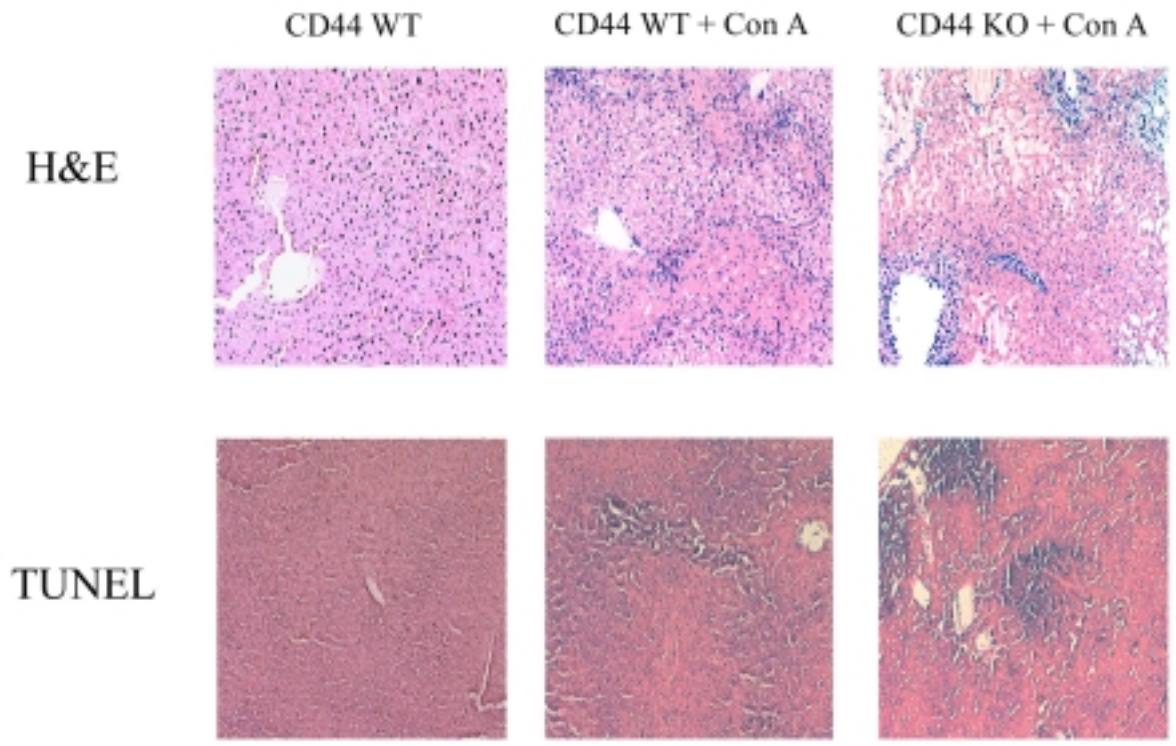

FIGURE 2.2: Nature of hapatocellular cell damage in Con A-induced hepatitis. Upper panel: Hematoxylin and eosin (H&E) staining of liver section harvested 48 h after Con A challenge (×200). Lower panel: TUNEL assay to detect apoptotic nuclei (×200) was conducted 16 h after Con A challenge. Dark purple area is indicative apoptosis.

Detection of TNF-α, IFN-γ, IL-2, Fas and FasL mRNA in the liver

Increased mRNA expression of cytokines such as TNF-α, IFN-γ and IL-2 have been considered to play a key role in the development of Con A-induced hepatitis (28). To this end, total liver RNA was isolated from CD44 WT and CD44 KO mice 2h and 8h after treatment of mice with Con A. mRNA was reverse transcribed and amplified by PCR using primers specific for TNF-α, IFN-γ, IL-2, Fas and FasL. Mouse β-actin was used as a housekeeping gene in the experiment. In these experiment, CD 44 KO mice were found to exhibit higher levels of mRNA for TNF-α, IFN-γ, and IL-2 when compared to CD44 WT mice (Fig. 3). However, mRNA of Fas and FasL expression in CD44 WT and CD44 KO mice were very similar. To quantitatively measure the mRNA expression in liver tissue, a semi quantitative PCR was conducted using AlphaImager™2000 digital imaging system (Fig. 4). Two hours (Fig.4A) and eight hours (Fig.4B) after Con A injection, the percentage of various cytokine mRNA when compared to β-actin levels was measured. The data shown in Fig 4 suggested that CD44 KO mice had increased levels of TNF-α, IFN-γ and IL-2 mRNA expression but not Fas and FasL when compared to the CD44 WT mice.

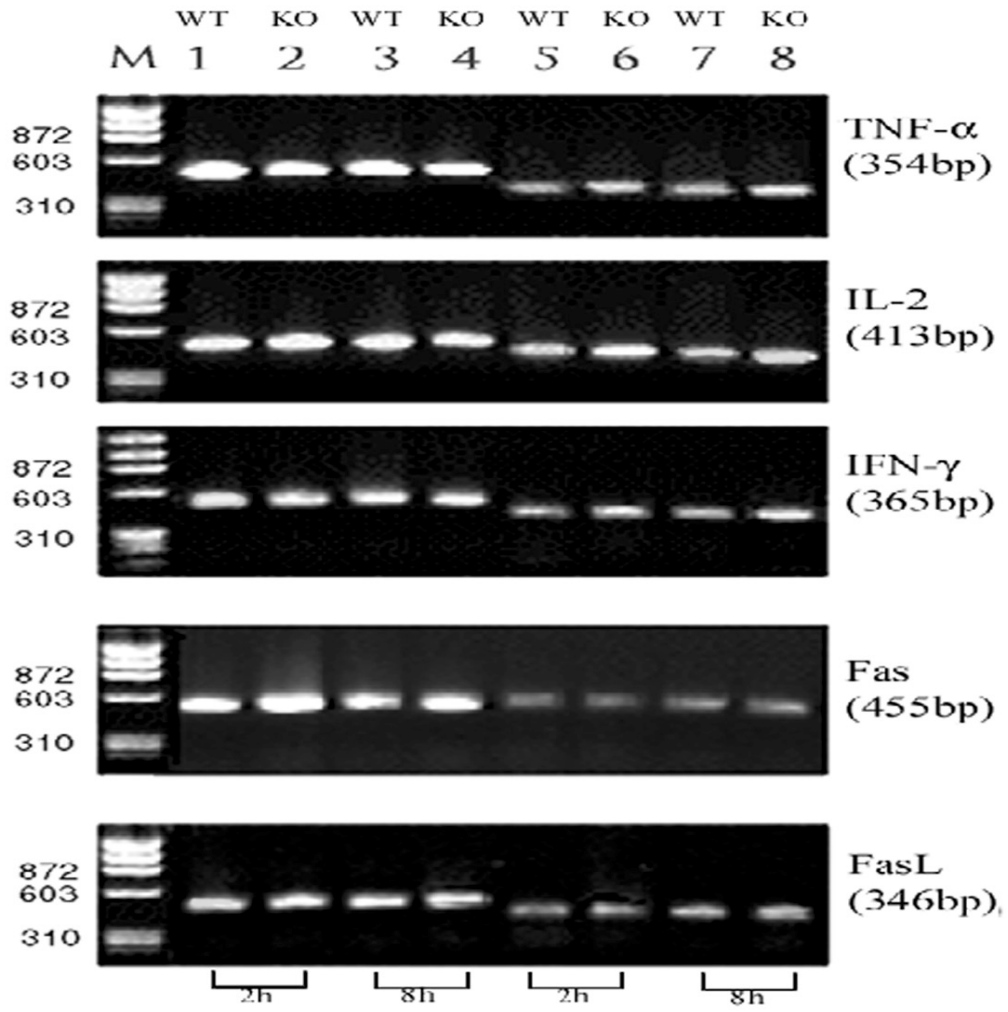

FIGURE 2.3: Detection of liver TNF-α, IFN-γ, IL-2, Fas and FasL mRNA in the liver. Total liver RNA was isolated from CD44 WT and CD44 KO mice 2h and 8h after treatment with Con A . mRNA was reverse transcribed and amplified by PCR using primers specific for TNF-α, IFN-γ, IL-2, Fas and FasL. Photograph of ethidium bromide-stained amplicons has in **Lane 1**, CD44 WT β-actin at 2h; **Lane 2**, CD44 KO β-actin at 2h; **Lane 3**, CD44 WT β-actin at 8h; **Lane 4**, CD44 KO β-actin at 8h; **Lane 5**, CD44 WT mRNA at 2h; **Lane 6**, CD44 KO mRNA at 2h; **Lane7**, CD44 WT mRNA at 8h; **Lane 8**, CD44 KO mRNA at 8h; **M**, Marker.

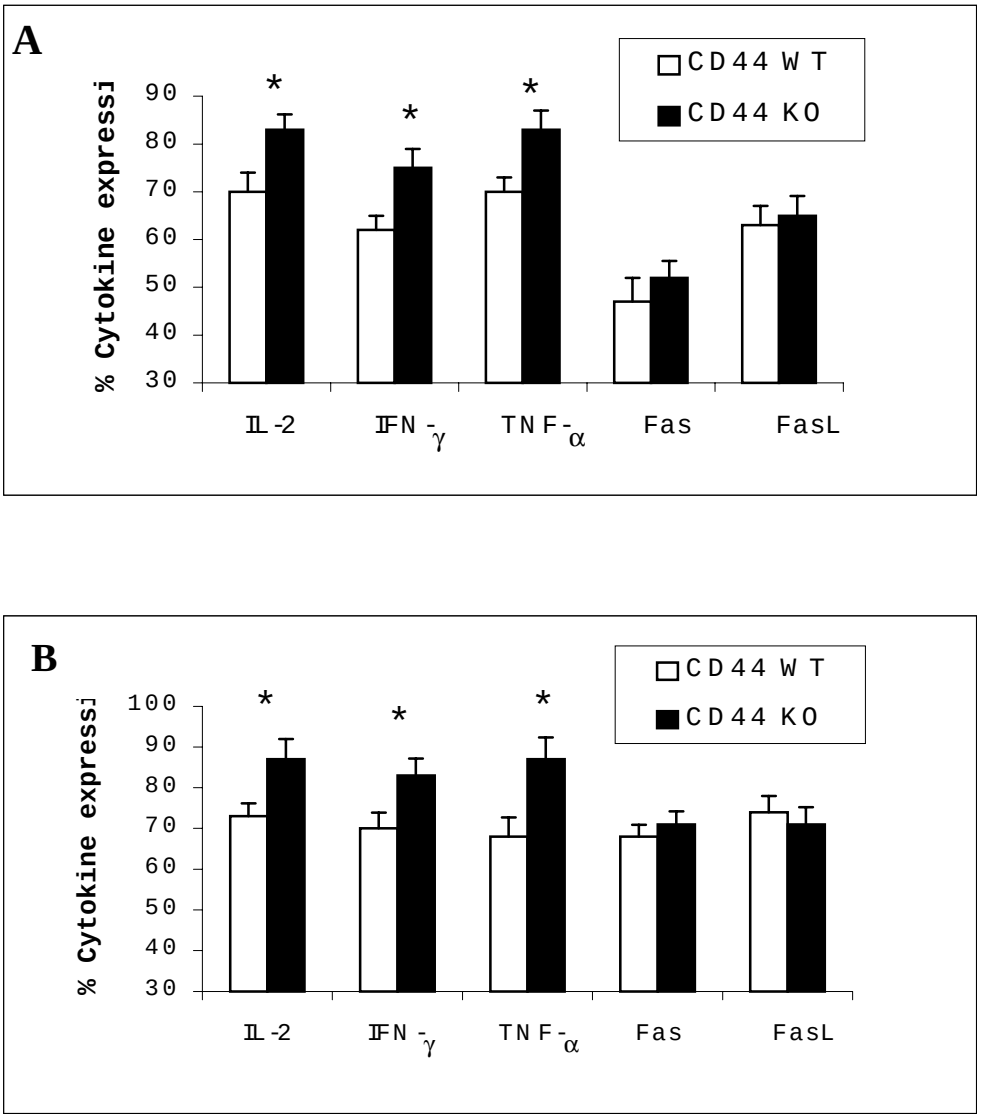

FIGURE 2.4: Semi quantitative PCR analysis for TNF-α, IFN-γ, IL-2, Fas and FasL mRNA in the liver of CD44 WT and CD44 KO mice. Two hours (Fig 4A) and 8 hours (Fig 4B) after Con A treatment, the percent of cytokine mRNA expression when compared to β-actin was measured using AlphaImager™2000 digital imaging system as described in Material and Methods. *: $P<0.05$ was considered to be statistically significant.

Spleen and liver infiltrating lymphocyte subpopulation

It has been suggested that $CD4^+$ T cells (1, 22) are involved in the development of Con A-induced hepatitis. In the current study, we wanted to find out whether the increased susceptibility of CD44 KO mice to hepatitis was caused by differential activation of T cell subsets when compared to the CD44 WT mice. To this end, lymphocyte subpopulation in the spleen and in the liver infiltration were screened using flow cytometry (Fig.5). At different time points after Con A treatment, spleen cells or liver infiltrating mononuclear cells from CD44 WT and CD44KO mice were harvested and the lymphocyte subpopulations was screened using flow cytometry (Fig. 5A-E). There was statistically no significant difference in the proportions of $CD4^+$, $CD8^+$, $CD3^+$, T cells as well as B cells and macrophages when analyzed 0-36 hrs after Con A injection between CD44 WT and CD44 KO mice. Similar results were also obtained when cells infiltrating the liver were screened (Fig. 5F). These data suggested that the increased hepatitis seen in CD44 KO mice was not caused by altered presence of lymphocyte/macrophage subpopulations.

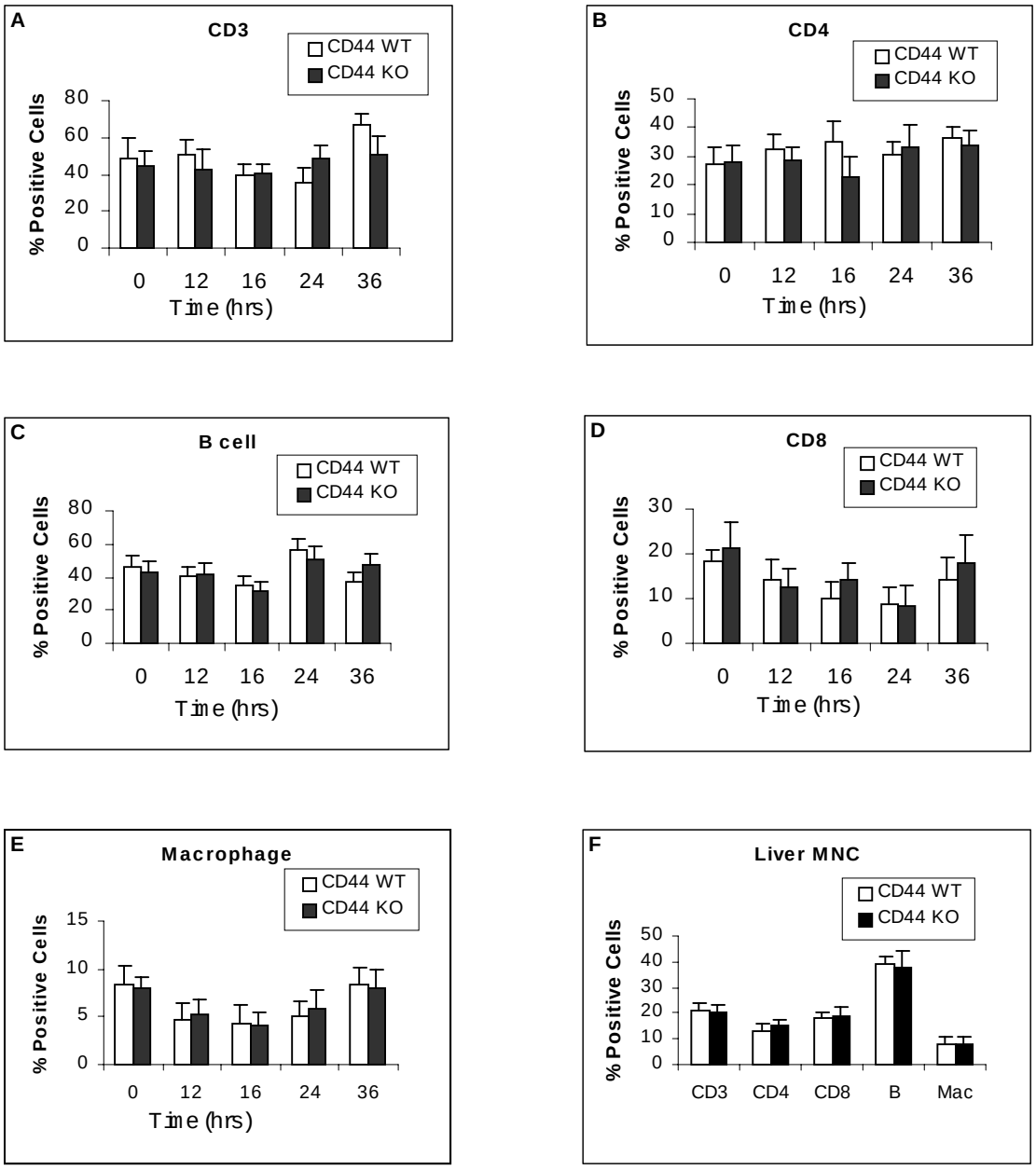

FIGURE 2.5: Enumeration of lymphocytes and macrophage in spleen and liver. Panel A-E: At different time point after Con A treatment, spleen cells from CD44 WT and CD44 KO mice were harvested and percentages of lymphocyte subpopulation and macrophages were determined using flow cytometry. Panel F: Sixteen hours after Con A treatment, mononuclear cells

infiltrating the liver were isolated, as described in the Material and Methods, and the percentage of lymphocyte subsets and macrophages were determined using flow cytometry.

Detection of apoptosis in T cells in vivo

Recent studies form our lab have demonstrated that CD44-deficient T cells are more resistant to activation induced cell death (unpublished data). We therefore tested the hypothesis that CD44 KO mice are more susceptible to Con A induced hepatitis because the Con A activated T cells fail to undergo apoptosis thereby continuing to elicit hepatitis. To this end, attempts were made to detect apoptotic T cells in CD44 WT and CD44 KO mice after Con A treatment (Fig. 6). In both groups of mice, early signs of apoptosis were detected at 8 hours after Con A injection. The apoptosis induction peaked at 24 hours and declined by 48 hours (Fig 6). Interestingly, at 16 and 24 hours following Con A injection, the apoptosis induction in T cells was markedly higher in CD44 WT mice when compared to the CD44 KO mice (fig 6). A representative experiment carried out 24 hour after Con A injection has been depicted in Fig 6B which indicates clearly that CD44 KO mice had significantly low percentage of apoptotic cells (27.8%) when compared to CD44 WT mice (60.2%).

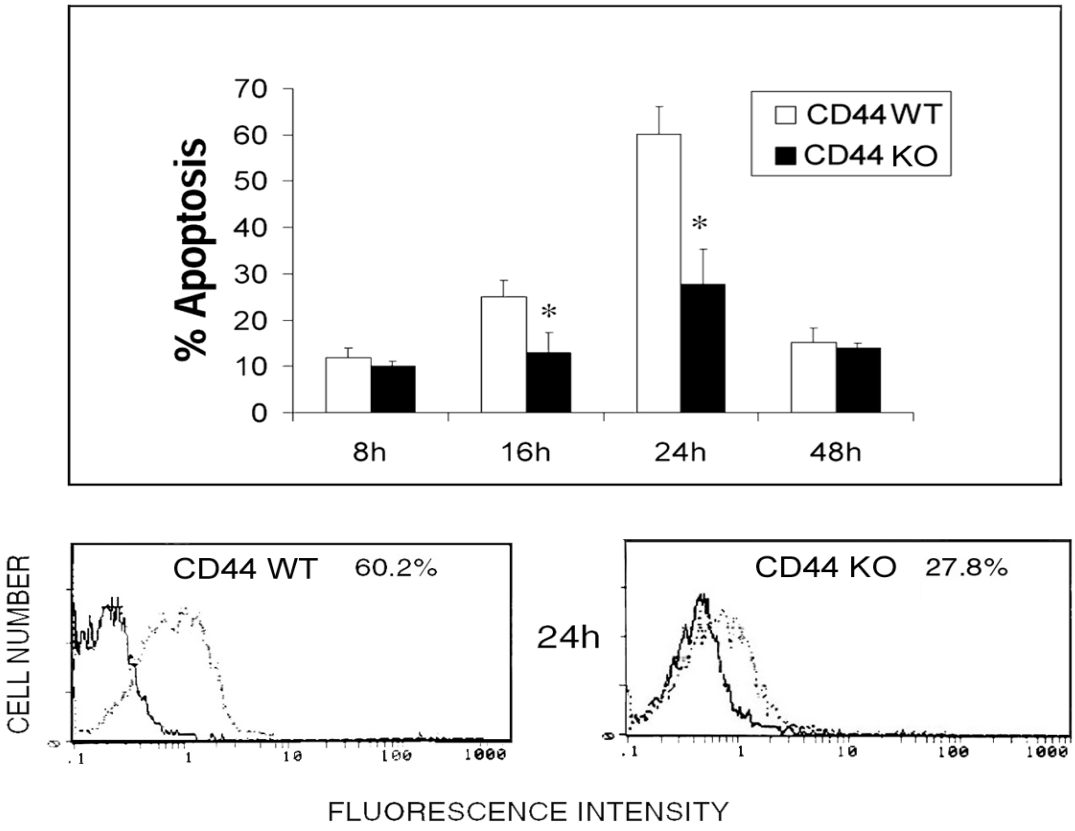

FIGURE 2.6: Detection of apoptosis *in vivo* after Con A treatment. At different time points following Con A injection, T cells form CD44 WT and CD44 KO mice were purified from the spleen. Next, one million cells were analyzed for apoptosis using TUNEL assay as described in the Material and Methods. Upper panel shows percentage of apoptotic T cells in spleen at various time intervals after Con A injection *: $P<0.05$ when compared to the CD44 WT mice. Lower panels show a representative experiment in which apoptosis in T cells was studied 24 hours after Con A injection. Bold histogram represents T cells stained with TdT alone and broken histogram shows T cells stained with TdT+FITC-dUTP. Percentage of apoptotic cells has been depicted in each histogram.

Detection of apoptosis in T cells in vitro

To further corroborate that T cells from CD44 KO mice were more resistant to apoptosis, T cells were cultured *in vitro* with Con A and the ability of cells to undergo activation induced cell death was studied. The result demonstrated that at 8, 16 and 24 hours after incubation with Con A, more apoptosis was seen in CD44 WT when compared to CD44 KO mice (Fig 7).

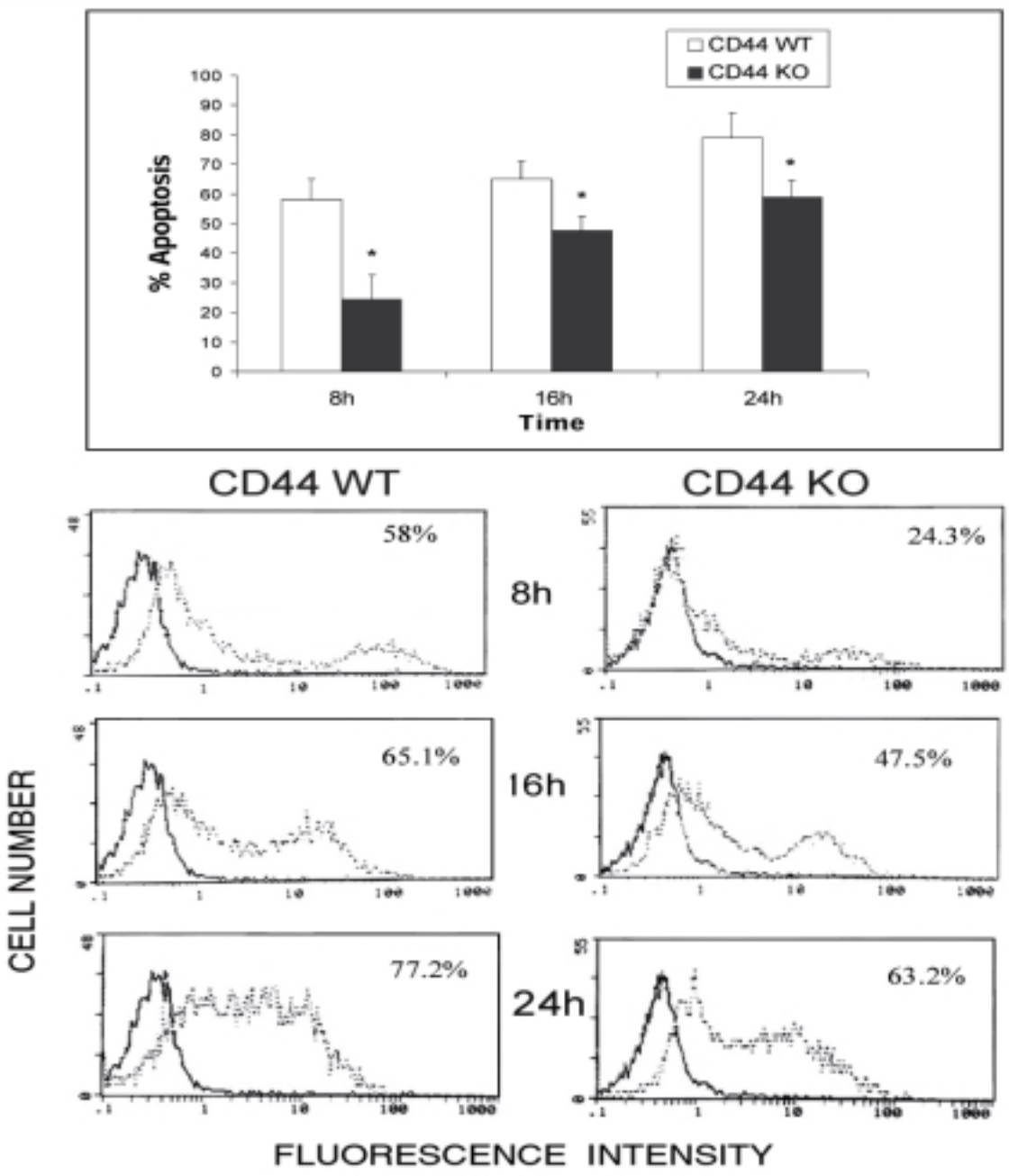

FIGURE 2.7: Detection of apoptosis in T cells *in vitro* after incubation with Con A. Purified T cells from the spleens of CD44 WT or CD44 KO mice were cultured with Con A (5µg/ml) for 8-48 hours. At each time point, the cells were harvested and analyzed for apoptosis using TUNEL assay. Upper panel shows percent apoptotic T cells at various time points. Vertical bars represent mean± SE of triplicate cultures. Asterix indicates statistically significant difference ($P<0.05$)

when compared to CD44 WT mice. Lower panels demonstrate a representative experiment showing apoptosis in T cells cultured with Con A. The data are presented as described in Fig 6.

Role of CD44 in hepatitis induced by adoptive cell transfer

It was not clear from the previous experiments whether CD44 expression on immune cells or hepatic cells was playing a critical role in hepatitis induction. To investigate this, adoptive experiments were conduced. Twenty four hours after Con A treatment, purified T cells from CD44 WT or CD44 KO mice were adoptively transferred into normal CD44 WT mice (1×10^8 cells/mouse *i.v.*). AST levels were measured 24 hours after the adoptive transfer. Transfer of Con A activated T cells form CD44 WT mice into normal CD44 WT mice triggered increased AST levels thereby indicating that Con A activated T cells were capable of inducing hepatitis. Interestingly, similar transfer of Con A-activated T cells from CD44 KO mice into normal CD44 WT mice induced higher levels of AST when compared to the WT→WT group (Fig 8). Transfer of Con A activated CD44 WT cells into CD44 KO mice induced similar AST levels as the WT→WT group (data not shown). Together, these data demonstrate that Con A activated CD44-deficient T cells were able to induce increased levels of AST when compared to $CD44^+$ T cells. Also, when Con A activated $CD44^+$ T cells were transferred into CD44 WT or CD44 KO mice the levels of AST were similar, thereby suggesting that the CD44 expression in the liver did not influence the hepatitis induction. Thus, CD44 expression on T cells rather than hepatocytes played a crucial role in Con A induced hepatitis.

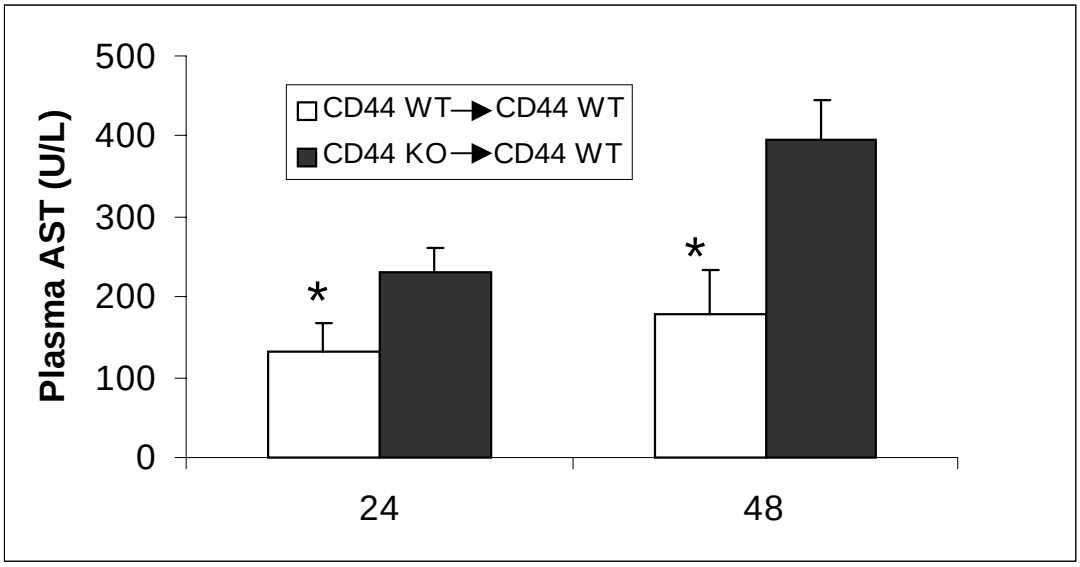

FIGURE 2.8: Effect of adoptive transfer of Con A activated T cells on induction of hepatitis. CD44 WT and CD44 KO mice were injected with Con A (12mg/kg body weight, i.v. in 100 μl of saline). Twenty four hours later whole spleen cells as mentioned in Material and Methods were purified and transferred into normal CD44 WT mice. Plasma AST levels from individual mice was determined at 24 and 48 hours after the adoptive transfer. Vertical bars represent mean±SE of 5 samples. Asterix indicated statistically significant difference ($P<0.5$) when compared to CD44 WT →CD44 WT group.

Discussion:

In the current study, we demonstrated using CD44 KO mice that CD44 plays a crucial role in Con A-induced hepatitis. CD44 KO mice exhibited increased hepatitis compared to CD44 WT mice. CD44 deficient T cells showed increased resistance to undergo apoptosis after Con A activation. Thus, prolonged survival of these cells generated increased levels of cytokines such as IL-2, INF-α and TNF-γ that may have contributed to more severe liver damage as seen in CD44 KO mice when compared to the CD44 WT mice. Furthermore, adoptive transfer experiments corroborated this observation by demonstrating that transfer of activated CD44 deficient T cells into wild type mice could induce higher levels of AST when compared to transfer of Con A activated CD44 WT T cells into CD44 WT mice. These data together demonstrated that CD44 is actively involved in the Con A-induced hepatitis.

Con A is a potent T cell mitogen with tropism for the liver. In mice, Con A induces an acute hepatitis that is a model of T lymphocyte-mediated liver injury (1). Previous studies have shown that Con A-induced hepatitis requires IL-2, INF-α, and TNF-γ (23, 24). In addition, Fas-Fas Ligand interaction (24) and perforin-granzyme system (25) were also shown to play an important role in Con A-induced hepatitis. In this study, we observed that the increased hepatitis seen in CD44-KO mice was partially due to increased production of cytokines such as TNF-α, IL-2 and IFN-γ, but not Fas or FasL. This may be because the Con A activated T cells that survive the process of activated induced cell death (AICD) may be producing TNF-α, IL-2 and IFN-γ but not FasL.

CD44 is a family of cell surface glycoproteins with proposed functions in extracellular matrix (EMC) binding, cell migration, lymphopoiesis, and lymphocyte homing (29). Gantner et al. (1995) have proposed that in Con A-induced hepatitis, liver-infiltrating T lymphocytes are recruited from the spleen and migrate to the liver where either activated macrophages or T cells may directly cause hepatocyte death. In the current study, we screened different subsets of lymphocytes in spleen and liver. However, we did not find any significant changes in CD44 KO mice compared to CD44 WT mice. These data excluded the possibility that increased hepatitis seen in CD44 KO mice was caused by presence of lymphocyte subsets. Previous studies have shown that CD44 is involved in the migration of lymphocytes to organs, including the liver (29). However, in an earlier study using CD44 KO mice, we observed in IL-2 induced vascular leak syndrome that the lymphocyte infiltration in lungs and liver was similar to CD44 WT mice (10). A histological study in the current investigation also showed that similar levels of infiltration of lymphocytes were seen in the livers of Con A injected CD44 WT and CD44 KO mice. These data suggested that increased hepatitis seen in CD44 KO mice was not caused by altered migration of lymphocytes to the liver.

T cells upon activation undergo apoptosis, a process termed as activation induced cell death (AICD) (20, 21). The role of AICD in Con A induced hepatitis has not been previously studied. In the Con A-induced hepatitis model, how T cells undergo apoptosis upon Con A activation may be crucial for mice to recover from the disease. CD44 has been shown to be upregulated following T cell activation after Con A injection thereby suggesting that CD44 could be a good candidate for inducing apoptosis (data not show). Indeed, we found increased resistance to apoptosis and prolonged survival of T cells in CD44 KO mice when compared to

CD44 WT mice both *in vivo* and *in vitro*. These data also indicated that CD44 on activated T cells may participate in AICD.

CD44 is expressed on a wide range of lymphoid and nonlymphoid (29). Thus not clear whether CD44 expression in the liver tissue or on lymphocytes was playing a role in Con A-induced hepatitis. To this end, we conducted the adoptive transfer experiment and found that activated T cells were enough to induce hepatitis in both WT and CD44 KO mice. In fact, T cells from Con A injected CD44 KO mice when transferred into CD44 WT mice induced increased plasma AST levels when compared to similar cells transferred from CD44 WT mice into CD44 WT mice. It should be noted that the plasma AST levels in adoptive transfer experiments were not as high as the AST levels seen in mice injected directly with Con A. This may be because the transferred cells may not home well. In fact in a recent report, the authors injected the cells into the spleen to induce a significant degree of hepatitis (4). It is also possible that liver $NK1^+$ T cells play a significant role in Con A induced hepatitis (26). Thus, the T cells from the spleens may play a secondary role.

In summary, the current study demonstrates that CD44 KO mice are more susceptible to Con A-induced hepatitis when compared to WT mice. Increased expression of IL-2, INF-γ and TNF-α was observed in the liver of CD44 KO mice but not WT mice following Con A injection. However, the expression of Fas and FasL as well as the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T cells, macrophages and B cells following Con A injection were similar in spleen of WT and CD44 KO mice. In spleens of CD44 KO mice, decreased apoptosis was demonstrated when compared to WT mice following treatment with Con A *in vitro* or *in vivo*. Adoptive transfer of

spleen cells from CD44 KO mice to WT mice induced increased levels of plasma AST whereas spleen cells from WT mice when transferred to WT or CD44 KO mice led to decreased AST levels.

In the current study, it was noted that the livers from Con A injected mice exhibited marked induction of apoptosis. These data are consistent with the previous studies that FasL is involved in Con A-induced hepatitis (24). In addition, TNF-α has also been shown to induce hepatotoxicity following Con A injection (27). The mechanism by which CD44 participates in activation induced cell-death is not clear. We have shown previously that T cells upon activation express increased levels of CD44 (16,19). Hyaluronic acid has been shown to serve as an important ligand for CD44. Thus, interaction between CD44 and HA on activated T cells may trigger apoptosis. Therefore, T cells deficient in CD44 may fail to undergo apoptosis and continue to produce cytokines thereby causing enhanced hepatitis. If CD44 plays an important role in Con A-induced cell death, use of Abs against CD44 that act as agonist should help in deleting activated cells thereby reducing the hepatotoxicity.

Reference

Austyn, J. M. and Wood, K. J. 1994. Principles of cellular and molecular immunology. Oxford University Press Inc., New York.

Aruffo, A., I. Stamenkovic, M. Melnick and C. B. Underhill. 1990. Seed CD44 is the principal cell surface receptor for hyaluronate. *Cell 61*:1303.

Beasley R.P., L.Y. Hwang, C.C. Lin, and C.S. Chein. 1981. Hepatocellular carcinoma and hepatitis B virus. A Prospective study of 22,707 men in Taiwan. Lancet. 2:1129.

Bradley, M. J., A. Zeytun, A. Rafi-Janajreh, P.S. Nagarkatti, and M. Nagarkatti. 1998. Role of spontaneous and IL-2 induced NK activity in the cytotoxicity and rejection of Fas+ and Fas- tumor cells. *Blood.* 92:4248.

Cao Q., R. Batey, G. Pang, A. Russell, and R. Clancy. 1998. IL-6, IFN-gamma and TNF-alpha production by liver-associated T cells and acute liver injury in rats administered concanavalin A. *Immunol Cell Biol.* 76:542.

Charles A., and Janeway, Jr. 1997. Immunobiology, the immune system in health and disease.Freeman and Company. New York.

Cheng, J., T. Zhou, C. Liu, J.P. Shapiro, M.J. Brauer, M.C. Kiefer, P.J. Barr, and J. D. Mountz. 1994. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science*, 263:1759.

Chisari F.V., C. Ferrari, and M.U. Mondelli. 1989. Hepatitis B virus structure and biology. *Microb Pathol*. 6:311.

Cohen, J. J., R.C. Duke, V.A. Fadok, and K.S. Sellins. 1992. Apoptosis and programmed cell death in immunity. *Annu Rev Immunol*. 10:267.

Decker K., D. Keppler. 1974. Hepatitis Galactosamine: Key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev Physiol Biochem Pharmacol*. 71:77.

Denning, S.M., P.T. Le, K.H. Singer, and B.F. Hayned. 1990. Antibodies against CD44p80, lymphocyte homing receptor molecule augment human peripheral blood T cell activation. *J Immunol* 144:7.

Flanagan, B.F., R. Dalchau, A.K. Allen, A. S. Daar and J. W. Fabre. 1989. Chemical composition and tissue distribution of the human CDw44 glycoprotein. *Immunol* 67:167.

Galandrini R; N. Albi, G. Tripodi, D. Zarcone, A., Terenzi, A. Moretta, C.E. Grossi and A. Velardi. 1993. Antibodies to CD44 trigger effector functions of T lymphocyte clones. *J Immunol* 150:4225.

Gantner F., M. Leist, A.W. Lohse, P.G. Germann, and G. Tiegs. 1995. Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. *Hepatology*. 21:190.

Gerschenson L.E., and R.J. Rotello, 1992. Apoptosis: a different form of cell death. *FASEB J*. 6:2450.

Hammond DM, P.S. Nagarkatti, L.R. Gote, A. Seth, M.R. Hassuneh, ans M. Nagarkatti. 1993 Double-negative T cells from MRL-lpr/lpr mice mediate cytolytic activity when triggered through adhesion molecules and constitutively express perforin gene. *J Exp Med*. 178:2225.

Haynes, B.F., M. J. Telen, L. P. Hale and S. M. Denning. 1989. CD44-A molecule involved in leukocyte adherence and T-Cell activation. *Innunol Today* 10:423.

Watanabe H., K. Ohtsuka, M. Kinura, Y. Ikarashi, K. Ohmori, A. Kusumi, T. Ohteki, S. Seki and T. Abo. 1992 Details of an isolation method for hepatic lymphocytes in mice. *J Immun Meth* 146:145.

Horvitz, H. R., S. Shaham, and M.O. Hengartner. 1994. The genetics of programmed cell death in the nematode Caenorhabditis elegans. Cold Spring Harb. *Symp. Quant. Biol.* 59:377.

Huet, S., H. Groux, B. Caillou, H. Valentin, M. Prieur and A. Bernard. 1989. CD44 contributes to T cell activation. *J Immunol* 143:798.

Gerschenson L.E., and R.J. Rotello. 1992. Apoptosis: a different form of cell death. *FASEB J* . 6:2450.

Kamath A.B., I. Camacho, P.S. Nagarkatti, and M. Nagarkatti. 1999. Role of Fas-Fas ligand interactions in 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD)-induced immunotoxicity: increased resistance of thymocytes from Fas-deficient (lpr) and Fas ligand-defective (gld) mice to TCDD-induced toxicity. *Toxicol Appl Pharmacol*. 160:141.

Kamath A.B., H. Xu, P.S. Nagarkatti, and M. Nagarkatti. 1997. Evidence for the induction of apoptosis in thymocytes by 2,3,7,8-tetrachlorodibenzo-p-dioxin in vivo. *Toxicol Appl Pharmacol*. 142:367.

Kaneko Y., M. Harada, T. Kawano, M. Yamashita, Y. Shibata, F. Gejyo, T. Nakayama, and M Taniguchi. 2000. Augmentation of Vα14 NKT Cell-mediated Cytotoxicity by Interleukin 4 in an Autocrine Mechanism Resulting in the Development of Concanavalin A-induced Hepatitis. *J. Exp. Med*. 191:105-114.

Kincade, P.W. 1992. Cell interaction molecules and cytokines which participate in B lymphopoiesis . *Baillieres-Clin-Haematol.* 5:575.

Kuby, J. 1997. Immunology. Third Edition, W.H. Freeman and Company. New York.

Lesley J., R. Hyman, and P.W. Kincade. 1993, CD44 and its interaction with extracellular matrix.Adv Immunol. 54:271.

Lohsi AW. Meyer zum Bushenfelde K-H. 1994 Experimental hepatitis. In: Cohen IR, Miller A, eds. Autoimmune disease models. *A guidebook. San Diego: Acde, oc Press*, 1991.

Messadi, D.V. and C. N. Bertolami. 1993. CD44 and hyaluronan expression in human cutaneous scar fibroblasts. *Am-J-Pathol* 142:1041.

Misuhara H., E. O'Neill, N. Seki, T. Ogawa, C. Kusunoke, K. Otsuke, S. Satoh, M. Niwa, H. Senoh, and H. Fujiwara. 1998. T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by inbterleukin 6. *J Exp Med.* 179:1529.

Miyake, K., K. L. Medina, S. I. Hayashi, S. Ono, T. 1990. Hamaoka and P. W. Kincade. Monoclonal antibodies to Pfp-1/CD44 block lymph-hemopoiesis in long-term bone marrow cultures. *J Exp* 171:477.

Murakami, S., K. Miyake, C. H. June, P. W. Kincade and& R. J. Hodes. 1990. IL-5 induces a Pgp-1 (CD44) bright B cell subpopulation that is highly enriched in proliferative and Ig secretory activity and binds to hyaluronate. *J Immunol* 145, 3618.

Nagata, S. 1996. Fas ligand and immune evasion. *Nature Med*. 2:1306.

Okamoto T., Y. Nakano, W. Asakura, T. Kobayashi, N. Tsuzuike, K. Hara. 1998. Expression of cytokine mRNA in extrahepatic organs in a mouse concanavalin A-hepatitis model. *Jpn J Pharmacol.* 77:219.

Goossens P.L., H. Jouin, G. Marchal and G. Milon. 1990. Isolation and flow cytometric analysis of the free lymphomyeloid cells present in murine liver. *J Immun Meth* 132:137.

Rafi-Janajreh A.Q., D. Chen, R. Schmits, T.W. Mak, R.L. Grayson, D.P. Sponenberg, M. Nagarkatti, and P.S. Nagarkatti. 1999. Evidence for the involvement of CD44 in endothelial cell injury and induction of vascular leak syndrome by IL-2. *J Immunol.* 163:1619.

Rieux-Laucat, F. Deist, C. Hivroz, I.A. Roberts, K.M. Debatin, A. Fischer, and J.P. De Villartay. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science,* 268:1347.

Rothman, B., M. Blue, K. Kelley, D. Wunderlich, D. Mierz and T. 1991. Aune. Human T cell activation by OKT3 is inhibited by a monoclonal antibody to CD44. *J Immunol*. 147:2493.

Schwartzman R.A., and J.A. Cidowski. 1993. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr Rev*. 14:133.

Schmits R., J. Filmus, N. Gerwin, G. Senaldi, F. Kiefer, T. Kundig, A. Wakeham, A. Shahinian, C. Catzavelos, J. Rak, C. Furlonger, A. Zakarian, J.J. Simard, P.S. Ohashi, C.J. Paige, J.C. Gutierrez-Ramos, T.W. Mak. 1997. CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood.* 90:2217.

Seth, A., L. Gote, M. Nagarkatti and P.S. Nagarkatti. 1991. T cell receptor-independent activation of cytolytic activity of cytotoxic T Lymphocytes mediated through CD44 and gp90MEL-14. *Proc Natl Acad Sci USA* 88:7877.

Shub, D. A. 1994. Bacterial viruses. Bacterial altruism. *Curr Biol*, 4:555.

Stamenkovic, I., M. Amiot, J. M. Pesando and B. Seed. 1989. Lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell.* 342:78.

Steller, H. 1995. Mechanisms and genes of cellular suicide. *Science*, 267:1445.

Tagawa Y., K. Sekikawa, and Y. Iwakura. 1997. Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. *J Immunol*. 159:1418.

Tiefs, G., J. Hentschel, and A. Wendel. 1992. A T cell-dependent experimental liver injury in mice inducible by concanavalin A. *J. Clin. Invest*. 90:196.

Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science*, 267:1456.

Toyabe S., S. Seki, T. Iiai, K. Takeda, K. Shirai, H. Watanabe, H. Hiraide, M. Uchiyama, and T. Abo. 1997. Requirement of IL-4 and liver NK1+ T cells for concanavalin A-induced hepatic injury in mice. *J Immunol.* 159:1537.

Underhill, C. CD44: The hyaluronan receptor. *J Cell Sci* 103, 293-298;1992. Vaux. D. L. and Strasser, A. 1996.The molecular biology of apoptosis. *Proc Natl Acad Sci U S A*, 93:2239.

Watanabe Y., M. Morita, T. Akaike. 1996. Concanavalin A induces perforin-mediated but not Fas-mediated hepatic injury. *Hepatology*. 24:702.

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