

CD44-Hyaluronic acid interactions in IL-2 induced Vascular Leak Syndrome

By
Amjad Mustafa

Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in
partial fulfillment of the requirements for the Degree of
Master of Science
in
Biology

Approved by

Dr. Prakash Nagarkatti

Dr. Robert Duncan

Dr. Mitzi Nagarkatti

Dr. David Popham

June 15, 2001
Blacksburg, VA

Keywords: CD44, Hyaluronate, Vascular Leak Syndrome

CD44-Hyaluronic acid interactions in IL-2 induced Vascular Leak Syndrome

By

Amjad Mustafa

Dr. Prakash S. Nagarkatti (Chairman)

(Abstract)

Immunotherapy with IL-2 is accompanied by severe toxicity leading to development of vascular leak syndrome (VLS). Previous studies from our laboratory demonstrated that CD44 knockout mice exhibit marked decrease in IL-2 induced VLS, thereby suggesting a role for CD44 in VLS. In the current study we tested whether use of mAbs against CD44 or hyaluronic acid (HA), the ligand for CD44, can abrogate IL-2 induced VLS. Administration of IL-2 (75,000 U/mouse, three times a day for 4 days) into C57BL/6 mice triggered significant VLS in the lungs and liver. Interestingly, HA caused a marked increase in IL-2-induced VLS in the lungs and liver. In contrast, use of anti-CD44 mAbs reduced IL-2-induced VLS in the lungs and liver. The change in VLS seen following HA or anti-CD44 mAbs treatment was not due to any defect in lymphocyte migration or homing to various organs because histopathological studies in these mice demonstrated significant and often greater perivascular infiltration of lymphocytes when compared to mice treated with IL-2 alone. However, HA treatment exhibited a marked increase in IL-2-induced lymphokine-activated killer (LAK) cell activity while anti-CD44 mAbs treatment led to a significant decrease in IL-2-induced LAK cell activity. These studies demonstrate that HA or anti CD44 mAbs may serve as a useful tool to selectively alter the LAK activity as well as to prevent the toxicity induced by IL-2. Altering CD44-HA interactions in vivo may offer a novel therapeutic approach to prevent endothelial cell injury by cytotoxic lymphocytes in a variety of clinical diseases.

Acknowledgements

I would like to thank my advisor, Dr. Prakash S. Nagarkatti and coadvisor, Dr. Mitzi Nagarkatti, for the opportunity to pursue my research interest in immunology. I appreciate their support and guidance throughout this study. I would also like to thank Dr. Robert Duncan for his assistance with the histology as well as the other member of my committee, Dr. David Popham for their time and advice during my graduate research.

Thanks also to lab members, Dr. Ahmed Zytun, for his help and advice, when things did not work according to the plan, Mr. Dawie Chen and Yoon, for their help.

Special thanks to my mother, Deya Issa, for her encouragement, advice and for the many sacrifices she has made over many years. Thanks also due to my friends Dr. Saad Alshahrani and Dr. Aref Alderbas for their constant encouragement and friendship. I would like to thank my brothers, Munir Mustafa and Nash Isa, who provided constant encouragement and advice from miles away.

Table of contents

Chapter 1: General Introduction, Specific Aims

Abbreviation	1
Introduction	2
Specific Aims	5

Chapter 2: Review of Literature

Adhesion Molecules.....	6
Significance of the Extracellular Matrix.....	9
Significance of CD44.....	12
Role of CD44 in tumor cell metastasis	17
Role of CD44 on macrophage functions.....	18
Hyaluronic Acid and its role in immunoregulation	19
The role of hyaluronic acid and anti- CD44 mAbs in preventing Vascular Leak Syndrome induced by Interleukin-2	23
References.....	24

Chapter 3: Agonistic effect of Hyaluronic acid and Antagonistic effect of anti-CD44 on IL-2 induced vascular Leak Syndrome.

Abstract.....	30
Introduction.....	30
Materials and Methods.....	32
Mice.....	32
Interleukins and reagents.....	32
Quantitation of Vascular Leak Syndrome.....	33
Effect of HA in IL-2 induced VLS	33
Use of anti-CD44 mAbs to block VLS	33
Histology	34
Studies on NK/LAK cells	34
JAM test	34
Statistical analysis	35
Results	
The effect of HA on IL-2 induced VLS	36
Use of mAbs against CD44 on IL-2 induced VLS	36
Histopathological studies in organs exhibiting VLS.....	37

LAK activity in mice undergoing VLS	40
Discussion	48
Abbreviations	51
References	52
Curriculum Vitae	56

List of Figures and Tables

Figure 1 Effect of HA on IL-2 induced VLS	38
Figure 2 Effect of anti-CD44 mAbs on IL-2 induced VLS	39
Figure 3 Histological studies on lungs and livers in PBS treated mice or HA alone treated mice or anti-CD44 mAbs alone treated mice.....	41
Figure 4 Histological studies on lungs in mice undergoing IL-2 induced VLS following HA treatment or anti-CD44 mAbs treatment	42
Figure 5 Histological studies on livers in mice undergoing IL-2 induced VLS following HA treatment or anti-CD44 mAbs treatment	43
Table 1 Perivascular infiltration of lymphocytes in wild-type mice treated with IL-2 alone or IL-2 + HA or IL-2 + anti-CD44 mAbs.....	44
Figure 6 Effect of HA on LAK activity in mice undergoing IL-2 induced VLS.....	46
Figure 7 Effect of anti-CD44 mAbs on LAK activity in mice undergoing IL-2 induced VLS	47

ABBREVIATIONS

APC, antigen presenting cell; CAMs, cell-adhesion molecules; CD, Cluster Designation CTL, cytotoxic T lymphocytes; DN, double-negative; ECM, extracellular matrix ELISA, enzyme-linked immunosorbent assay; HA, hyaluronic acid; HEVs, high-endothelial venules ;IFN- γ , interferon-gamma; Ig, Immunoglobulin; IL-1, interleukin-1; IL-2, interleukin-2; IL-4, interleukin-4; LAD, Leukocyte-adhesion deficiency; mAb, monoclonal antibody; NK, natural killer; TCR, T cell receptor; VLS, vascular leak syndrome; Vas, vascular addressins.

Chapter 1:

General introduction and Specific aims

Introduction

The immune system consists of a network of closely interacting cells and a variety of molecules that they produce. The main function of the immune system is to protect the host from pathogens and cancer. Immunity is the immune response against foreign molecules called antigen. Immune response can be divided into innate immunity and adaptive or acquired immune response.

Innate Immunity is the natural resistance. Such resistance may be based on species, race, strain etc. For example, humans are resistant to canine distemper whereas, dogs are resistant to mumps. A number of internal and external defenses contribute to innate immunity. For example, lysozyme in tears and skin as a barrier are parts of innate immunity that prevent infections. Phagocytosis is an important mechanism of innate immunity in which specialized cells called phagocytes engulf and kill foreign objects such as bacteria. Examples of phagocytic cells include monocytes, macrophages, and neutrophils. These constitute first line of defense. Innate immunity is nonspecific (1).

Adaptive or acquired immune response is specific and has memory. It is mediated by cells called lymphocytes. Because of specificity and memory, this response can generate life long immunity. There are two important types of lymphocytes: T lymphocytes and B-lymphocytes. T lymphocytes are called so because they originate in the thymus whereas, B cells originate in the bone marrow. These cells use distinct receptors to recognize an antigen. The B cells express immunoglobulin molecules (antibody) and use these as their receptors, whereas, T cells use a distinct receptor called, T cell-receptor (TCR). The main difference between the T and B cell receptors is that the B cell receptor can directly bind to the antigen whereas the T cell receptor binds to the antigen only after it is processed and presented by antigen presenting cells along with specialized molecules encoded by the major histocompatibility complex (MHC) (1).

The immune response can also be divided into:

- 1) Humoral immunity: Contributed by B cells and the antibodies that they produce.
- 2) Cell-mediated immunity: Mediated by T cells. There are two types of T cells: T- helper cells (TH) which help either B cells or the phagocytic macrophages. The second set of cells is called T-cytotoxic cells whose function is to recognize a cancer cell or any cell infected with a virus and destroy it.

In addition to the T cells, B cells and macrophages, there are natural killer (NK) cells. NK cells constitute important cells involved in protection against viral infections and cancer. Unlike T and B cells, NK cells do not have any specific receptors. Thus NK cells are considered to be nonspecific (1).

Lymphocytes and other leukocytes express a large number of different molecules on their surface which can be used to distinguish ('mark') cell subsets. Many of these cell markers can now be identified by specific monoclonal antibodies. A systematic nomenclature has been developed in which the term CD (Cluster Designation) refers to groups (clusters) of monoclonal antibodies, each cluster binding specifically to a specific molecule. The CD system derives from analysis of monoclonal antibodies against human leukocyte antigens produced mainly in mice. Monoclonal antibodies with similar characteristics are grouped together and given a CD number. However, it is now customary to use the CD marker to indicate the molecule recognized by each group of monoclonal antibodies.

Cytotoxic cells express a class of receptors, known as cytotoxic triggering molecules that initiate lytic processes when they bind ligands expressed by a target cell. Not all surface molecules on killer cells are cytotoxic triggers. The best-known triggering molecules are the TCR and Fc γ Rs on natural killer cells, monocytes, and granulocytes. However, under certain conditions, adhesion molecules that are not directly related to antigen binding, such as CD2, CD44, and CD69, can also serve as a cytotoxic triggers (3, 4).

Studies from our lab and elsewhere have shown that CD44 is a triggering molecule on some murine and human T cell clones and human NK cells (4). CD44 is a widely distributed cell surface glycoprotein whose principal ligand has been identified as hyaluronic acid (HA), a major component of the extracellular matrix (ECM). Recent studies have demonstrated that activation through CD44 leads to induction of effector function in T cells and macrophages (4). At sites of chronic inflammation as seen in certain infections, autoimmune diseases, allograft rejection, graft-versus-host (GVH) disease and treatment of cancer patients with high doses of interleukin-2, significant damage to endothelial cells has been known to occur, which leads to the toxicity or pathogenesis associated with the disease (5). Studies from our laboratory and elsewhere demonstrated that cytotoxic lymphocytes mediate lysis of target cells when activated through CD44. Cytotoxic T lymphocytes (CTL), double-negative (DN) T cells and NK cells upon activation express high levels of CD44 and mediate efficient MHC-unrestricted TCR-independent lysis following ligation of CD44. Such CD44-mediated cytotoxicity may play an important role in protection against viral infections and cancer. However, it could also cause non-specific tissue injury (5). For example, dysregulation in the interaction between activated cytotoxic lymphocytes expressing CD44 and endothelial cells bearing the appropriated ligand such as the hyaluronic acid, could lead to endothelial cell lysis (5, 61). Studies from our laboratory demonstrated that following IL-2 therapy, cytotoxic lymphocytes activated through CD44 mediate lysis of endothelial cells leading to the induction of vascular leak syndrome (VLS) (61).

Hypothesis:

In the current study we tested the hypothesis that treatment of mice with HA or anti-CD44 mAbs would alter the severity of VLS induced by IL-2 by influencing the migration of lymphocytes and/or cytotoxicity mediated against endothelial cells.

Specific Aims:

1. To test whether treatment of mice with HA or anti-CD44 mAbs would alter IL-2 induced VLS.
2. To address whether such treatment would alter perivascular lymphocytic infiltration in lungs and liver
3. To study whether treatment with HA or anti-CD44 mAbs would alter the cytotoxicity mediated by NK/LAK cells.

Chapter 2:

Review of Literature

I. Adhesion Molecules

The vascular endothelium serves as an important “ gatekeeper,” regulating the movement of blood-borne molecules and leukocytes into the tissues. In order for circulating leukocytes to enter inflammatory tissue or peripheral lymphoid organs, the cells must adhere to and pass between the endothelial cells lining the walls of blood vessels, a process called extravasation. Endothelial cells express leukocyte-specific cell-adhesion molecules (CAMs). Some of these membrane proteins are expressed constitutively, others are only expressed in response to localized concentrations of cytokines produced during an inflammatory response. Recirculating lymphocytes, monocytes, and granulocytes bear receptors that bind to CAMs on the vascular endothelium, enabling these cells to extravasate into the tissues.

In addition to their role in leukocyte adhesion to vascular endothelial cells, many adhesion molecules also serve to increase the strength of the functional interactions between cells of the immune system. Various adhesion molecules have been shown to contribute to the TH-antigen presenting cells, TH-B cell, and CTL-target cell interactions. Adhesion molecules play an important role in cell-cell and cell-extracellular matrix interactions (6). Such interactions are crucial to all developmental processes (7).

The adhesive interactions of cells with other cells and with the extracellular matrix are crucial to all developmental processes, but have a central role in the functions of the immune system throughout life. Three families of cell-surface molecules regulate the migration of lymphocytes and the interactions of activated cells during immune responses: 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) (8).

The integrin family of adhesion molecules serves as receptors for ICAM-1, ICAM-2, and VCAM-1. The integrins are heterodimeric proteins (consisting of an α and a β chain) that facilitate leukocyte adherence to the vascular endothelium or other cell-to-cell interactions. Different integrins are expressed by different populations of leukocytes, allowing these cells to bind to different CAMs expressed on the vascular endothelium. The importance of integrin molecules in leukocyte extravasation is demonstrated by leukocyte-adhesion deficiency (LAD), an autosomal recessive disease characterized by recurrent bacterial infections and impaired healing of wounds. The deficiency stems from abnormal synthesis of the chain of the integrin heterodimer present on all leukocytes. Leukocytes lacking integrin molecules cannot extravasate from the blood vessels to the tissues. As a result, an inflammatory response cannot develop in the tissues, and affected individuals have more frequent and more severe bacterial infections than normal individuals.

The selectin family of membrane glycoproteins have a characteristic extracellular structure consisting of three domains: a lectin domain, a domain having homology to epidermal growth factor, and a number of repeats related to complement-regulatory proteins. These molecules are sometimes referred to as LEC-CAMs in reference to their three domains. The selectin family serves as adhesion molecules on vascular endothelial cells or as adhesion-molecule receptors on circulating leukocytes. Selectins bind to specific carbohydrate groups by means of their distal lectin domain.

Adhesion molecules are a part of every process involving cell contact from thymic selection to antigen priming, from antigen recognition to cell activation, from cytotoxicity to lymphocyte recirculation (9). Adhesion molecules work in a cooperative fashion and their activity can be regulated by expression or modulated after display on the cell surface (10). Adhesion molecules are expressed by a wide variety of cells and these cell surface receptors are essential to the interactions between the cytoskeleton and the extracellular matrix (11). Cell surface receptors recognizing extracellular molecules are the principal mechanism used by cells to sense their environment (12). During an immune response, several families of adhesion molecules

participate in a cascade of binding events that lead to the binding of leukocytes, both to each other and to other cell types (i.e. endothelium and epithelium) (13).

Recent studies have shown that the function of an adhesion receptor cannot be determined from its expression alone. Adhesion receptors are thought to be "selected" to perform distinct effector functions based on their cell-background and factors present in the local environment (13). Therefore, adhesion receptors that are expressed on different cell types may be in different states of "activation-readiness" and may continue to be selected by conditions in the surrounding environment to bind to tissue-specific ligands and mediate leukocyte effector functions (13).

Some regions of vascular endothelium found in postcapillary venules of various lymphoid organs are composed of specialized cells with a plump, cuboidal ("high") shape; such regions are called high-endothelial venules, or HEVs. Each of the secondary lymphoid organs, with the exception of the spleen, contains HEVs. High-endothelial venules express a variety of cell adhesion molecules. Like other vascular endothelial cells, HEVs express CAMs of the immunoglobulin superfamily and selectin family. In addition, HEVs express some adhesion molecules that are distributed in a tissue-specific manner. These tissue-specific adhesion molecules have been called vascular addressins (VAs) because they serve to direct the extravasation of different populations of recirculating lymphocytes to particular lymphoid organs. The tissue-specific distribution of these addressin molecules can be demonstrated by differences in binding of monoclonal antibodies to HEVs. For example, some monoclonal antibodies bind only to vascular addressins in the HEVs of lymph nodes, whereas other monoclonal antibodies bind selectively to vascular addressins of Peyer's patches.

The adhesion of lymphocytes to HEVs or regions of inflamed endothelium and their subsequent extravasation is a multistep process involving a cascade of adhesion-molecule interactions. The first step usually involves the interaction of a homing receptor on a lymphocyte with a tissue-specific vascular addressin. Although this initial interaction is quite weak, the slow rate of blood flow in postcapillary venules, particularly in regions of HEVs, reduces the likelihood that the sheer force of the flowing blood will dislodge the tethered lymphocyte. In the

second step, cellular adhesion is strengthened by binding of an integrin receptor on the lymphocyte with a CAM on the HEV. This latter interaction might involve binding of the integrin molecule VLA-4 to VCAM-1, or binding of the integrin LFA-1 to ICAM-1 or ICAM-2. This generates an activating signal that causes the lymphocyte to up-regulate its expression of integrin molecules. The increased expression of integrin provides multiple adhesion receptors, strengthening the interaction between the lymphocyte and HEV. The final step in the adhesion cascade is extravasation the lymphocyte through the endothelium into the tissue. This step requires a reduction in the strength of adhesion between the lymphocyte and endothelium so that the lymphocyte can extravasate through the endothelium (1).

There has been a growing interest in lymphocyte interactions with extracellular matrix (ECM) components. Interaction of lymphocyte adhesion receptors with ECM has been shown to play a central role in regulating the migration, differentiation, and functions of the cells of the immune system (14). The interplay between the cell-surface and matrix molecules that regulate adherence, and chemoattractant gradients that direct cell migration, control the localization of cells (8). Thus the cells of the immune system depend on regulated interactions with other cells to activate and direct the response to infection (8). Recently, studies involving the interactions of T lymphocytes with antigen-bearing cells has exposed both antigen-specific receptors and a group of cell adhesion molecules.

Studies from our lab demonstrated that activated cytotoxic T lymphocytes (CTL) can mediate efficient lysis of target cells when activated through adhesion molecule, CD44 (15). Furthermore, similar observations were made using CD4-CD8- T cells found in MRL-Ipr/lpr mice, (16) thereby suggesting that CD44 can serve as a signaling molecule.

II. Significance of the Extracellular Matrix (ECM)

Leukocytes are mobile units of the immune system. The process of leukocytes migration from blood vessels to inflamed tissues involves two major steps: (1) extravasation through the vessel wall and (2) movement through the underlying basement membrane and ECM. The ECM

is a complex macromolecular mesh composed of proteoglycans and adhesive glycoproteins, such as collagen, laminin, and fibronectin (17). The proteoglycans consist of one or more glycosaminoglycans –linear polymers of repeating disaccharides-covalently bound to a protein core. Hyaluronic acid (HA) is a glycosaminoglycan consisting of repeating disaccharide units of (1-4)-D-glucuronic acid-beta-(1-3)-D-N-glycosaminoglycan. The components of the ECM serve many roles. They serve a mechanical role in supporting and maintaining tissue structure. They also are involved in a variety of cell functions including development, migration, and proliferation (18).

There are a number of lymphocyte ECM receptors which have recently been identified. These include members of the integrin family of adhesion molecules such as CD44 and CD26 (19). Lymphocytes have been found to interact with hyaluronic acid which is a major glycosaminoglycan that forms the carbohydrate gel in which ECM glycoproteins are immersed. Adhesion of many cell types to ECM components is a constitutive event. Cells adhere to an ECM ligand only if the correct receptor is expressed on the cell surface (19). However, it has been shown that despite the expression of the appropriate receptor(s), lymphocytes have revealed minimal adhesion to ECM components. Like many cell adhesion receptors, CD44 is broadly distributed, and its ligand, hyaluronan, is a common component of ECM and extracellular fluids. Yet a great variety of responses have been reported to result from CD44 ligation. These include cell adhesion, cell migration, induction (or at least support) of hematopoietic differentiation, effects on other cell adhesion mechanisms, and interaction with cell activation signals (29). This diversity of responses indicates that downstream events following ligand binding by CD44 may vary depending on the cell type expressing CD44 and on the environment of that cell (29).

Lymphocyte differentiation can result in stable changes in the level of expression of adhesion molecules that are associated with increased adhesion to ECM components. Lymphocyte activation has been shown to have rapid effects on adhesion to ECM (18). If the time period of lymphocyte activation is increased the overall levels of receptor cell surface expression is increased which results in ECM binding.

CD44 is expressed on cells in the early stages of hematopoiesis and has been shown to participate in at least some aspects of the hematopoietic process. In mature lymphocytes, CD44 is upregulated in response to antigenic stimuli and may participate in the effector stage of immunological responses. Along with other adhesion receptors that show alterations in expression after activation, CD44 probably contributes to differences in the recirculation patterns of different lymphocyte subpopulations. CD44 ligand-binding function on lymphocytes is strictly regulated, such that most CD44-expressing cells do not constitutively bind ligand. Ligand-binding function may be activated as a result of differentiation, inside-out signaling, and/or extracellular stimuli (29). This regulation, which in some situations can be rapid and transient, potentially provides exquisite specificity to what would otherwise be a common interaction. CD44 is not a single molecule, but a diverse family of molecules generated by alternate splicing of multiple exons of a single gene and by different posttranslational modifications in different cell types (20). It is not yet clear how these modifications influence ligand-binding function. The significance of the multiple isoforms of CD44 is not understood, but association of some isoforms with malignancies has been observed (29).

Adhesion molecules play an important role in T cell recognition of antigen. The adhesion molecules facilitate the interaction of the T cells with other cells expressing foreign antigen and they deliver necessary costimulatory signals that synergize with the primary activation signal provided by the T cell receptor (22). It has also been proposed that the ECM plays an important role in lymphocyte differentiation. Studies have demonstrated the ability of CD44 mAb's to inhibit B cell differentiation in long-term bone marrow cultures (23) Also, B cell adhesion to bone marrow stromal cells is mediated by B cell CD44 binding to HA on stromal cells. This collectively suggests that the CD44-HA interactions may play a critical role to lymphohemopoiesis in the bone marrow. Since HA composes more than 40% of the total glycosaminoglycan produced by thymic epithelial cells it is thought that CD44-HA interactions may be important in thymic differentiation (24).

III. Significance of CD44

CD44 is a ubiquitous multistructural and multifunctional cell surface adhesion molecule involved in cell-cell and cell-matrix interactions (25). CD44 (also known as Pgp-1, Ly-24, extracellular matrix receptor III and Hermes) is expressed on a wide variety of cell types, including T cells, thymocytes, B cells, granulocytes, macrophages, erythrocytes, neural cells, epithelial cells, and fibroblasts (26).

Twenty exons are involved in the genomic organization of this molecule. The first five and the last 5 exons are constant, whereas the 10 exons located between these regions are subjected to alternative splicing, resulting in the generation of a variable region (25). Differential utilization of the 10 variable region exons, as well as variations in N- glycosylation, O- glycosylation, and glycosaminoglycanation (by heparan sulfate or chondroitin sulfate), generate multiple isoforms (at least 20 are known) of different molecular sizes (85-230 kDa)(25). The smallest CD44 molecule (85-95 kDa), which lacks the entire variable region, is standard CD44 (CD44s)(25). Examination of the cDNA sequence of CD44 showed homology between the amino-terminal portion of CD44 to chick and rat cartilage link proteins and provided evidence that CD44 has an ECM ligand. Subsequent studies have shown that HA is a ligand for CD44 (27). Hyaluronic acid, an important component of the extracellular matrix (ECM), is the principal but by no means the only, ligand of CD44. Other CD44 ligands include the ECM components collagen, fibronectin, laminin, and chondroitin sulfate. Mucosal addressin, serglycin, osteopontin, and the class II invariant chain (Ii) are additional, ECM-unrelated, ligands of the molecule (25). In many, but not in all cases, CD44 does not bind HA unless it is stimulated by phorbol esters, activated by agonistic anti- CD44 antibody, or deglycosylated (e.g., by tunicamycin)(25).

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) (28). The amino terminal portion of CD44 is homologous to cartilage link proteins, which promote proteoglycan- and collagen-dependent extracellular matrix adhesion.

CD44 is a multifunctional receptor involved in cell-cell and cell-ECM interactions, cell traffic, lymph node homing, presentation of chemokines and growth factors to traveling cells, and transmission of growth signals. CD44 also participates in the uptake and intracellular degradation of HA, as well as in transmission of signals mediating hematopoiesis and apoptosis. After immunological activation, T lymphocytes and other leukocytes transiently upregulate CD44 isoforms expressing variant exons (designated CD44v).

A CD44 isoform containing the last 3 exon products of the variable region (CD44V8-10, also known as epithelial CD44 or CD44E), is preferentially expressed on epithelial cells. The longest CD44 isoform expressing in tandem eight exons of the variable region (CD44V3-10) was detected in keratinocyte (25). 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 (29).

The homing function of CD44 was extensively explored in the late 1980s and early 1990s. Depletion of CD44⁺ cells from bone marrow by anti-CD44 mAb (using a "panning" procedure) prevented their ability to reconstitute the thymus of irradiated mice (60). This shows that CD44 is a homing receptor for migrating thymus progenitor cells. In contrast, the migration of lymphocytes into the lymph nodes and into other organs was normal, despite the removal of their CD44 receptor by anti-CD44 mAb treatment (57). Traffic of mouse lymphocytes to lymph nodes and their adhesion to HEV, as indicated by examination of frozen lymph node sections, was blocked, however, by Mel-14- or LFA-1-specific mAb treatment (60,57). These findings indicate that the HEV recognizing receptor of the mouse lymphocyte is selectin or integrin, rather than CD44. Unlike mouse lymphocytes, anti-CD44 mAb (e.g., Hermes-1) inhibits binding of human lymphocytes to lymph node HEV, suggesting that CD44 is the homing receptor of human cells (60).

In addition to its adhesive function, the CD44 receptor also serves as an accessory molecule, cooperating with other molecules in the transmission of growth signals delivered from the cell membrane to the cell nucleus (reviewed by 29). Several anti-CD44 mAbs (including V6- or V9-specific mAbs), or their Fab fragments, act in conjunction with immobilized anti-CD2 or anti-CD3 mAbs (usually at suboptimal levels) to enhance T-cell proliferation or IL-2 production (4,29, 60). Addition of anti-CD28 mAb to this assay system markedly augments these cellular activities (49; 60). The CD44-supported proliferative response is cyclosporin-A sensitive (60). Other anti-CD44 mAbs promote T-cell proliferation or IL-2 production, even if they are the sole stimulator in the *in vitro* assay system (4,29). In some cases, the promoting effect is dependent on the presence of macrophages in the *in vitro* system (29; 60). On the other hand, some anti-CD44 mAbs or their Fab fragments inhibit CD3-dependent cell proliferation or IL-2 production (60,29). The same anti-CD44 mAbs that inhibit the proliferation of CD3-activated T cells induced palmitoylation of the ligated CD44, suggesting that lipid modification of this molecule interferes with signaling pathways (60). Collectively, these findings emphasize the significance of "crosstalk" between CD44 and other cell surface molecules, leading to positive or negative growth-transducing signals.

Triggering of CTL or natural killer (NK) cell cytolytic activities with anti-CD44 mAbs has been observed (29). In this context, it was reported by our laboratory and others (3,4) that the lytic activity of CTL against the Fc γ receptor-positive EL4 or P815 mouse cell lines is activated by anti-CD44 mAb, used to link the CD44 receptor of the effector cell to the Fc γ receptor of the target cells (a procedure known as redirected killing). The anti-CD44 mAb triggers the CTL to release the trypsin-like esterase granzyme. Genestein, a protein tyrosine kinase inhibitor, blocked both the CD44-dependent proliferation and the cytotoxic activity of the effector cells (4). It was also found that CD44 activation of CTL causes tyrosine phosphorylation of several proteins within the 70- to 150-kDa range, but not of the 21 -kDa ζ chain of the T-cell receptor. The same anti-CD44 did not trigger the cytotoxic activity of NK cells (4). However, it has also been reported that the CD44-dependent redirected killing of target cells with anti-CD44 bispecific mAb is conditional upon IL-2 or IL-12 co-stimulation of the NK cells (60).

It was further demonstrated that CD44 is a potent signaling receptor. Early studies using anti-CD44 mAb to trigger the receptor in lieu of a physiological ligand established that CD44 is a costimulatory molecule on T cells (49, 50, 51, 52, 53). It is now known that similar effects can be triggered through CD44 following HA binding (45, 54, 55). The findings reported in one study (37) added to our understanding of CD44 as a signaling receptor and further support the notion that the function of CD44 as a signaling molecule is as important as its function as a cell adhesion receptor (50, 56).

Recently, three groups have reported that anti-CD44 mAb have potent anti-inflammatory activity in vivo. Administration of an anti-CD44 mAb (IM7) was found to prevent cutaneous delayed-type hypersensitivity (DTH) responses (57). This same anti-CD44 mAb was found to prevent the progression of ongoing collagen induced arthritis, blocking leukocyte infiltration and tissue swelling (58, 59). In these two studies the investigators reported that the anti-CD44 mAb mediated the rapid release of CD44 from the surface of CD44 positive leukocytes presumably preventing the CD44-HA mediated recruitment of leukocytes. The findings by a study (37) and those reported by others on the role of CD44 as a signaling molecule suggest that the potent anti-inflammatory effects resulting from the mAb mediated shedding of CD44 from leukocytes may prevent not only leukocyte recruitment but also prevent their activation. This dual effect might account for the potent anti-inflammatory activity of this anti-CD44 mAb in vivo.

Whereas lymph node homing of lymphocytes via CD44-dependent binding to HEV has been observed in humans but not in mice, the ability of lymphocytes or lymphoma cells to adhere to endothelial cells, in a CD44 dependent manner, has been demonstrated in both species (71,72). According to one report (73), the binding of lymphocytes to endothelial cells is conditional upon activation of the lymphocytes by anti-CD44 mAb. Another report showed that the CD44-dependent lymphocyte-endothelial cell interaction occurred only if the lymphocytes were activated by both phorbol dibutyrate and ionomycin, and the endothelial cells were stimulated by IL-1 (71). This finding suggests that inflamed endothelium can activate the CD44-dependent binding of lymphocytes. The interaction between cell surface CD44 and endothelium may be

vital to the hematogenic spread of tumors. In addition to CD44, cell surface selectin and integrins also are involved in the adherence of leukocytes to endothelial cells, an event that is essential to intra- and extravasation, as well as to cell traffic along the blood vessels (74).

It is likely that expression of CD44 by leukocytes is also requisite for events following endothelial adhesion, including penetration into the ECM of lymphoid tissues and subsequent locomotion in the matrix. It has been suggested that cell movement in the ECM is guided by local chemotactic signals (62), while "stop signals" halt the movement of cells, anchoring them to ECM components (63). Cell surface adhesion molecules, especially β 1 integrins (64,65) and HA binding proteins (including CD44) (63), are involved in cell adherence to ECM. However, HA receptors, including CD44, are used not only to anchor cells (such as chondrocytes, fibroblasts, epithelial cells, and hematopoietic cells) to the ECM, but also to facilitate the assembly and organization of the pericellular matrix (63). In vitro experiments have revealed that chondrocytes (63) and synovial cells (66) exploit the hyaluronan receptor to bind HA, which, in turn, interacts with cartilage proteoglycan (aggrecan) to form the pericellular matrix. This HA-mediated cell-proteoglycan interaction might be essential to the organization and retention of aggrecan molecules within the cartilage ECM, and it could also protect tumor cells from immune surveillance.

CD44 ligand-binding function on lymphocytes is strictly regulated, such that most CD44-expressing cells do not constitutively bind ligand (29). 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 (29). The affinity for hyaluronate can be experimentally controlled and depends on the cytoplasmic domain of CD44 (10).

The hyaluronan binding function of CD44 has been assayed in cell lines and in fusion proteins, termed CD44-Igs, consisting of the external domain of CD44 coupled to the hinge, CH2 and CH3 regions of human IgG1. These studies have shown that hyaluronan binding by

CD44 is regulated by the cells in which it is expressed, and that at least part of this regulation is determined by cell specific posttranslational modifications, especially N- glycosylation, of CD44 itself (20). Variant isoforms of CD44 determined by alternative splicing of 11 optional exons in the middle of the gene determine additional functions of CD44, as well as contributing to the regulation of hyaluronan binding. (20)

Role of CD44 in tumor cell metastasis:

Many cancer cell types as well as their metastases express high levels of CD44. Whereas some tumors, such as gliomas, exclusively express standard CD44, other neoplasms, including gastrointestinal cancer, bladder cancer, uterine cervical cancer, breast cancer and non-Hodgkin's lymphomas, also express CD44 variants. Hence CD44, particularly its variants, may be used as diagnostic or prognostic markers of at least some human malignant diseases (25). Furthermore, it has been shown in animal models that injection of reagents interfering with CD44-ligand interaction (e.g., CD44s- or CD44v-specific antibodies) inhibit local tumor growth and metastatic spread. These findings suggest that CD44 may confer a growth advantage on some neoplastic cells and, therefore, could be used as a target for cancer therapy (25). Several lines of evidence indicate that interaction between the hyaluronan receptor CD44, expressed on tumor cells, and host tissue stromal hyaluronan can enhance growth and invasiveness of certain tumors. Disruption of CD44-hyaluronan interaction by soluble recombinant CD44 has been shown to inhibit tumor formation by lymphoma and melanoma cells transfected with CD44. Studies indicate that hyaluronan oligomers injected at concentrations as low as 1 mg/ml can markedly inhibit B16F10 melanoma growth, providing a potentially attractive reagent for the control of local tumor development (32). Many studies have investigated the pattern of CD44 distribution in tumours and some observations suggest that certain cells do not use CD44 in tumorigenesis or in the production of metastases. However, the data are extremely conflicting, and further studies are needed to establish the prognostic value of CD44 and its variant isoforms. It is hoped that identification of CD44 variants expressed on cancer but not on normal cells will lead to the development of anti-CD44 reagents restricted to the neoplastic growth.

Role of CD44 on macrophage functions:

Macrophages, which constitutively express CD44, are active phagocytic cells capable of ingesting and digesting exogenous antigens. A number of antimicrobial and cytotoxic substances produced by activated macrophages are responsible for the intracellular destruction of phagocytosed microorganisms. In addition, these toxic substances can be released from macrophages to mediate potent antitumor activity.

Macrophages bind Ab-coated tumor target cells and are responsible for Ab-dependent cell-mediated cytotoxicity using Fc γ RII (CD32), which is constitutively expressed, and Fc γ RI (CD64), which is induced by INF γ or granulocyte CSF. In addition, macrophages can exhibit cytotoxic activities in the absence of Abs, and cytokine-activated macrophages are able to mediate spontaneous tumor cell lysis.

Studies have shown that murine bone marrow-derived macrophages are stimulated by HA to synthesize mRNA for IL-1 β and tumor necrosis factor α (29). This activity was inhibited by CD44-specific mAb suggesting that CD44-HA interaction on the macrophage cell surface might initiate a cytokine cascade. Stimulation of macrophages with CD-44 specific Abs can result in release of cytokines: TNF, IL-1 and macrophage colony stimulating factor (29). It is possible that monoclonal antibody (mAb) 9F3, and IM7 directed against murine CD44 expressed on macrophages, can trigger the lytic activity of macrophages and redirect macrophage-mediated lysis to Fc receptor-positive target cells.

It has been reported that lymphoid cell lines (31), B cell hybridomas (23), and IL 5-activated B cells (33) 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 (23). These results along with earlier data showing inhibition by anti-CD44 *mAb's* of B cell

lymphopoiesis in long-term bone marrow cultures (23) suggests that the CD44-HA interaction may be important in B cell differentiation.

The role of CD44 as a hyaluronan receptor (43, 44, 45) has been known for many years and is consistent with the hypothesis that CD44 is a cell adhesion molecule (46). In recent years many studies designed to explore the role of CD44 as a cell adhesion receptor have given rise to a large body of data demonstrating that this is a major function of CD44. For example, the results of two recent studies provide evidence that CD44-HA interactions mediate the binding of lymphocytes to cultured endothelial cells (47) and tonsillar stromal cells (48). These findings indicate that CD44 significantly contributes to the recruitment of leukocytes to sites of inflammation and to their migration through lymphatic tissues.

It is not possible to review in this short introduction the full complexity of the CD44 antigen: its multiple isoforms, its multiple ligands, and its varied and complex biological activities. A recent report has raised new and interesting questions regarding the function of CD44 (37). For example, are all CD44 isoforms capable of binding HA fragments? Does the interaction between CD44 and HA fragments in other cell types lead to a signaling event? In particular, are the previously reported angiogenic properties of HA fragment mediated by CD44 molecules expressed on vascular endothelial cells? Is the ability of CD44 to bind HA fragments regulated in the same way as the interaction between CD44 and the HA polymer? It is important to point out that much remains to be learned about the function of the CD44 antigen and one of its ligands, HA.

IV. Hyaluronic Acid and its role in immunoregulation:

Hyaluronic acid is one of a group of polysaccharides typically found in the connective tissues of vertebrates, which were formerly known as acid mucopolysaccharides and are now designated glycosaminoglycans. Glycosaminoglycans are unbranched single-chain polymers of disaccharide units containing N-acetylhexosamine and hexose. The second sugar is a hexuronic

acid in all except keratan sulphate, which contains galactose instead. Glycosaminoglycans other than HA share several other characteristics. All contain sulphate groups, and their polysaccharide chains are relatively short (<50 kDa, commonly 15-20 kDa). Their synthesis takes place in the endoplasmic reticulum and Golgi bodies, and they are substituted in peptide cores, often with a variety of other saccharides, to form proteoglycans. Different kinds of sulphated glycosaminoglycans can be joined to a common peptide core, which with accompanying variations in the peptides and other saccharides provides for great variety in proteoglycan structure and consequently, in their potential for reactivity with other components of extracellular matrix and with various cells. Most proteoglycans are notable for one or more strong associations with fixed matrix structures or cells and are relatively immobile.

HA is quite distinct from other glycosaminoglycans in most of these respects. Its primary structure contains a linear unbranched polysaccharide consisting of repeating disaccharide units of (1-4)-D-glucuronic acid-beta-(1-3)-D-N-glycosaminoglycan. HA is a major component of connective tissue and differs from other glycosaminoglycans because it is not covalently linked to a core protein (34), which is consistent with its synthesis in the plasma membrane rather than the Golgi. Although it consists of a single polysaccharide chain like other glycosaminoglycans, its molecular weight (relative molecular mass; M_r) usually reaches the millions; in normal synovial fluid, for example, the weight-average is about 7×10^6 , which if straightened would extend to $> 15 \mu\text{m}$. Its uniformity of structure would seem at first sight to restrict its biological roles, but this limitation is overcome by the numbers of specific HA binding sites that have evolved in other matrix molecules and on cell surfaces. Moreover, it has other distinctive attributes arising from its extraordinary molecular mass, which underlie its distinctive role in extracellular matrix.

HA is a prominent molecule in the extracellular matrix and has recently been reported to serve as a ligand for CD44. The ECM fills the space between cells and the components of the ECM perform a key role in the structure and organization of the ECM (35). They also communicate with the cell interior and thus modulate cell adhesion, proliferation, and differentiation (29). HA is enriched when there is rapid tissue proliferation, regeneration and repair (36). The highest HA

concentrations are found in typical connective tissues such as umbilical cord, synovial fluid, skin and the vitreous body. Notable amounts are also present in lung, kidney, brain and muscle but very little in liver. The lowest concentration is found in blood serum. Hyaluronan is synthesized in the plasma membrane by a membrane-bound protein whose genetic code has recently been determined in bacteria, mouse and human (60). This adds sugar units from nucleotide precursors to the chain on the cytoplasmic aspect of the membrane and translocates the growing chain to the pericellular space. It is synthesized by fibroblasts, chondrocytes, and mesothelial cells (60). The production of HA by human lung fibroblasts is stimulated by cytokines TNF, IFN- γ , and IL-1 and further augmented by a combination of TNF and IFN-alpha, or of TNF and IL-1, as was shown in an *in vitro* assay (60). Hyaluronate is an important component of the ECM, filling the intercellular spaces. Within the ECM, HA noncovalently interacts with proteoglycans, the binding stabilized by a link protein (60) Because of the striking physicochemical properties of hyaluronan solutions, various physiological functions have been assigned to it, including lubrication, water homeostasis, filtering effects and regulation of plasma protein distribution. In addition, HA regulates cell-cell adhesion and the cell's spatial orientation and traffic, as well as its growth and differentiation (60). Consequently, HA is involved in various biological processes such as inflammation, wound healing, and tissue remodeling (60), as well as morphogenesis (60). Hyaluronan also interacts with the cell surface to form a "coat," which may act as a protective cellular barrier (60).

Studies have presented evidence that the binding of low molecular weight fragments of hyaluronan to alveolar macrophages via CD44 elicits the expression of a number of pro-inflammatory chemokines (37). These observations extend earlier findings showing that HA fragments are capable of activating NF- κ B (38) and induce the expression of insulin-like growth factor-1 by murine macrophages (39). These studies bring together two disparate and previously unconnected observations regarding the expression and biological activity of HA fragments. The first observation is that HA fragments are present at abnormally high levels in the joints of patients with rheumatoid arthritis (RA) and in other inflammatory conditions (40, 41). These HA fragments are thought to arise primarily as a result of activated leukocyte driven extracellular matrix degradation at sites of inflammation and their presence has been proposed as a biological marker of disease. The second observation is that HA fragments but not HA polymers have

angiogenic activity, a process which has been proposed to play a key role in the maintenance and progression of RA and other chronic inflammatory diseases (42). The findings by Nobel and his colleagues suggest that the high levels of HA fragments found in inflamed tissues bind to leukocytes and other CD44 expressing cells and trigger a cascade of signaling events which are involved in maintaining and/or amplifying the inflammatory response. It is of interest to note that the biological activities elicited by the HA fragments are distinct from those elicited by the HA polymers. While HA polymers are components of extracellular matrix and a substrate for CD44 mediated cell adhesion, HA fragments are signaling molecules which alert the immune system that significant tissue damage has occurred at a site of inflammation.

In addition, HA supports the migration of invasive tumors (60). Indeed, tumor invasion is sometimes observed in regions with high concentrations of HA, such as the medullary and papillary interstitium of renal tissue, the submucosa of the gastrointestinal tract, around the centrilobular veins, and beneath the capsule of the liver (60). Some tumors synthesize and release HA into their immediate environment (60), while others stimulate the production of HA by surrounding fibroblasts (60). Zhang and colleagues (1995) reported that intravenously injected mouse melanoma cells (BI6-FI) expressing a high level of HA on their surfaces formed a greater number of lung metastases than did melanoma cells bearing low amounts of surface HA. Collectively, these findings emphasize the central role of HA in metastasis.

In animals and man, the half-life of hyaluronan in tissues ranges from less than 1 to several days. It is catabolized by receptor-mediated endocytosis and lysosomal degradation either locally or after transport by lymph to lymph nodes which degrade much of it. The remainder enters the general circulation and is removed from blood, with a half-life of 2-5 min, mainly by the endothelial cells of the liver sinusoids.

V. The role of hyaluronic acid and anti- CD44 antibodies in preventing Vascular Leak Syndrome induced by Interleukin-2

The major dose-limiting toxicity of interleukin-2 (IL-2) which is used in the treatment of cancer is vascular leak syndrome (VLS). VLS is characterized by an increase in vascular permeability accompanied by extravasation of fluids and proteins resulting in interstitial edema and organ failure (37). Previous studies from our laboratory and elsewhere demonstrated that cytotoxic lymphocytes use CD44 in mediating endothelial cell injury. Cytotoxic T lymphocytes, double-negative T cells and natural killer (NK) cells upon activation express high levels of CD44 and mediate efficient MHC –unrestricted TCR-independent lysis following ligation of CD44. Such CD44-mediated cytotoxicity may play an important role in protection against viral infections and cancer (5). However, dysregulation in the interaction between activated cytotoxic lymphocytes expressing CD44 and endothelial cells bearing the appropriated ligand such as the hyaluronate (HA) may cause endothelial cell injury.

We hypothesize that blocking CD44-HA interaction in vivo using exogenous anti-CD44 mAbs or exogenous hyaluronic acid may prevent endothelial cell injury by cytotoxic lymphocytes. In the current study we tested this hypothesis.

VII. References

1. Roitt, I., Brostoff, J., Male, D. (1998) Immunology, fifth edition. Mosby International Ltd, pp 1-41.
2. Pericle F., Sconocchia, G., Titus, J. A., Segal, D.M. (1996) CD44 is a cytotoxic triggering molecule on human polymorphonuclear cells. *J Immunol* 157:4657-63. 1996.
3. 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 gp90. *Proc. Natl. Acad. Sci. USA* 88: 7877.
4. Galandrini R, Albi N, Tripodi G, Zarccone D, Terenzi A, Moretta A, Grossi CE, Velardi A. (1993) Antibodies to CD44 trigger effector functions of human T cell clones. *J Immunol.* 1993 15;150(10):4225-35.
5. Rafi-Janajreh AQ, Nagarkatti PS, Nagarkatti M. (1998) Role of CD44 in CTL and NK cell activity. *Front Biosci.* Jul 1;3:D665-71.
6. Grossi, C.E., Zarccone, D., Tenca, C., De-Rossi, G. Mandelli, F. (1992) Expression of Adhesion molecules in Lymphoproliferative Disorders. *Leukemia.* Suppi 4: 35-7.
7. Wegner, C.D., Gundel, R.H., Letts, L.G. (1993) Efficacy of monoclonal antibodies against adhesion molecules in animal models of asthma. *Agents-Actions-Suppl* 43: 151-62.
8. Springer, T. A. (1 990) Adhesion receptors of the immune system. *Nature* 346, 425433.
9. Pitzalls.C. (1 993) Adhesion and migration of inflammatory cells. *Clin-ExpRheumatol.* 1 Suppl 8:S71-6.
10. Kincade, P.W. (1992) Cell interaction molecules and cytokines which participate in B lymphopoiesis. *Baillieres-Clin-Haematol.* 5(3): 575-98.
11. Benton, L.D., Khan, M., Greco, R.S. (1993) Integrins, adhesion molecules and surgical research. *Surg-Gynecol-Obstet.* 177(3): 311-27.
12. Humphries, M.J., Mould, A.P., Tuckwelf, D.S., (1993) Dynamic aspects of adhesion receptor function--integrins both twist and shout. *Bioessays.* 15(6): 391-75.
13. Hogg, N., Landis, R.C. (1993) Adhesion molecules in cell interactions. *Current Opinion in Immunol* 5(3): 383-90.

14. Palma-Carlos, A.G., Palma-Carlos, M.L. (1993) Adhesion molecules in immunity and inflammation. *Allerg-Immunol-Paris*. 25(1): 4-9.
15. Nagarkatti, P., Seth, A., Nagarkatti, M., Muthusamy, N., Rychilk, B., Subbarao, B. (1 989) A Specific Defect in the Proliferative Capacity of B Cells from Old Mice Stimulated with Autoreactive T Cells. *Cellular Immunology*. 120, 102-113.
16. Hammond, D.M., Nagarkatti, P., Gote, L., Seth, A., Hassuneh, M., Nagarkafti, M. (1 993) Double-negative T Cells from MRL-Ipr/lpr Mice Mediate Cytotoxic Activity when Triggered through Adhesion Molecules and Constitutively Express Perforin Gene. *J. Exp. Med.* 178, 2225-2230.
17. Lider O., Hershkovich R., Kachalsky S.G. (1995) Interactions of migrating T lymphocytes, inflammatory mediators, and the extracellular matrix. *Crit Rev Immunol*. 15(3-4):271-83. Review.
18. Shimizu, Y., van Seventer, G.A., Horgan, K.J., and Shaw, S. (1990) Regulated expression and function of three VLA (BI) integrin receptors on T cells. *Nature (London)* 345: 250-253.
19. Shimizu, Y., Shaw, S., (1991) Lymphocyte interactions with extracellular matrix *Faseb J*. 5: 2292-2299.
20. Lesley, J., Hayman, R. (1998) CD44 structure and function. *Front Biosci*. Jul 1;3:D616-30.
21. Yurchenco, P.D., and Schiffny, J.C. (1990) Molecular architecture of basement membranes. *FASEB*. 4:1577-1590.
22. Makgoba, M. W., Sanders, M. E., and Shaws, S. (1989) The CD2-LFA-3 and LA-1ICAM-1 pathways: relevance to T-cell recognition, *Immunol. Today*. 10, 417-422.
23. Miyake, K., Underhill, C. B., Lesley, J., and Kincaid, P. W. (1990) Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 172: 69-75.
24. Britz, J. S., and Hart, G. W. (1983) Biosynthesis of glycosaminoglycans by epithelial and lymphocytic components of murine thymus. *J. Immunol* 130, 1848-1855.
25. Noar, D., Sionov, R. V., Ish-Shalom, D. (1997) CD44: structure, function, and association with the malignant process. *Adv Cancer Res*. 71:241-319. Review

26. Bevilacqua, M.P. (1993) Endothelial-Leukocyte Adhesion Molecules. *Annu. Rev. Immunol* 11:767-804.
27. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* 61: 1303-1313.
28. Underhill, C., (1992) CD44: The hyaluronan receptor. *J. of Cell Science*. 103. 293-298.
29. Lesley J., Hyman, R., Kincade, P. (1993) CD44 and Its Interactions with Extracellular Matrix. *Advances In Immunology*. 54: 271-335.
31. Lesley J., Schulte, R., and Hymann, R. (1990), Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. *Exp. Cell Res*. 187:224-233.
32. Zeng C., Toole B. P., Kinney S. D., Kuo J. W., Stamenkovic I. (1998) Inhibition of tumor growth in vivo by hyaluronan oligomers. *Int J Cancer*. 77(3):396-401.
33. Murakami, S., Miyake, K., June, C. H., Kincade, P. W., and Hodes, R. J., (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: 3618-3627.
34. Iwata, H. (1993) Pharmacologic and Clinical Aspects of Intraarticular Injection of Hyaluronate. *Clinical Orthopaedics and Related Research*. 289: 285-291.
35. Tate, D.J., Oliver, P.D., Miceli, M.V., Stem, R., Shuster, S., Newsome, D.A. (1993) Age-dependent change in the hyaluronic acid content of the human chorioretinal complex. *Arch-Ophthalmol*. 111(7): 963-7.
36. Stem, M., Stem, R. (1992) An ELISA-like assay for hyaluronidase and hyaluronidase inhibitors. *Matrix*. 12(5): 397-403.
37. McKee, C.M., Penno, M.B., Cowman, M., Bao, C., and Noble, P.W. (1996) Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J. Clin. Invest*. 98: 2403-2413
38. Noble, P.W., McKee, C.M., Cowman, M., and Shin, H. (1996) Hyaluronan fragment activates an NF- κ B/I- κ B α autoregulatory loop in murine macrophages. *J. Exp. Med*. 183: 2373-2378.

39. Noble, P.W. , F.R. Lake , P.M. Henson , and D.W.H. Riches (1993) Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor- α -dependent mechanism in murine macrophages. *J. Clin. Invest.* 91: 2368-2377
40. Bjermer, L. , R. Lundgren , and R. Hallgren (1989) Hyaluronan and type III procollagen peptide concentrations in bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis. *Thorax.* 44: 126-131
41. Poole, A. , and P. Dieppe (1994) Biological markers in rheumatoid arthritis. *Semin. Arthritis Rheum.* 23S: 17-31.
42. West, D.C. , I.N. Hampson , F. Arnold , and S. Kumar (1985) Angiogenesis induced by degradation products of hyaluronic acid. *Science (Wash. DC).* 228:1324-1326.
43. Aruffo, A., I. Stamenkovic, M. Melnick, C.B. Underhill, and B. Seed. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell.* 61(7): 1303-1313.
44. Culty, M., K. Miyake, P. W. Kincade, E. Silorski, E.C. Butcher, and C. Underhill. 1990. The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. *J. Cell Biol.* 111:2765-2774.
45. Miyake, K. , C.B. Underhill , J. Lesley , and P.W. Kincade (1990) Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 172: 69-75.
46. Jalkanen, S., R.F. Bargatze, J. de los Toyos, and E.C. Butcher. 1987. Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95-kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. *J. Cell Biol.* 105:983-990.
47. DeGrendele, H.C. , P. Estess , L.J. Picker , and M.H. Siegelman (1996) CD44 and its ligand hyaluronate mediate rolling under physiologic flow: a novel lymphocyte-endothelial cell primary adhesion pathway. *J. Exp. Med.* 183: 1119-1130.
48. Clark, R.A. , R. Alon , and T.A. Springer (1996) CD44 nd hyaluronan-dependent rolling interactions of lymphocytes on tonsillar stroma. *J. Cell Biol.* 134: 1075-1087.
49. Huet, S. , H. Groux , B. Caillou , H. Valentin , M. Prieur , and A. Bernard (1989) CD44 contributes to T cell activation. *J. Immunol.* 143: 798-801.

50. Shimizu, Y. , G.A. Van Seventer , R. Siraganian , L. Wahl , and S. Shaw (1989) Dual role of the CD44 molecule in T cell adhesion and activation. *J. Immunol.* 143: 2457-2463.
51. Denning, S.M. , P.T. Le , K.H. Singer , and B.F. Haynes (1990) Antibodies against the CD44 p80, lymphocytes homing receptor molecule augment human peripheral blood T cell activation. *J. Immunol.* 144: 7-15.
52. Rothman, B.L. , M.L. Blue , K.A. Kelley , D. Wunderlich , D.V. Mierz , and T.M. Aune (1991) Human t cell activation by OKT3 is inhibited by a monoclonal antibody to CD44. *J. Immunol.* 147: 2493-2499.
53. Pierres, A. , C. Mawas , and D. Olive (1992) A unique CD44 monoclonal antibody identifies a new T cell activation pathway. *Eur. J. Immunol.* 22: 413-417.
54. Galandrini, R. , E. Galluzzo , N. Albi , C.E. Grossi , and A. Velardi (1994) Hyaluronate is costimulatory for human T cell effector functions and binds to CD44 on activated T cells. *J. Immunol.* 153: 21-31.
55. Lesley, J. , N. Howes , A. Perschl , and R. Hyman (1994) Hyaluronan binding function of CD44 is transiently activated on T cells during an in vivo immune response. *J. Exp. Med.* 180: 383-387.
56. 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. *Immunol. Today.* 10(12):423-428.
57. Camp, R.L. , A. Scheynius , C. Johansson , and E. Puré (1993) CD44 is necessary for optimal contact allergic responses but is not required for normal leukocyte extravasation. *J. Exp. Med.* 178: 497-507.
58. Verdrengh, M. , R. Holmdahl , and A. Tarkowski (1995) Administration of antibodies to hyaluroan receptor (CD44) delays the start and ameliorates the severity of collagen II arthritis. *Scand. J. Immunol.* 42: 353-358.
59. Mikecz, K. , F.R. Brennan , J.H. Kim , and T.T. Glant (1995) Anti-CD44 treatment arogates tissue oedema and leukocyte infiltration in murine arthritis. *Nat. Med.* 1: 558-563
60. Noar, D., Sionov, R. V., Ish-Shalom, D. (1997). CD44: Structure, Function, and Association with the Malignant Process. *Adv Cancer Res* 71: 242-319. Review

61. Rafi-Janajreh AQ, Chen D, Schmits R, Mak TW, Grayson RL, Sponenberg DP, Nagarkatti M, Nagarkatti PS. 1999. Evidence for the involvement of CD44 in endothelial cell injury and induction of vascular leak syndrome by IL-2. *J Immunol.* 1999 Aug 1;163(3):1619-27.
62. Dustin, M. L., Springer T. A. (1991). Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu Rev Immunol* 9:27-66.
63. Knudson, C. G., and Knudson, W. (1993). Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J.* 7, 1233-1241.
64. Helmer, M. E. (1990) .VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu. Rev. Immunol.* 8, 365-400.
65. Yamada, K. M. (1991). Adhesive recognition sequences. *J. Biol. Chem.* 266, 12809-12812.
66. Mikecz, K., Brennan, F. R., Kim, J.H., Glant, T.T. (1995). Anti-CD44 treatment abrogates tissue oedema and leukocyte infiltration in murine arthritis. *Nature Medicine* 1, 558-563.

Chapter 3.

Agonistic effect of Hyaluronic acid and Antagonistic effect of anti-CD44 mAbs on IL-2 Induced vascular Leak Syndrome.

Abstract

Immunotherapy with IL-2 is accompanied by severe toxicity leading to development of vascular leak syndrome (VLS). Previous studies from our laboratory demonstrated that CD44 knockout mice exhibit marked decrease in IL-2 induced VLS, thereby suggesting a role for CD44 in VLS. In the current study we tested whether use of mAbs against CD44 or hyaluronic acid (HA), the ligand for CD44, can abrogate IL-2 induced VLS. Administration of IL-2 (75,000 U/mouse, three times a day for 4 days) into C57BL/6 mice triggered significant VLS in the lungs and liver. Interestingly, HA caused a marked increase in IL-2-induced VLS in the lungs and liver. In contrast, use of anti-CD44 mAbs reduced IL-2-induced VLS in the lungs and liver. The change in VLS seen following HA or anti-CD44 mAbs treatment was not due to any defect in lymphocyte migration or homing to various organs because histopathological studies in these mice demonstrated significant and often greater perivascular infiltration of lymphocytes when compared to mice treated with IL-2 alone. However, HA treatment exhibited a marked increase in IL-2-induced lymphokine-activated killer (LAK) cell activity while anti-CD44 mAbs treatment led to a significant decrease in IL-2-induced LAK cell activity. These studies demonstrate that HA or anti CD44 mAbs may serve as a useful tool to selectively alter the LAK activity as well as to prevent the toxicity induced by IL-2. Altering CD44-HA interactions in vivo may offer a novel therapeutic approach to prevent endothelial cell injury by cytotoxic lymphocytes in a variety of clinical diseases.

INTRODUCTION

A major drawback of immunotherapy with interleukin 2 (IL-2) is development of vascular leak syndrome (VLS). Manifestations of VLS include fluid retention, increase in body weight,

peripheral edema, pleural and pericardial effusions, ascites, anasarca and, in severe form, signs of pulmonary and cardiovascular failure (1).

Studies from our laboratory and elsewhere have suggested that IL-2 induced VLS may result from actual damage to the endothelial cells caused by cytotoxic lymphocytes (2, 3). In contrast, some types of endothelial cell damage may result from participation of neutrophils and complement components (4). The fact that cytotoxic T lymphocytes are involved in the induction of VLS was demonstrated in a recent study from our laboratory in which administration of a CTL clone into a syngeneic irradiated mouse along with IL-2 led to a significant induction of VLS in vivo (5). Furthermore, we demonstrated that CD44 knockout (KO) mice were more resistant to IL-2 induced apoptosis and toxicity when compared to CD44 wild-type mice (6). IL-2 administration increased the expression of CD44 on lymphocytes activated killer (LAK) cells which in turn bind to the endothelial cells that express hyaluronic acid (HA) and mediate cytotoxicity (6, 7).

Endothelial cell injury is one of the most widely noted phenomena in a variety of clinical diseases. For example, in murine lymphocytic choriomeningitis viral infection, massive delayed-type hypersensitivity reaction occurs in the cerebrospinal fluid. It has been speculated that virally activated CD8⁺ T cells kill the endothelial cells, leading to massive extravasation of monocytes and CD4⁺ T cells via the subarachnoid space (8). In multiple sclerosis, damage to the blood-brain barrier has been known to occur after injury to the endothelial cells by cytotoxic T cells (9). Also, in autoimmune disease models involving vasculitides, the lesions have been associated with infiltration of lymphocytes and macrophages at the vascular wall structure (10). Similarly, in arteriosclerosis, endothelial cell damage and inflammatory cell activation have been shown to contribute to the further development of the cardiovascular disease (11, 12). Moreover, endothelial cell damage by host-derived lymphocytes is known to cause allograft rejection (13, 14). Thus identification of mechanisms by which immune cells cause endothelial cell damage may provide useful therapeutic modalities to prevent toxicity and pathogenesis in a wide range of clinical diseases.

CD44 is a family of cell surface glycoproteins with proposed functions in extracellular matrix (ECM) binding, cell migration, lymphopoiesis, and lymphocyte homing (15). The principal ligand of CD44 has been identified as hyaluronic acid (HA), a major component of the ECM. CD44 has been implicated in a variety of immunological functions including T cell (7, 16) and, B cell (17) activation and functions. We and others have also shown that CD44 can serve as a cytotoxic-triggering molecule on CTL and NK/LAK cells (7, 18). Thus, the interaction between CD44 expressed on cytotoxic lymphocytes and HA expressed on endothelial cells may lead to killing of the latter cells (19).

Having demonstrated that CD44 is involved in the endothelial cell injury during IL-2 treatment (6), we tested whether administration of HA or anti-CD44 mAbs would abrogate endothelial cell damage and vascular leak. In the current study we report that HA and anti-CD44 mAbs have opposite effects when administered with IL-2. While anti-CD44 mAbs block IL-2 mediated VLS, HA enhances the toxicity by increasing the vascular leak. These studies have significant clinical impact in the treatment of vascular injury.

Materials and Methods

Mice

Adult female C57BL/6 mice were purchased from the National Institutes of Health (Bethesda, MD).

Interleukins and reagents

Recombinant IL-2 was kindly provided by Hoffmann-La Roche (Nutley, NJ) and by Dr. C. Reynolds (National Institutes of Health, Bethesda, MD). HA from human umbilical cord was purchased from Sigma Chemical Co. (St. Louis, MO)

Quantitation of VLS

Vascular leak was studied by measuring the extravasation of Evans blue, which when given i.v. binds to plasma proteins, particularly albumin, and following extravasation can be detected in various organs as described previously (6, 20). VLS was studied by injecting IL-2 as previously described (3, 6). Groups of five mice were injected i.p. with 75,000 U of rIL-2 or PBS as a control, three times a day for 3 days. On day 4, they received one injection of IL-2 in the morning and 2 h later, were injected i.v. with 0.1 ml of 1% Evans blue in PBS. After 2 h, the mice were sacrificed, and the heart was perfused with heparin in PBS. The lungs and liver, where maximum extravasation is known to occur, were harvested and placed in formamide at 37°C overnight. The Evan's blue in the organs was quantitated by measuring the absorbance of the supernatant at 650 nm with a spectrophotometer. The VLS seen in IL-2-treated mice was expressed as the percent increase in extravasation compared to that seen in PBS-treated controls and was calculated as: $(\mu\text{g of dye in the organ of IL-2-treated mice}) - (\mu\text{g of dye in the organ of PBS-treated controls}) / (\mu\text{g of dye in the organ of PBS-treated control}) \times 100$. Each mouse was individually analyzed for vascular leak, and the data from five mice were pooled and expressed as the mean \pm SEM percent increase in VLS in IL-2-treated mice compared with that in PBS treated controls.

Effect of HA in IL-2 induced VLS

C57BL/6 wild-type mice were treated with IL-2 as described above along with i.p. injections of 2mg/mouse/day of human umbilical cord HA (mol. Wt. 5-8 million Dalton , Sigma Chemical Co. St. Louis, MO) for a period of 4 days. The control mice received IL-2 and for a similar duration. On day 4, the mice were studied for the degree of VLS as described above.

Use of anti-CD44 mAbs to block VLS

C57BL/6 wild-type mice were treated with IL-2 as described above along with 500 μg of anti-CD44 (9F3) Abs/mouse/day for a period of 4 days. The control mice received IL-2 and 500 μg of normal rat IgG/mouse/ day for a similar duration. The VLS seen in these IL-2-treated mice was studied by extravasation of the dye as described above.

Histology

For histopathological studies, groups of five separate mice were injected with IL-2 or PBS and treated with HA or anti-CD44 mAbs as described earlier and on day 4, lungs and liver were fixed in 10% buffered neutral formalin solution. The organs were processed routinely, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Perivascular infiltration was scaled by counting the number of lymphocytes infiltrating the vessel and perivascular space and averaging the minimum and maximum range for each group (3). Three samples were used each for lung, and the liver.

Studies on NK/LAK cells

NK/LAK activity in mice undergoing IL-2 induced VLS was studied using YAC-1 cells as targets. Wild-type mice were injected with IL-2 or IL-2 and treatment as described previously. On day 4, spleen cells were passed over nylon wool and non-adherent cells were tested for cytotoxicity against ³H-labeled YAC-1 target cells.

JAM TEST

JAM test was used to study the ability of LAK cells to induce apoptosis in tumor cells. DNA fragmentation was assessed by labeling the target YAC-1 cells with ³H thymidine as described by Matzinger(21) and modified in our laboratory (22). Briefly, target cells consisting of YAC-1 Cells were labeled with 5 μ Ci/mL of ³H-thymidine (ICN, Irvine, CA) by incubating in a humidified atmosphere with 5% CO₂ at 37°C for 6 hours. Excess ³H-thymidine was washed off, and 10 x 10³ target cells were mixed with varying numbers of effector LAK cells in 96-well plates (Costar, Cambridge, MA). The plates were incubated at 37°C for 12 hours, and the cells were harvested onto glass-fiber filters using a semiautomatic cell harvester. The filters were dried and added to liquid scintillation fluid, and radioactivity was counted in a beta counter (Tm analytical).

The mean percentage of DNA fragmentation was calculated from triplicate culture using the following formula: % DNA fragmentation = $(S - E)/S \times 100$, where S is retained DNA in the absence of killers (spontaneous) and E is experimentally retained DNA in the presence of killers.

Statistical analysis

The VLS data in different strains of mice were compared using Student's t test, and $p < 0.05$ was considered statistically significant.

Results

The effect of HA on IL-2 induced VLS

To investigate the effect of HA on IL-2 induced VLS, groups of five C57BL/6 adult mice were injected intraperitoneally with 75,000 U IL-2 or PBS as a control, three times a day for 4 days. In addition to IL-2, one group received one injection of hyaluronic acid (2mg/day for four days). Two hours after the last injection, on the fourth day, all mice were injected with 0.1ml of 1% Evans blue dye in PBS, and the VLS was studied by determining the extravasation of Evans blue in the lungs and livers.

Fig.1A shows a representative experiment in which the wild-type (BL/6) mice displayed significant VLS following IL-2 (75000 U) administration in the lungs and liver when compared with the PBS-treated group. However, in the HA+ IL-2 treated group, there was a statistically significant increase in VLS in the lungs and livers when compared to the group treated with IL-2 alone. Similar results were observed with a lower dose of IL-2 (10,000 U) (Fig.1B). These experiments were repeated three times with consistent results. These data suggested that HA aggravates IL-2 induced VLS in the lungs and liver.

Effect of mAbs against CD44 on IL-2 induced VLS

To investigate the effect of anti-CD44 mAbs on IL-2 induced VLS, groups of five C57BL/6 adult mice were injected intraperitoneally with 75,000 U IL-2, three times a day for 4 days. In addition to IL-2, these mice received injections of anti-CD44 mAbs (500 ug /day for four days) or the control antibodies consisting of similar concentrations of normal rat IgG. Two hours after the last injection, on the fourth day, all mice were injected with 0.1ml of 1% Evans blue dye in PBS, and the VLS was studied by determining the extravasation of Evans blue in the lungs and livers. As shown in Fig. 2A, injection of mAbs against CD44 significantly inhibited VLS in the lungs and liver when compared to the VLS induced in mice injected with IL-2 + control Abs. The inhibition caused by anti-CD44 mAbs in VLS was also noted with a lower dose of IL-2

(10000U) as shown in Fig.2B. These data suggested that anti-CD44 mAbs served as CD44 antagonist capable of blocking IL-2 induced VLS.

Histopathological studies in organs exhibiting VLS

Inasmuch as CD44 is involved in lymphocyte homing to organs, histopathologic studies were conducted to investigate whether the decrease or the increase in VLS was due to the changes in the ability of lymphocytes to migrate to the lungs and liver. Mice were injected with PBS or IL-2 in addition to the treatments with HA or anti-CD44 mAbs, as described in Fig.1 and 2 respectively. On day 4, the organs were harvested and processed for histopathological analysis following staining with hematoxylin and eosin.

The PBS treated mice, the HA alone treated mice and the anti-CD44 mAbs treated mice did not exhibit any perivascular lymphocytic infiltration in the lungs and liver (Fig. 3). In contrast, treatment with IL-2 alone at 10,000U (Fig. 4A, 5A) or 75,000U (Fig. 4B, 5B) caused significant perivascular lymphocytic infiltration and increased edema in the lungs (Fig. 4A, B) as well as in the liver (Fig. 5A, B), consistent with earlier studies (6). When HA was administered along with 10,000U of IL-2 (Fig. 4C, Fig. 5C) or 75,000U of IL-2 (Fig. 4D, Fig. 5D), there was a marked increase in the perivascular infiltration and edema in both the lungs (Fig. 4C, D) and the liver (Fig. 5C, D).

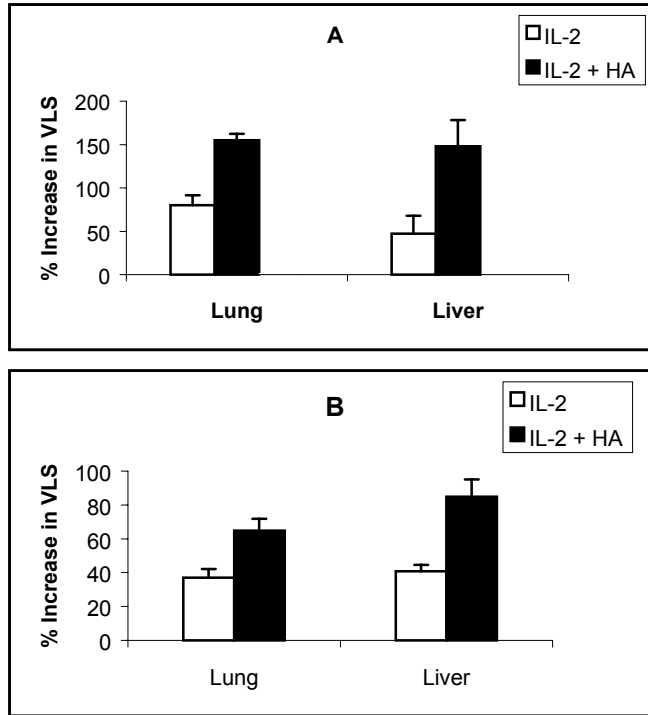


FIGURE 1. Increased VLS in IL-2 treated mice following HA administration. Groups of five C57BL/6 wild-type mice were injected i.p. with 75,000 U of IL-2 alone three times daily for 3 days and once on day 4 or IL-2 and 2 mg HA/mouse/day for 4 days (A). The control mice received PBS alone for a similar duration. Two hours later the mice were injected with 1 % Evans blue, and VLS was studied by determining the extravasation of Evans blue dye in the lungs and liver. B shows a similar experiment, except that the mice were treated with low dose of IL-2 (10,000U). vertical bars represent the percent increase in VLS \pm SEM seen following IL-2 alone or IL-2 and HA treatment when compared to that seen in the PBS-treated controls as described in *Materials and Methods*.

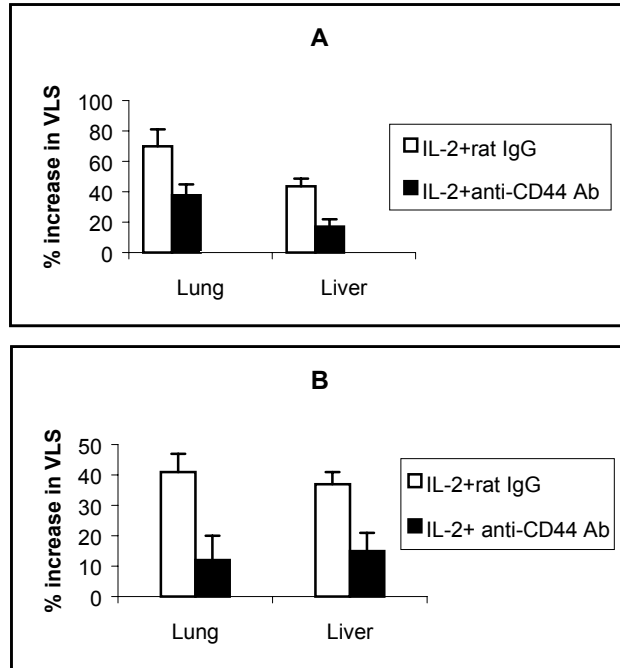


FIGURE 2. Effects of administration of mAbs against CD44 on VLS induction in wild-type mice. In A, groups of five C57BL/6 mice were injected with IL-2 (75,000 U) as described in Fig. 1. The IL-2 injected mice in addition received 500 μ g of anti-CD44 mAbs or normal rat IgG mouse/day for 4 days. Panel B shows a similar experiment, except that the mice were treated with low dose of IL-2 (10,000 U) along with 500 μ g of anti-CD44 mAbs or normal rat IgG /mouse/day for 4 days. The VLS was determined as described in Fig. 1.

In contrast, administration of anti-CD44 mAbs along with 10,000U of IL-2 (Fig. 4E, Fig.5E) or 75,000U of IL-2 (Fig. 4F, Fig. 5F) caused similar levels of perivascular infiltration as mice treated with IL-2 alone. However, the anti-CD44 mAb + IL-2 treated groups had significantly less edema therefore less toxicity both in the lungs (Fig. 4E, F) and liver (Fig. 5E, F). The degree of infiltration was also measured by counting the number of lymphocytes infiltrating each vessel and averaging the range for each group (Table 1). These results showed that HA + IL-2 treated mice had higher levels of perivascular infiltration and increase in edema than mice treated with IL-2 (75,000U) alone. In contrast, treatment with anti-CD44 mAbs + IL-2 (75,000U) caused similar levels of perivascular infiltration as the mice treated with IL-2 (75,000U) alone. Together these data suggested that HA increases the VLS caused by IL-2 whereas anti-CD44 mAbs decrease the VLS.

LAK activity in mice undergoing VLS

To test the effect of HA or anti-CD44 mAb treatment on IL-2 induced LAK cytotoxicity and to further corroborate the results noted in previous experiments, splenic T cells collected from HA or anti-CD44 mAb treated mice undergoing IL-2 induced VLS were tested for cytolytic activity against YAC-1 tumor targets. The data shown in Fig 6(A) indicated that PBS treated mice exhibited spontaneous cytotoxicity against YAC-1 targets. This spontaneous cytotoxicity was not altered when mice were injected with HA (Fig 6A). After IL-2 administration, the mice exhibited a significant increase in LAK activity (Fig. 6B). Furthermore, IL-2 + HA treatment led to a significant increase in LAK cytotoxicity against YAC-1 targets when compared to treatment with IL-2 alone. Similar results were noted using lower doses of IL-2 (10,000U) (Fig 6 C). In contrast to treatment with HA, anti-CD44 mAbs treatment in IL-2 injected mice caused a significant decrease in LAK cytotoxicity when compared to mice treated with IL-2 alone (Fig. 7B).

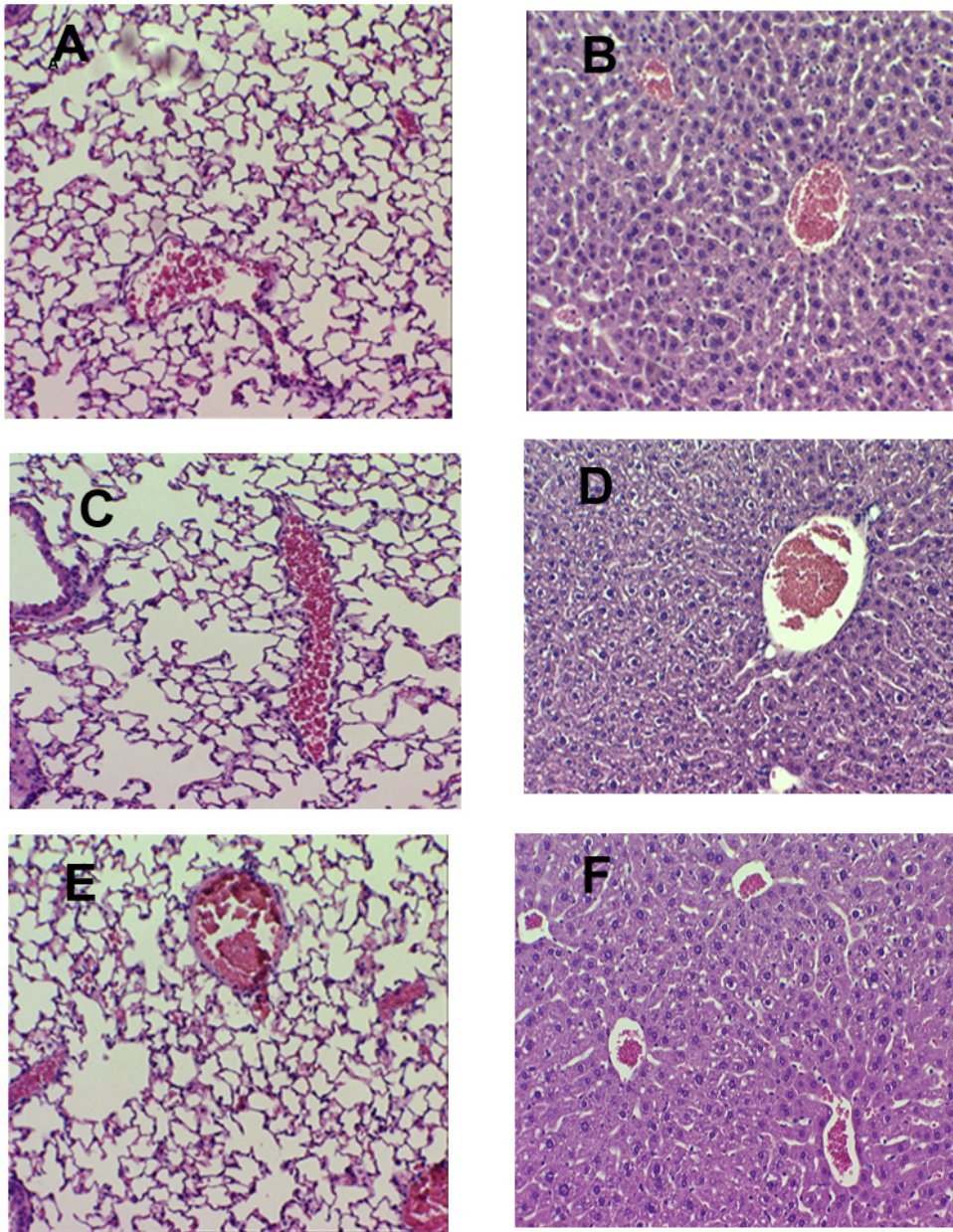


Figure 3. Histopathologic studies on lungs and livers in mice. No edema or cellularity (lymphocytes) can be seen in perivascular space in Lungs or liver from mice treated with PBS (A, B respectively) or HA alone (C, D) or anti-CD44 mAbs alone (E, F). Lungs and livers were harvested and preserved in 10% formalin solution. Sections were stained with hematoxylin and eosin. The sections were viewed at $\times 250$ and representative images were captured as shown.

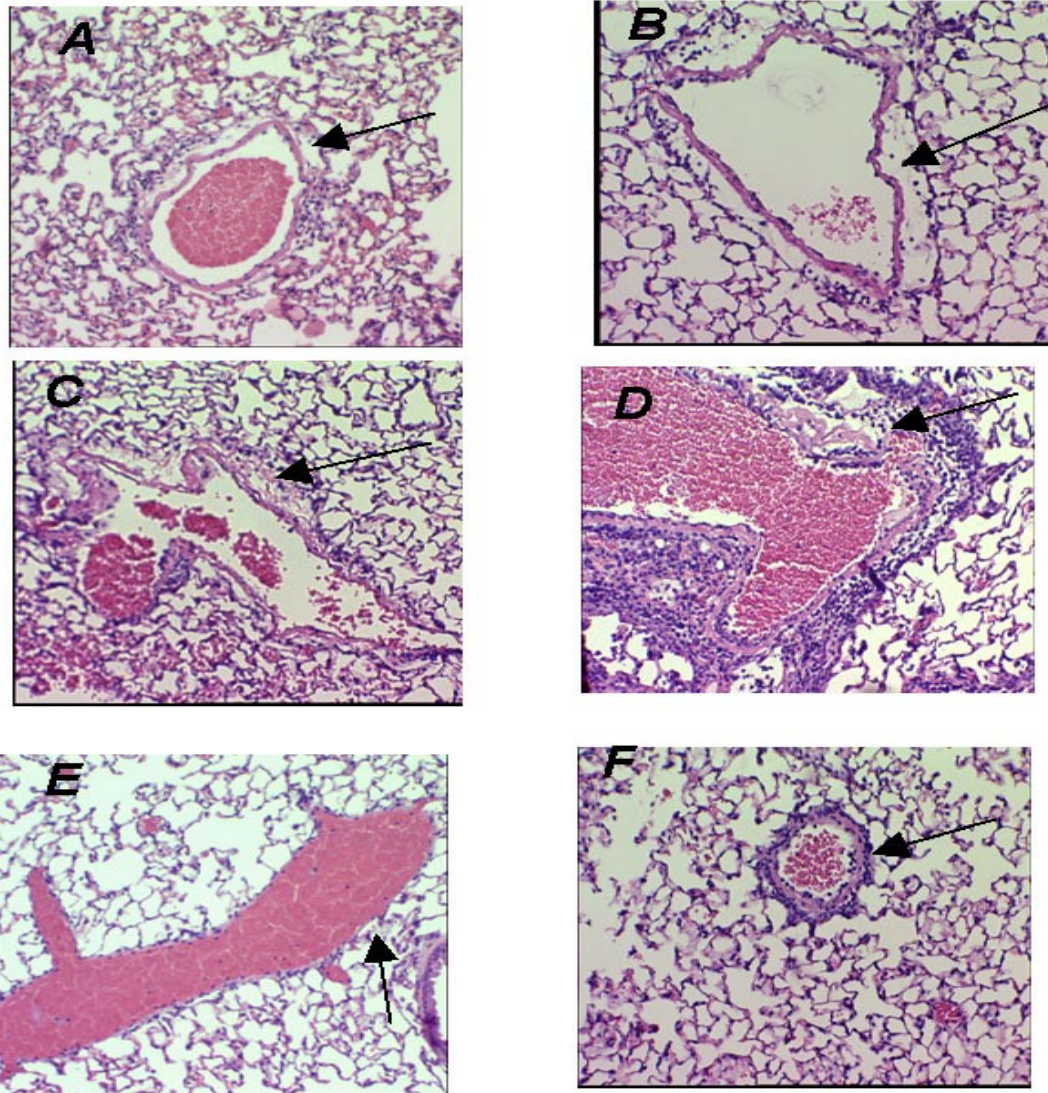


Figure 4. Histopathologic studies on lungs in mice. Edema and mild cellularity (lymphocytes) can be seen in perivascular space in Lungs from mice treated with IL-2 (10,000U) alone (A) or IL-2 (75,000U) alone (B) and, to a higher extent, in lungs from mice treated with IL-2 (10,000U) and HA (C) or IL-2 (75,000U) and HA (D). In contrast, little or no edema and mild cellularity can be seen in perivascular space in lungs from mice treated with IL-2 (10,000U) and 9F3 mAbs (E) or IL-2 (75,000U) and 9F3 mAbs (F). Lungs were harvested and preserved in 10% formalin solution. Sections were stained with hematoxylin and eosin. The sections were viewed at $\times 250$ and representative images were captured as shown. Perivascular infiltration, consisting mostly of lymphocytes, and expansion of the perivascular space is indicated by arrows.

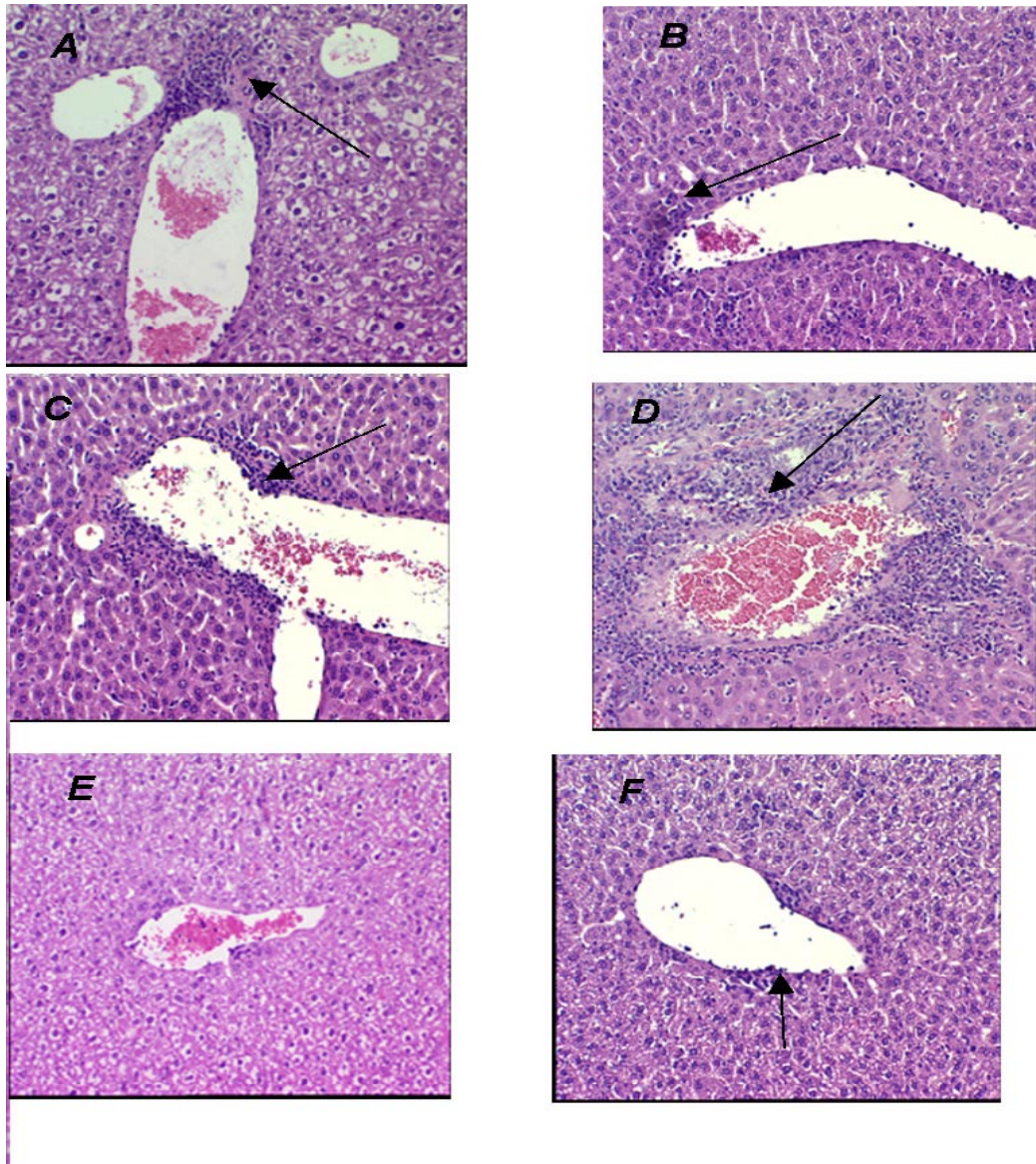


Figure 5. Histopathologic studies on liver in mice. Livers from mice treated with IL-2 (10,000U) alone (A) or IL-2 (10,000U) and HA (C) , IL-2 (10,000U) and 9F3 mAbs (E) , IL-2 (75,000U) alone (B) , IL-2 (75,000U) and HA (D), or IL-2 (75,000U) and 9F3 mAbs (F) were harvested and preserved in 10% formalin solution. Sections were stained with hematoxylin and eosin. The sections were viewed at $\times 250$ and representative images were captured as shown. Perivascular infiltration, consisting mostly of lymphocytes, is indicated by arrows

Table 1. Perivascular infiltration of lymphocytes in wild-type mice treated with IL-2 alone or IL-2 + HA or IL-2 + anti-CD44 mAbs .

Organ	Treatment ^a		
	IL-2 alone	IL-2 + HA	IL-2 + anti-CD44 mAbs
Lung ^b	3.06 ± 0.34	* 7.94 ± 0.44	3.65 ± 0.28
Liver ^b	2.70 ± 0.23	* 4.12 ± 0.37	2.53 ± 0.22

^aMice were injected with IL-2 (75000U) to induce VLS or IL-2 and HA or anti-CD44 mAbs and the organs were processed for histopathological studies as described in *Materials and Methods*.

The perivascular infiltration was measured by counting the number of lymphocytes infiltrating each vessel.

^b The number of lymphocytes infiltrating a venule. The data represent the mean ± SEM obtained from three samples per mouse (three mice were analyzed each organ). Asterix shows statistically significant difference when compared to the controls.

Similar results were seen using lower doses of IL-2 (10,000U) (Fig. 7C). Together these data suggested treatment of IL-2 administered mice with HA caused an enhancement in LAK activity while treatment with anti-CD44 mAbs caused a significant decrease in cytotoxic activity. These data are consistent with increased and decreased VLS induced by HA and anti-CD44 mAbs respectively in the lungs and liver.

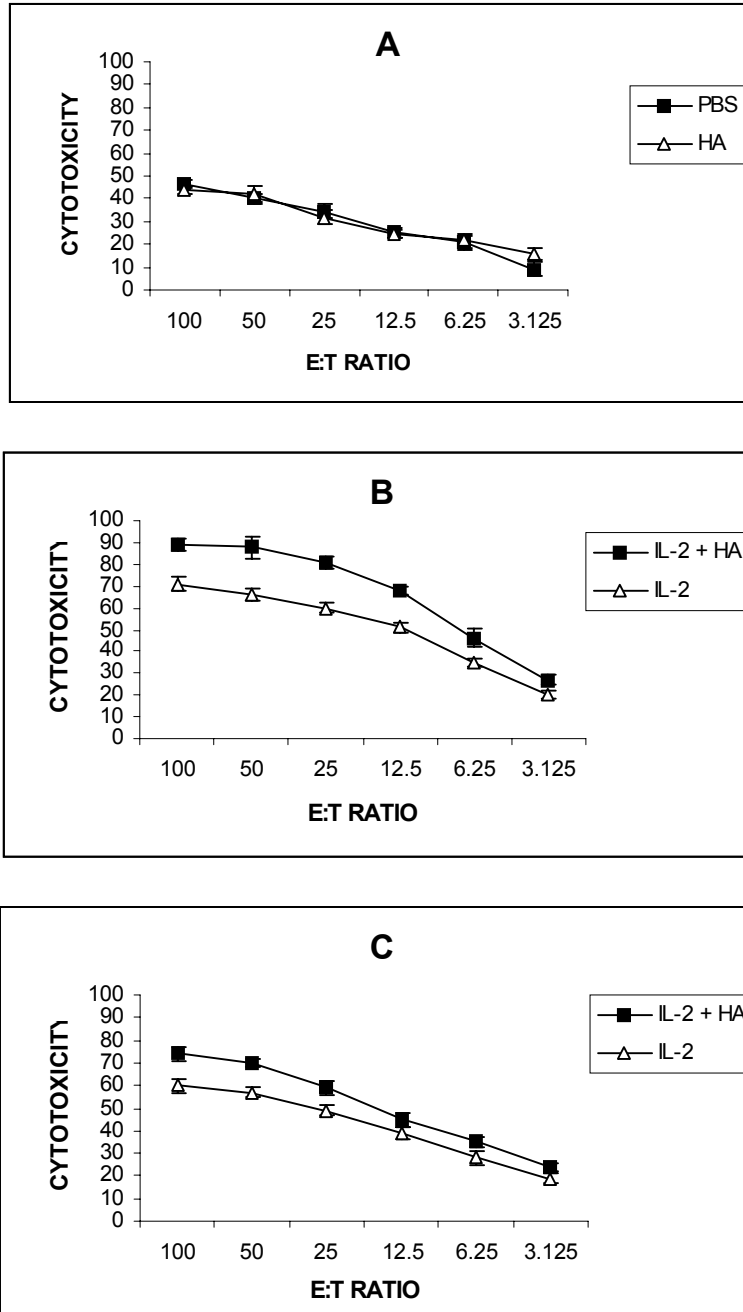


Figure 7. In panel A, mice injected with PBS alone or HA alone, were tested for NK activity against YAC-1. In panel B, mice were injected with IL-2 alone (75,000U) or IL-2 +HA as described in Fig 2. Panel C is identical to panel B except that lower concentrations (10,000U) of IL-2 were used. On day four, the LAK activity was tested against YAC-1 targets. The data indicate mean percent cytotoxicity of triplicate cultures \pm SEM.

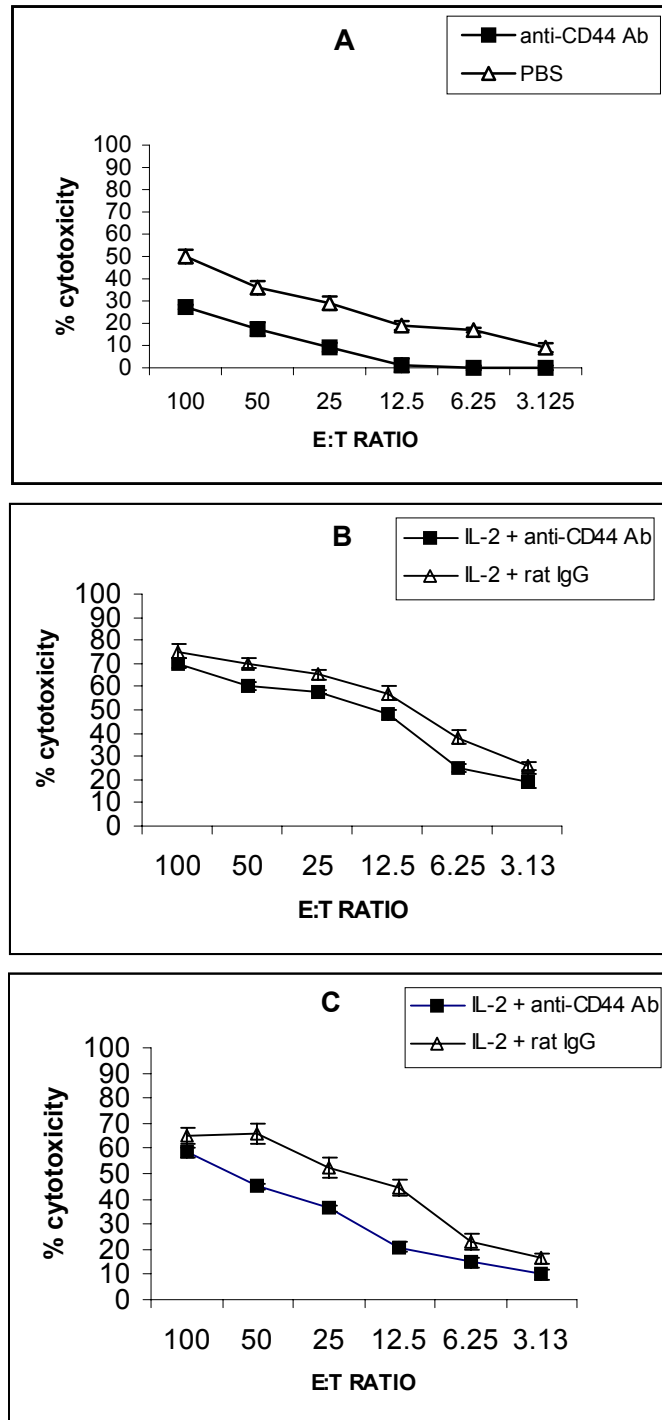


Figure 7. In panel A, mice were injected with anti-CD44 mAbs or rat IgG as described in Fig 2 and tested for NK activity against YAC-1 targets. In panel B, mice were injected with IL-2 (75,000U) + anti-CD44 mAbs or IL-2 + rat IgG as described in Fig 2. Panel C is identical to panel B except that lower dose of IL-2 (10,000U) was used. The data indicate mean percent cytotoxicity of triplicate cultures \pm SEM.

Discussion

Administration of IL-2, although promising in the treatment of certain types of cancer, triggers severe side effects in patients who develop a capillary leak syndrome resulting in anasarca and multiorgan system dysfunction (23,24). Previous studies from our laboratory have suggested that CD44 is actively involved in the induction of VLS by IL-2 (6). The principal ligand of CD44 has been identified as hyaluronic acid (HA), a major component of the ECM. In the current study we therefore tested whether administration of HA would suppress the VLS. Contrary to our belief, we observed that IL-2-induced VLS in the lungs and liver was markedly increased following HA treatment when compared to treatment with IL-2 alone. Furthermore, histopathological studies demonstrated that HA-treated mice exhibited more perivascular lymphocytic infiltration and increased edema in the lungs and livers. Also, IL-2 +HA treated mice exhibited increased LAK activity when compared to IL-2 alone treated groups. Others and we have shown that activated CTL, NK cells from wild-type mice, and double-negative T cells found in mice with the *lpr* mutation mediate efficient lysis of target cells when activated through CD44 (7, 18). Inasmuch as, endothelial cells express the ligands for CD44, it is likely that dysregulated interaction between cytotoxic lymphocytes and endothelial cells can cause endothelial cell lysis. IL-2 treatment activates the LAK cells to express higher levels of perforin and Fas ligand as well as CD44, which may account for their ability to migrate to various organs and cause endothelial cell lysis (3, 25). The current study suggests that HA acts as agonist and activates the LAK cells to mediate increased lysis of the target cells. The fact that treatment with HA enhanced the cytotoxicity of NK/LAK cells from IL-2 injected but not from those mice that did not receive IL-2, suggests that HA can influence only activated but not naïve NK/LAK cells.

The exact mechanism by which HA increases the cytolytic activity of LAK cells and causes increased VLS is not clear. Previous studies presented evidence that the binding of low molecular weight fragments of HA to alveolar macrophages via CD44 elicits the expression of a number of pro-inflammatory chemokines (26). These observations extend earlier findings showing that HA fragments are capable of activating NF- κ B (27) and induce the expression of insulin-like growth factor-1 by murine macrophages (28). These studies have significant impact

on the biological activity of HA fragments. Moreover, the role of HA in autoimmune diseases has also been studied. It was shown that HA fragments are present at abnormally high levels in the joints of patients with rheumatoid arthritis (RA) and in other inflammatory conditions (29, 30). These HA fragments are thought to arise primarily as a result of activated leukocyte driven extracellular matrix degradation at sites of inflammation and their presence has been proposed as a biological marker of disease. The second observation is that HA fragments but not HA polymers have angiogenic activity, a process which has been proposed to play a key role in the maintenance and progression of RA and other chronic inflammatory diseases (31). Studies have suggested (27, 28) that the high levels of HA fragments found in inflamed tissues bind to leukocytes and other CD44 expressing cells and trigger a cascade of signaling events which are involved in maintaining and/or amplifying the inflammatory response. It is of interest to note that the biological activities elicited by the HA fragments may vary based on their size. For example, while high molecular weight HA polymers are components of extracellular matrix and a substrate for CD44 mediated cell adhesion, processed lower molecular weight HA fragments may serve as signaling molecules (32). It should be noted that in the current study we used high molecular weight HA with a molecular weight of 5 to 8 million Da. In contrast, low molecular weight HA had no significant effects on IL-2 induced VLS (data not shown).

In contrast to HA, in the current study, we noted that Abs against CD44 acted as antagonist, thereby blocking IL-2 induced VLS. We noted a significant inhibition of VLS in the lungs and liver caused by anti-CD44 mAbs when compared to the controls. Interestingly, the inhibition was not due to the inability of the lymphocytes to migrate to the lungs and liver but may have resulted from decrease in LAK activity. This conclusion was supported by histological studies and cytotoxicity. The decreased VLS seen following anti-CD44 mAbs treatment correlated with a significant decreased in edema in the lungs and livers as seen from histological studies.

A number of studies have shown that treatment of T cells with antibodies specific for CD44 in conjunction with other activation signals can result in either enhancement or inhibition of lymphocyte response. Recent reports have described the effects of an antibody specific (IM7) for CD44 that has beneficial effects in two murine models of autoimmune disease. Both

experimental allergic encephalomyelitis (EAE) and collagen-induced arthritis were ameliorated by treatment with anti-CD44 mAbs (33, 34). In contrast, the same antibodies failed to prevent infiltration of T cells in the thyroid and in fact exacerbated experimental autoimmune thyroiditis (35).

These studies together suggest that antibodies to CD44 may play varying roles in terms of their ability to prevent lymphocyte homing and activation. This effect may be further complicated by the fact that CD44 is expressed in a variety of isoforms and the binding of the Abs to specific sites on CD44 may determine their ability to act as agonist/antagonist in lymphocyte homing and activation. It is interesting to note that anti-CD44 mAbs or CD44-receptor globulins have been shown to prevent the growth and metastasis of melanomas (36). These studies combined with the data presented in the current research suggest that combination therapy with IL-2 and anti-CD44 mAbs may greatly benefit tumor immunotherapy by reducing IL-2 induced toxicity and preventing tumor metastasis. Such a possibility is currently being tested in a murine melanoma model in our laboratory.

ABBREVIATIONS

CTL, cytotoxic T lymphocytes; ECM, extracellular matrix; HA, hyaluronic acid; IL-2, interleukin-2; KO, knockout; LAK cell, lymphokine-activated killer cell. mAb, monoclonal antibody; NK, natural killer; TCR, T cell receptor; VLS, vascular leak syndrome.

References

1. Baluna R; Vitetta ES. Vascular leak syndrome: a side effect of immunotherapy. *Immunopharmacology* 1997 Oct; 37(2-3): 117-32.
2. Damle, N., L. Doyle, J. Bender, and E. Bradley. Interleukin 2-activated human lymphocytes exhibit enhanced adhesion to normal vascular endothelial cells and cause their lysis. *J. Immunol* 1987; 138:1779.
3. Rafi, A., A. Zeytun, M. Bradley, P. Sponenberg, R. Grayson, M. Nagarkatti, and P. Nagarkatti. Evidence for the involvement of Fas ligand and perform in the induction of vascular leak syndrome. *J. Immunol* 1998; 161:3077.
4. Ward, P. A.. Role of complement, chemokines, and regulatory cytokines in acute lung injury. *Ann. NY Acad. Sci.* 1996; 796:104.
5. Hammond-McKibben, D., A. Seth, P. Nagarkatti, and M. Nagarkatti. Characterization of factors regulating successful immunotherapy using a tumor-specific cytotoxic T lymphocyte clone: role of interleukin-2, cycling pattern of lytic activity and adhesion molecules *Int. J. Cancer* 1995; 60:828.
6. Rafi-Janajreh AQ, Chen D, Schmits R, Mak TW, Grayson RL, Sponenberg DP, Nagarkatti M, Nagarkatti PS. Evidence for the involvement of CD44 in endothelial cell injury and induction of vascular leak syndrome by IL-2. *J Immunol.* 1999 Aug 1; 163(3):1619-27.
7. Seth, A., L. Gore, M. Nagarkatti, and P. S. Nagarkatti. T cell receptorindependent activation of cytolytic activity of cytotoxic T lymphocytes mediated through CD44 and gp96/EL-14. *Proc. Natl. Acad. Sci. USA* 1991; 88:7877.
8. Doherty, P. C., J. E. Allan, F. Lynch, and R. Ceredig. Dissection of an inflammatory process induced by CD8⁺ T cells. *Immunol. Today* 1990; 11:55.
9. Tsukada, N., M. Matsuda, K. Miyagi, and N. Yanagisawa. Cytotoxicity of T cells for cerebral endothelium in multiple sclerosis. *J. Neurol. Sci.* 1993; 117:140.

10. Moyer, C. F., and C. L. Reinisch. The role of vascular smooth muscle cells in experimental autoimmune vasculitis. *Am. J. Pathol.* 1983; 117:380.
11. Fox, W. M., A. Hameed, G. M. Hutchins, B. A. Reitz, W. A. Baumgartner, W. E. Beschorner, and R. H. Hruban. Perforin expression localizing cytotoxic lymphocytes in the intimas of coronary arteries with transplant-related accelerated arteriosclerosis. *Hum. Pathol.* 1993; 24:477.
12. Van der Wal, A. C., P. K. Das, A. J. Tigges, and A. E. Becker. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am. J. Pathol.* 1992; 141:1427.
13. Kupiec-Weglinski JW. Graft rejection in sensitized recipients. *Ann Transplant* 1996;1(1):34-40.
14. Rafi-Janajreh AQ, Nagarkatti PS, Nagarkatti M. Role of CD44 in CTL and NK cell activity. *Front Biosci.* 1998 Jul 1; 3:D665-71.
15. Lesley, J., R. Hyman, and P. Kincade. CD44 and its interactions with extracellular matrix. *Adv. Immunol* 1993; 54:2713.
16. DeGrendele HC, Estess P, Siegelman MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 1997 Oct 24; 278(5338):672-5.
17. Rafi A, Nagarkatti M, Nagarkatti PS. Hyaluronate-CD44 interactions can induce murine B-cell activation. *Blood* 1997 Apr 15;89(8):2901-8.
18. Sconocchia G, Titus JA, Segal DM. CD44 is a cytotoxic triggering molecule in human peripheral blood NK cells. *J Immunol* 1994 Dec 15;153(12):5473-81.
19. Hammond DM, Nagarkatti PS, Gote LR, Seth A, Hassuneh MR, Nagarkatti M. 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* 1993 Dec 1;178(6):2225-30.
20. Udaka, K., Y. Takeuchi, and H. Movat. Simple method for quantitation of enhanced vascular permeability. *Proc. Soc. Exp. Biol Med* 1970; 133:1384.
21. Matzinger P: The JAM test. *J Immunol Methods* 1991; 145:185.

22. Zeytun A, Hassuneh M, Nagarkatti M, Nagarkatti PS. Fas-Fas ligand-based interactions between tumor cells and tumor-specific cytotoxic T lymphocytes: a lethal two-way street. *Blood* 1997 Sep 1;90(5):1952-9.
23. Rosenberg, S., M. T. Lotze, L. M. Muul, S. Leitman, A. E. Chang, S. E. Ettinghausen, Y. L. Matory, J. M. Skibber, E. Shiloni, and J. T. Vetto, et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N. Engl. J. Med.* 1985; 313:1485.
24. Lotze, M. T., Y. L. Matory, A. A. Rayner, S. E. Ettinghausen, J. T. Vetto, C. A. Seipp, and S. A. Rosenberg. Clinical effects and toxicity of interleukin-2 in patients with cancer. *Cancer* 1986; 58:2764
25. Bradley, M., A. Zeytun, A. Rafi-Janajreh, P. S. Nagarkatti, and M. Nagarkatti. Role of spontaneous and IL-2 induced NK activity in the cytotoxicity and rejection of Fas' and Fas- tumor cells. *Blood* 1998; 92: 4248.
26. McKee, C.M. , M.B. Penno , M. Cowman , C. Bao , and P.W. Noble. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J. Clin. Invest.* 1996; 98: 2403-2413.
27. Noble, P.W. , C.M. McKee , M. Cowman , and H. Shin. Hyaluronan fragment activate an NF- κ B/I- κ B α autoregulatory loop in murine macrophages. *J. Exp. Med.* 1996; 183: 2373-2378
28. Noble, P.W., F.R. Lake , P.M. Henson , and D.W.H. Riches. Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor- α -dependent mechanism in murine macrophages. *J. Clin. Invest.* 1993; 91: 2368-2377
29. Bjermer, L., R. Lundgren, and R. Hallgren. Hyaluronan and type III procollagen peptide concentrations in bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis. *Thorax.* 1989; 44: 126-131
30. Poole, A., and P. Dieppe. Biological markers in rheumatoid arthritis. *Semin. Arthritis Rheum.* 1994; 23S: 17-31
31. West, D.C. , I.N. Hampson , F. Arnold , and S. Kumar. Angiogenesis induced by degradation products of hyaluronic acid. *Science (Wash. DC)* 1985; 228: 1324-1326
32. Termeer CC, Hennies J, Voith U, Ahrens T, Weiss JM, Prehm P, Simon JC.

Oligosaccharides of hyaluronan are potent activators of dendritic cells. *J Immunol* 2000 Aug 15;165(4):1863-70.

33. Brennan FR, O'Neill JK, Allen SJ, Butter C, Nuki G, Baker D. CD44 is involved in selective leucocyte extravasation during inflammatory central nervous system disease. *Immunology* 1999 Nov;98(3):427-35.

34. Nedvetzki S, Walmsley M, Alpert E, Williams RO, Feldmann M, Naor D. CD44 involvement in experimental collagen-induced arthritis (CIA). *J Autoimmun* 1999 Aug;13(1):39-47.

35. Parish NM, Brennan FR, Cooke A. 1999. Anti-CD44 treatment does not prevent the extravasation of autoantigenic T cells to the thyroid in experimental autoimmune thyroiditis. *Immunology*. Jul;97(3):533-9.

36. Zawadzki V, Perschl A, Rosel M, Hekele A, Zoller M. Blockade of metastasis formation by CD44-receptor globulin. *Int J Cancer* 1998 Mar 16;75(6):919-24.

Amjad Mustafa
P.O.Box 10854
Blacksburg, VA 24060
(540) 953-2023

JOB EXPERIENCE

- **COLUMBIA/HCA MONTGOMERY REGIONAL HOSPITAL. 4/93-11/98**
MEDICAL LABORATORY TECHNOLOGIST (GENERALIST)
Perform all lab tests, procedures and analyses.
Perform Quality controls
Columbia Montgomery Regional Hospital
3700 south main St.
Blacksburg , VA 24060
TEL:(540) 953 - 5125
- **CONSULTING MEDICAL LABORATORIES MT (GENERALIST). 6/91 to 8/92**
PERFORM ALL ROUTINE AND SPECIALIZED lab tests.
- **JAHRA HOSPITAL /MINISTRY OF PUBLIC HEALTH , Kuwait 8/89-8/90**
MT (GENERALIST). HEMATOLOGY AND BLOOD BANK.

EDUCATION

- **Graduate student, biology department, Virginia Tech .**
- **BACHELOR OF SCIENCE IN MEDICAL LABORATORY TECHNOLOGY, 5/89**
UNIVERSITY OF KUWAIT, KUWAIT.(evaluated as equivalent to US BS.)
- **Graduate teaching Assistantship(GTA) Spring, 2001.**
- **Graduate teaching Assistantship(GTA) Fall, 2000.**
- **Graduate teaching Assistantship(GTA) Spring, 2000.**
- **Graduate teaching Assistantship(GTA) Spring, 1999.**
- **Graduate teaching Assistantship(GTA) Fall, 1999.**

COARSE WORK

Advanced Medical Biochemistry
Biochemisry for Life Science
Cell Interaction Immune system I
Cell Interaction Immune system II
Molecular Biology for Life Science
Introduction for Clinical Reseach
Bioinformatics
Molecular Biology Of The Cell
Molecular Biology Lab.
Immunology

QCA. 3.81

- **Many continuing education courses in the field of medical laboratory technology.**

RELATED BACKGROUND

CLINICAL HEMATOLOGY
URINALYSIS
HISTOPATHOLOGY
RIA
CELL CULTURE

CLINICAL CHEMISTRY
SEROLOGY
CLINICAL MICROBIOLOGY
BLOOD BANK
IMMUNOCHEMISTRY

CERTIFICATIONS 1. **MT. AMERICAN SOCIETY OF CLINICAL PATHOLOGISTS (ASCP), USA**

2. **Board certified by NATIONAL CERTIFICATION AGENCY (NCA)
Clinical Laboratory Scientist (CLS) 7/89 U.S.A**

REFERENCES AVAILABLE UPON REQUEST