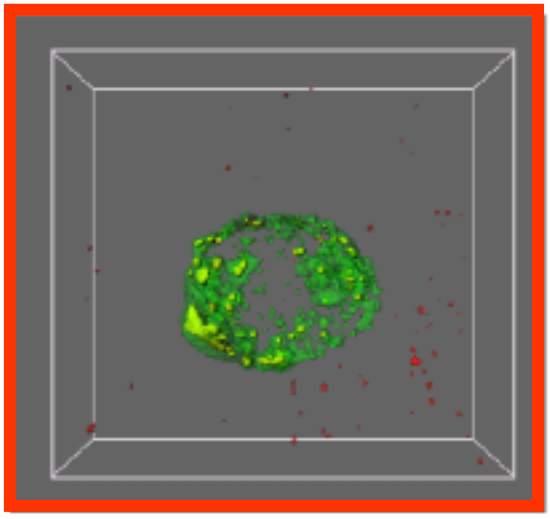
Universität Basel

REGULATION OF FAS MEDIATED APOPTOSIS BY CD44



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“En torno de la esencia está la morada de la ciencia.”

Platon

A mis padres y hermanos,
A Michael,
A mis amigos.

Table of contents

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Introduction

Wanted: dead or alive?

Many diseases reflect a deregulation of programmed cell death (also known as apoptosis). Apoptosis is essential for normal life and in some disorders such as cancer and autoimmunity this vital process is deficient, in others such as neurodegenerative diseases cell death is excessive. The common aim of many researchers is to understand how this balance between life and death is regulated with the hope that we might one day control the equilibrium between proliferation and apotosis which is vital for preserving health.

Most of the people see cancer as a modern and unpredictable plague that separates us from our nearest and dearest. For the patient suffering from this disorder, cancer is a terrifying disease invading his body and often only treatable with unspecific therapies with a high risk of relapse. For the cellular biologist, a cancer cell is defined as being immortal but paradoxically cancer is a cause of mortality and approximately one person of five, in the developed countries of the world die of cancer. In the context of cell biology, cancer is specially interesting because it alters the fundamental rules of behavior of the cells.

Autoimmune diseases are characterized by the activity of autoreactive lymphocytes and/or autoantibodies targeting self tissue for destruction. These disorders are clinically and molecularly very heterogeneous but a common feature is once more the deregulation of apoptosis. Most of these disorders do not have a specific treatment and patients are often treated with immunosuppressors with unpleasant side effects.

Many proteins from every compartment of the cell play a role in the complex regulation of apoptosis. Growth factors and their receptors, as well as their membrane, cytoplasmic and nuclear downstream effectors have been described as pro-apoptotic and/or anti-apoptotic. Among these numerous players the cell adhesion molecule CD44 has been identified, therefore, to understand the action of CD44 in the regulation of apoptosis is our wish in this thesis.

Introduction

I. CD44: more than a simple adhesion molecule

1. Structure and isoforms of CD44

The CD44 transmembrane glycoprotein was discovered more than 20 years ago and since then, more and more functions have been ascribed to this membrane receptor including its capacity to mediate inflammation and tumor growth and metastasis (1-4). CD44 receptor exists in different isoforms due to alternative splicing affecting the extracellular domain and to post-translational modifications such as O and N-glycosylations and chondroitin and heparan sulphate chains. CD44 proteins are all encoded by a single highly conserved gene located on chromosome 11 in humans (chromosome 2 in the mouse). CD44 pre-mRNA is encoded by 20 exons, 10 of which can be regulated by alternative splicing (Figure 1). In principle, all ten variant exons could be joined with each other in multiple combinations, resulting in more than 100 different isoforms. The inclusion of exon s9, normally absent in most CD44 transcripts, results in a CD44 short-tail form (reviewed by (5-7)).

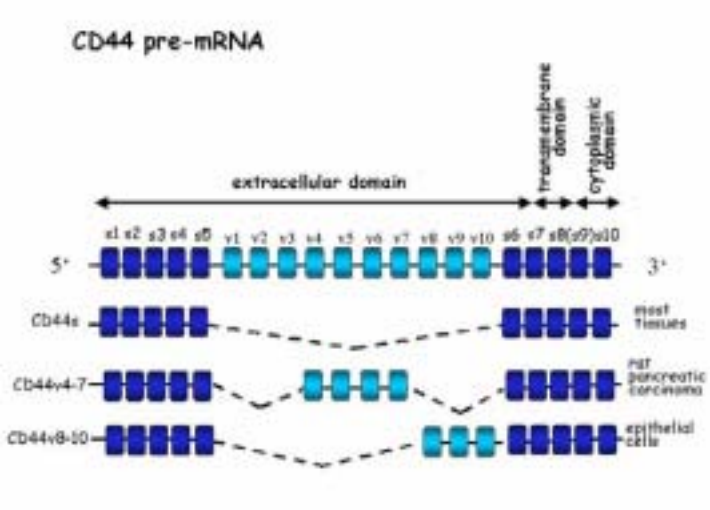

Figure 1. CD44 pre-mRNA is encoded by 20 exons, 10 of which are regulated by alternative splicing (variant exons). CD44 standard (CD44s) is the shorter form and is expressed in most of the tissues. The variant isoforms are only expressed under special conditions.

The shorter standard form of CD44 (CD44s) is ubiquitously expressed in vertebrates, in developing and adult organisms. In contrast, the larger variant isoforms (CD44v) are expressed only in epithelial tissues (8), and importantly, they are upregulated under special conditions such as leukocyte activation, inflammation and tumorigenesis. Control and regulation of CD44v expression depends, at least in part, on mitogenic signals and stimulation of the Ras-Mek-Erk pathway which triggers inclusion of variant exons into the mature RNA (reviewed by (5)).

- **The hyaluronan-binding, amino terminal domain**

The standard exons 2 and 3 of CD44 encode an amino-terminal globular protein domain known as the hyaluronan binding domain. This domain interacts with hyaluronan, a polysaccharide of the extracellular matrix (Figure 2) (5, 6, 9).

- **The stem structure**

The amino-terminal globular domain of the smallest CD44 isoform (CD44s) is separated from the plasma membrane by a short stem structure (46 aa). This structure can be enlarged by up to 381 aa if all alternatively spliced variant exons are included. This stem contains putative proteolytic cleavage sites, hence the extracellular portion of CD44 can be cleaved by matrix metallo-proteinases (10, 11). Inclusion of exon v3 provides a unique heparan sulphate addition site which is important for binding of heparan-sulphate dependent growth factors such as: fibroblast growth factor (FGF), hepatocyte growth factor (HGF) or the chemokine macrophage inflammatory protein-1 (MIP-1) (Figures 2) (reviewed by (5, 6, 12)).

- **The transmembrane and cytoplasmic domain**

The transmembrane region consists of 23 hydrophobic amino acids encoded by exon s8. The transmembrane domain might be responsible for the association of CD44 proteins with lipid rafts (13, 14). The carboxy terminal cytoplasmic domain encoded by exon s10 supports the binding of proteins with crucial functions in cytoskeletal organization and signaling (as discussed below, see paragraph “Intracellular interacting partners”) (reviewed by (5, 6)).

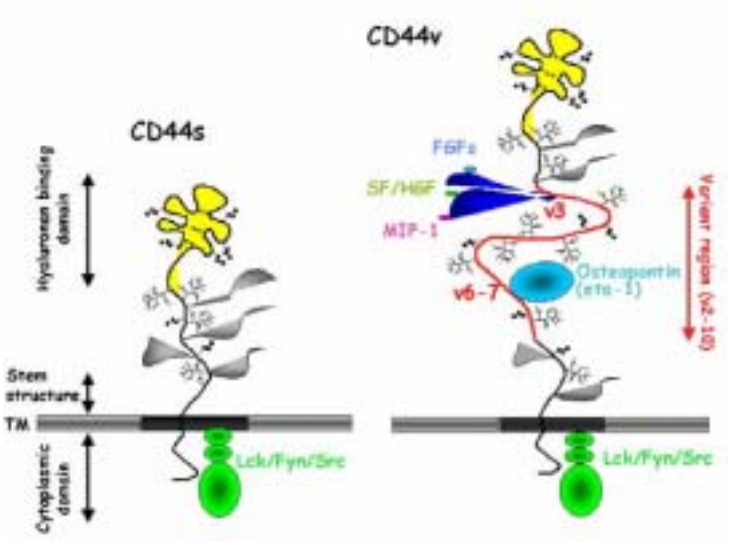

Figure 2. Left: Structure of CD44s. CD44 is highly glycosylated, O-glycosylations are represented by ramifications, black bullets represent the N-glycosylations. The condroitin sulphate chains (represented in gray) provide a binding site for extracellular matrix proteins such as fibronectin and laminin (9). Some non-receptor tyrosine kinases such as: Lck, Fyn and Src can interact with the cytoplasmic tail of CD44. Right: Structure of the largest human variant isoform CD44v2-10 (v1 is expressed in mice but not in human). The variant region is located in the extracellular part of the protein, between the globular domain and the stem structure. The variant 3 region contains binding sites for heparan sulphate chains where growth factors and cytokines concentrate.

2. Interacting partners and functions of CD44

Like any cell adhesion molecule, CD44 mediates cell-cell and cell-extracellular matrix interactions. CD44 can interact with soluble extracellular components and with the extracellular matrix. The main ligand of CD44 is hyaluronan, a hydrophilic, linear, extracellular polysaccharide crucial for normal development and life of vertebrates. *In vitro* interactions of CD44 with hyaluronan, collagen, laminin and fibronectin seem to promote matrix-dependent migration. The binding of CD44 to hyaluronan seems to be also involved in leukocyte rolling (reviewed by (2)). Beside adhesion and migration through the extracellular matrix, CD44 proteins participate in many other vital physiological processes including organ development, T cell differentiation, haematopoiesis and cytokine/chemokine binding. The latter is concentrated at the heparan sulphate chains located in the variant 3 region. From its initial identification as a transmembrane hyaluronan receptor mediating cell adhesion and migration, the list of

functions ascribed to CD44 has increased annually and includes activities independent of hyaluronan binding. CD44 is involved in a variety of physiological but also pathophysiological processes such as chronic inflammation and tumorigenesis (described below). The multifunctionality of CD44 is in part due to its extra and intracellular interactions with a broad number of different molecules.

Á Extracellular interacting partners

ü *Platform function*

CD44 proteins can function as specialized platforms for soluble extracellular molecules such as growth factors. Heparan sulphate chains attached to CD44 are able to bind them, concentrate them and to bring substrate and enzymes together.

Indeed it has been shown that CD44 recruits MMP9 (Matrix metalloproteinase 9) to the cell surface. This allows MMP9 to activate TGF-η (transforming growth factor η) which then triggers angiogenesis and invasion (15, 16) (Figure 3 A). MMP7 and its substrate, the pro-form of the heparan-binding epidermal growth factor (HBEGF), are also bound to the cell surface, probably through the heparan sulphate chains of the CD44 variant 3 region (Figure 3 B). The binding of heparan-binding growth factors to a heparan sulphate proteoglycan such as CD44 is a prerequisite for the activation of their high affinity receptors. Activated HBEGF is engaged in the activation of the ErbB receptor tyrosine kinase ErbB4 that signals for cell survival (17, 18).

The interaction of CD44 variants 6 and 7 with the cytokine osteopontin (Figure 2) promotes inflammation (19, 20) and cell survival of mouse bone marrow cells (21).

CD44 proteins containing the heparan sulphate modification in the variant 3 region can recruit members of the fibroblast growth factor (FGF) family and present them to the high affinity FGF receptor (22).

This platform function is probably important for physiological processes in which signaling events must be concentrated and integrated at the cell surface.

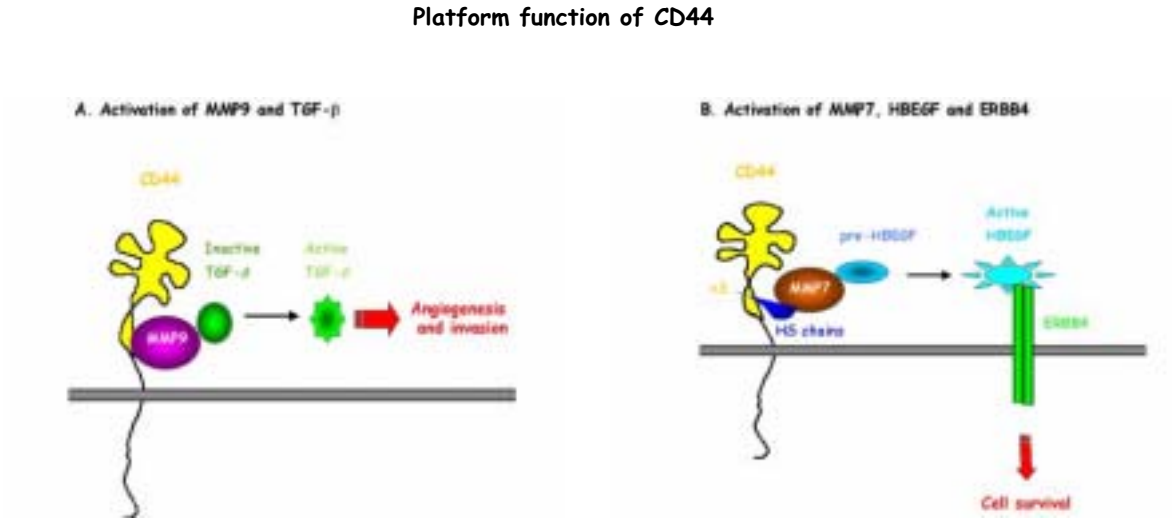

Figure 3. A. CD44 can assemble the matrix metalloproteinase 9 (MMP9) and TGF-η. This association results in the activation of TGF-η leading to neovascularization (15). **B.** CD44 proteins carrying the heparan sulphate chains of exon v3, interact with MMP-7 and its substrate, the heparan-binding epidermal growth factor. This process is required for ErbB4 activation (17).

ü *Co-receptor function*

An emerging concept in signal transduction is that cell-adhesion molecules can function as co-receptors and recent reports demonstrate CD44 to be a good example. Although CD44 itself lacks of kinase activity, it can cooperate with receptor tyrosine kinases and mediate their activation or modulate their kinase activity.

CD44v6 has been shown to associate with the tyrosine kinase receptor c-met and its ligand scatter factor/hepatocyte growth factor (SF/HGF) (Figure 4 A). The formation of the multimeric complex CD44v6/c-met/HGF is required for c-met autophosphorylation and signaling (23).

CD44 also functions as co-receptor for the ErbB receptor tyrosine kinase family and interacts with ErbB1, ErbB2, ErbB3 and ErbB4 in several cell lines. As discussed above, the function of the complex CD44-ErbB4 involves the processing of HBEGF by MMP7 which leads to cell survival (17).

A recent study describes the interaction of the epithelial cell adhesion molecule EpCAM with CD44v4-v7 (Figure 4 B) and demonstrates that this complex supports apoptosis resistance (24).

The association of CD44 with laminin ζ5 in melanoma cells inhibits tumor migration, invasion and angiogenesis (25).

Both, the platform and the co-receptor functions could be responsible for the action of CD44 in cells of the immune system during antigenic stimulation, and inflammation. These functions might also be implicated in the aberrant proliferation of tumor cells.

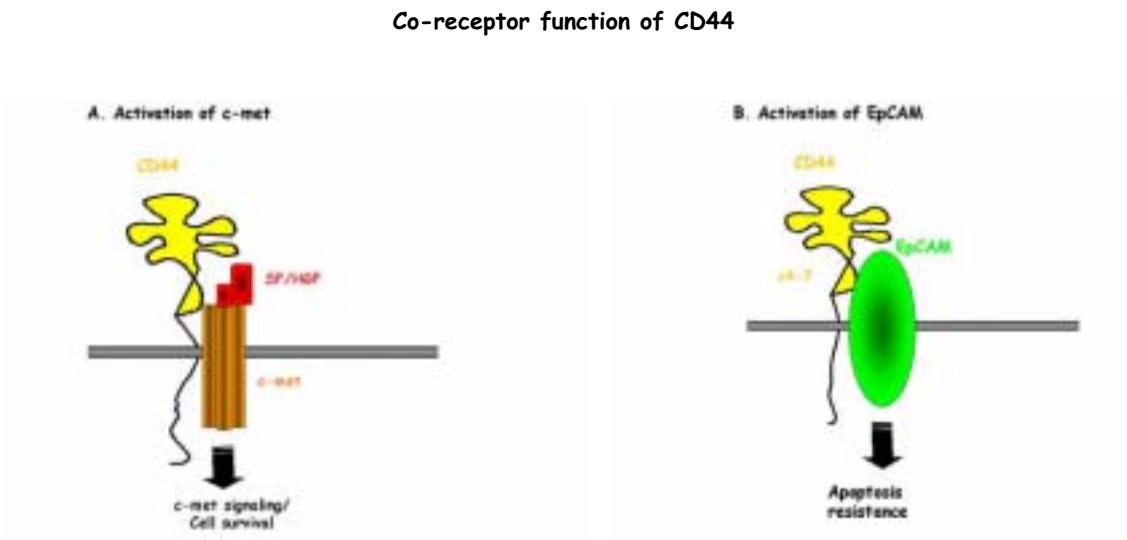

Figure 4. A. The variant 6 region is required for the scatter factor/hepatocyte growth factor (SF/HGF) dependent activation of c-met (23). **B.** The epithelial cell adhesion molecule EpCAM associates with CD44v4-v7 and this complex is involved in apoptosis resistance (24).

Á Intracellular interacting partners

ü *Interactions with signaling molecules*

Beside the extracellular interactions and its functional implications, many intracellular signaling components form complexes with the cytoplasmic tail of CD44. The most widely reported intracellular partners are GTPases of the Rho-family and non-receptor tyrosine kinases of the Src family (reviewed by (26)). CD44 co-

immunoprecipitates with Src, Lyn, Lck, Fyn and Hck, and activation of CD44 stimulates tyrosine phosphorylation of these kinases and their substrates (reviewed by (6)). However, it is not clear whether these interactions are direct or indirect and whether they have any functional significance.

CD44 associates with the integrin VLA-4 and this interaction is required for the extravasation of T cells into sites of inflammation (2).

Smad1 was also found to associate with the cytoplasmic domain of CD44. This interaction promotes the cellular response to the bone morphogenetic protein-7 (BMP-7) and is required for chondrocyte differentiation (27).

ü *Proteolytic processing of CD44*

As mentioned before, the extracellular stem region of CD44 is cleaved by matrix metalloproteinases leaving a C-terminal fragment embedded in the membrane. This proteolysis is required for further processing of the C-terminus fragment by presenilin-1/ν secretase and results in the secretion of a CD44η-like peptide and a CD44 intracellular domain fragment (ICD) (28). The CD44-ICD translocates to the nucleus and promotes transcription and one of its target genes is the gene encoding CD44 itself (29).

ü *Interaction with the cytoskeleton*

Many studies have shown that CD44 also interacts with cytoskeleton associated proteins, thus, modulating indirectly actin rearrangement.

The first intracellular partner identified was ankyrin (30). Ankyrin binding is regulated by GTP proteins and involves the activation of a Rho kinase. This association is implicated in mediating hyaluronan dependent cell adhesion and motility (31). CD44 can recruit annexin II which mediates interaction with the actin cytoskeleton (32). In culture cells, CD44 is strongly localized to the microvilli and regions of actin polymerization, which suggests that it associates with the actin cytoskeleton.

Because the CD44 cytoplasmic domain does not contain any actin-binding sites, this interaction is indirectly mediated by membrane-cytoskeleton crosslinker proteins (reviewed by (6)). Indeed, it has been demonstrated that the cytoplasmic domain of CD44

binds proteins with crucial functions in cytoskeletal organization and signaling such as the ERM (Ezrin, Radixin, Moesin) family members and the related protein merlin (Figure 5) (reviewed by (5, 6)).

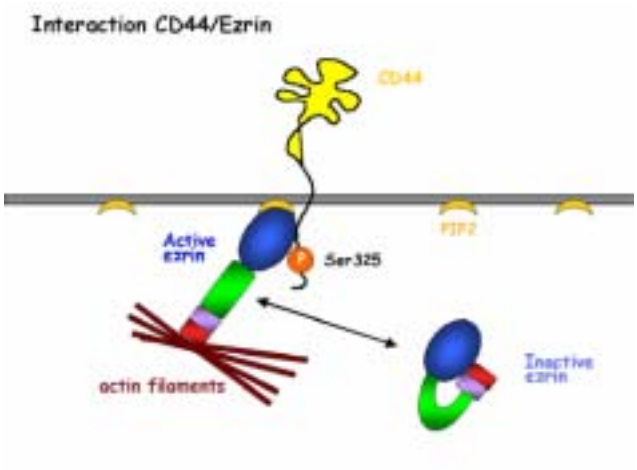

Figure 5. Model for the interaction of ezrin with the cytoplasmic domain of CD44. Inactive ezrin becomes activated by binding to phosphatidylinositol 4,5 biphosphate (PIP_2) (reviewed by (33)). When CD44 is phosphorylated at serine 325, it binds to active ezrin which links CD44 to actin cytoskeleton (34).

Structurally, the ERM proteins consist of a ~ 300 amino acid membrane binding domain (band 4.1, FERM) at the N-terminus, followed by an ζ4helical central region and#a C-terminal domain that contains an F-actin binding site (Figure 6). The closed, inactive form of the ERM proteins becomes activated by phosphorylation and binding to membrane phospholipids (33, 35-37). In this open, active conformation, the N-terminal domain can associate with membrane receptors such as CD44 and actin can bind to the C-terminal domain (38) (Figure 5). Ezrin is the prototype member of the ERM protein family. This ability of the ERM proteins to switch between an active and inactive conformation, provides a mechanism to make and break the CD44-cytoskeletal association. The interaction ezrin-CD44 is also regulated by protein kinase C (PKC). The dephosphorylation of serine 325 and phosphorylation of serine 291 at the cytoplasmic tail of CD44 by PKC results in the dissociation of ezrin and is required for cell motility (34).

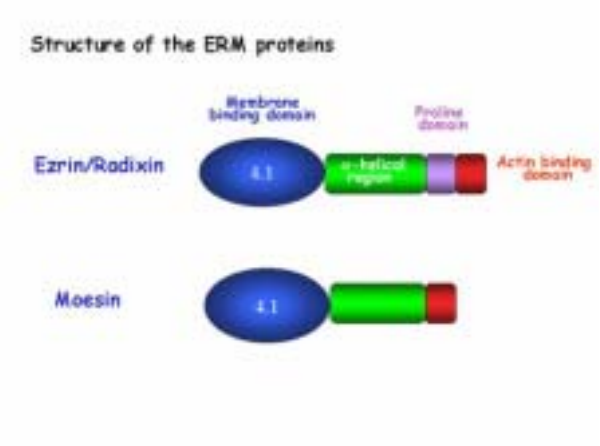

Figure 6. ERM proteins belong to the band 4.1 family and are composed by a membrane binding domain (band 4.1 or FERM domain) located at the N-terminus, followed by an ζ-helical domain, and the actin binding domain. Ezrin and radixin additionally possess a proline rich domain.

3. CD44 in disease

Á **CD44 and autoimmune diseases**

It is well known that CD44v are upregulated in inflammatory diseases in which they seem to inhibit apoptosis and promote proliferation of cells from the immune system. CD44v upregulation was observed in the following cases:

ü *Multiple sclerosis and experimental autoimmune encephalomyelitis*

Some reports also show that CD44v are involved in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) and that therapy with antibodies against specific CD44 variant isoforms can improve the course of the disease (39).

ü *Inflammatory bowel disease*

In a colitis mouse model it has been described that mice lacking CD44v6 and v7 show higher rates of apoptosis in the inflamed lesions compared to wild type mice (40). This observation indicates that CD44v might protect from autoimmunity by mediating apoptosis of inflammatory cells (Figure 12). Blockade of CD44v7 in a colitis model and in mononuclear cells from patients with Crohn's disease, triggers apoptosis (4, 41).

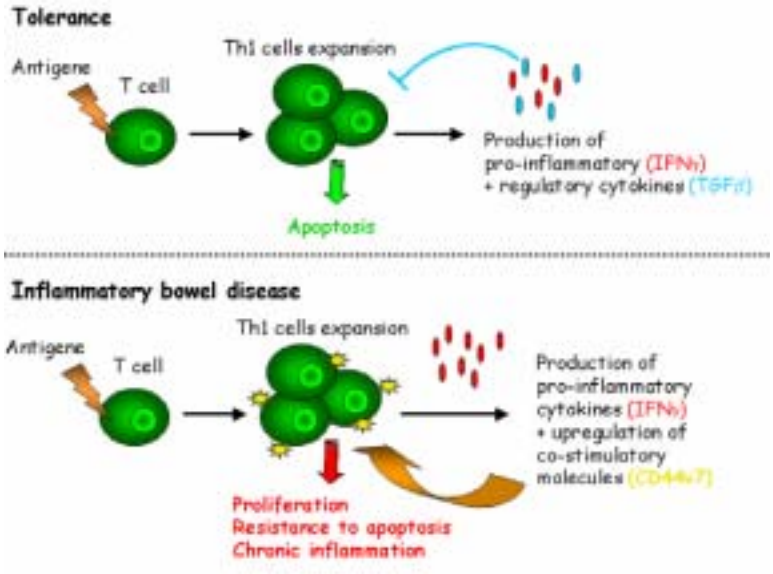

Figure 12. Model of inflammatory bowel disease and implication of CD44 in this disease. Co-stimulatory molecules such as CD44 are represented in yellow, inflammatory and regulatory cytokines in red and blue respectively. Activation of naïve T cells by antigen leads to proliferation of Th1 cells which secrete pro-inflammatory cytokines. In the absence of regulatory cytokines (TGFη), normally produced by regulatory T cells, Th1 cells continue to proliferate and upregulate stimulatory molecules like CD44v7. These cells become resistant to apoptosis and contribute to a persistent inflammation.

ü *Alopecia areata*

T cells from patients suffering from alopecia areata express higher amounts of CD44v7 which interestingly correlates with an increase in resistance to apoptosis (42).

ü *Rheumatoid arthritis*

CD44 also seems to play an essential role in human rheumatoid arthritis. In fact, joint synovium of patients with arthritis contains considerable amounts of various CD44 isoforms (reviewed by (43)). Treatment of collagen II-induced arthritic mice with an anti-panCD44 antibody, completely abrogates the disease (44).

ü *Diabetes*

Inflammatory cells in the islets causing autoimmune insulitis express CD44. Injection of anti-CD44 monoclonal antibody protects mice from diabetes (45).

ü *Delayed-Type Hypersensitivity responses*

Infiltrates cells in the ear upregulate CD44v10 and mice deficient in CD44v10 and v6/v7 show less edema and higher numbers of apoptotic cells.

Á CD44 and cancer

It is known that tumorigenesis is a complex process which implicates mutations in the tumor cells but also a contribution of environmental factors. Alterations in the expression and function of cell adhesion molecules such as CD44 correlate with the progression to tumor malignancy (46). While CD44s is expressed in most tissues, CD44 variant isoforms are aberrantly upregulated only under special conditions such as leukocyte activation, inflammation and tumorigeneses (4, 47-50). In most cancers expression of CD44 is misregulated. The pattern of CD44 expression is regulated by alternative splicing. The latter event is in part under the control of signaling pathways such as the Ras-MAP kinase cascade often activated in tumorigenesis (51, 52). CD44v have often been described to be involved in cancer. In fact, it was discovered in 1991, that CD44v4-7 isoforms are linked to the metastasis of a rat pancreatic carcinoma (1). Many studies have demonstrated since that the presence of diverse CD44v isoforms is associated with tumourigenesis, tumor cell invasion and metastasis.

ü *Colon cancer*

Upregulation of CD44 variant isoforms in colorectal carcinomas is related to tumor progression, predicts a poor prognosis (53) and confers resistance to apoptosis (54).

ü *Gastric and renal carcinomas*

Gastric carcinomas express CD44v9 and renal cell tumors upregulate v6 and v9 in the course of tumour progression. Over-expression of CD44v isoforms also correlates with an adverse prognosis in stomach and kidney carcinomas (reviewed by (55, 56)).

ü *Epithelium derived cancers*

Squamous cell carcinomas and some adenocarcinomas upregulate CD44v6 expression (57).

ü *Neuroblastoma*

Expression of the gene encoding the cell surface protein CD44 is repressed in neuroblastoma cells (58).

ü *Prostate cancer*

CD44s suppresses the metastatic ability of prostate cancer cells and this suppression does not require the binding to hyaluronic acid (59).

ü *Gynecologic cancers*

Breast, cervical, endometrial and ovarian cancer over-express CD44v isoforms which correlates with the metastatic potential of the malignant cells. CD44 has a role as a tumor marker in gynecological cancers (reviewed by (60)).

ü *Hematological cancers*

Expression of CD44v9 by myeloma cells is associated with an advanced clinical stage and disease progression (61-63) (Figure 13). Nearly all high-grade Non-Hodgkin´s lymphomas and acute myeloid leukemias (AML) express CD44v6 which correlates with an unfavorable course of the disease (3, 64). Anti-CD44 monoclonal antibodies induce differentiation of AML cell lines and inhibit their proliferation (65).

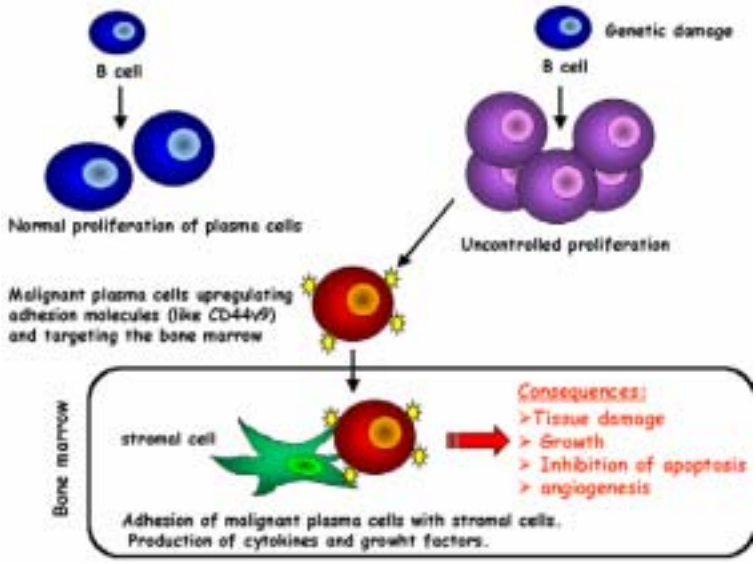

Figure 13. Model of multiple myeloma and implication of CD44 in this disease. When B cells are damaged, the plasma cells become malignant. These myeloma cells travel through the bloodstream and express adhesion molecules (represented in yellow) such as CD44v9 among others. The aberrant expression of adhesion receptors allows them to target bone marrow and attach to stromal cells. This interaction stimulates the production of cytokines (IL-6) and growth factors (VEGF) which promote proliferation, angiogenesis and inhibit apoptosis.

II. Apoptosis

1. General introduction to apoptosis

Á Discovery and features of apoptosis

The term apoptosis was coined in 1972 by John Kerr and describes the morphological manifestations of programmed cell death. It describes a distinct form of cell death in which cells actively commit suicide in a tightly regulated fashion. In contrast, necrosis is a passive degenerative process in which cells swell and lyse after irreversible tissue injury. Apoptosis is a fundamental process, evolutionary highly conserved, which is complementary but opposite to cell proliferation. The following changes characterize apoptosis: nuclear and cytoplasmic condensation, membrane blebbing, internucleosomal DNA fragmentation and the formation of apoptotic bodies (66) (Figure 7 and Table 1). Apoptosis is required for successful development and maintenance of tissue homeostasis. Excessive cell death may lead to compromised development or degenerative diseases whereas the diminished cell death may result in proliferative disorders. Drugs targeting the apoptotic pathways are a growing industry as they may be applied to the treatment of cancer, autoimmune and neurodegenerative diseases.

	Features of apoptosis
Cell size	Shrinkage.
Plasma membrane	Blebbed. Apoptotic bodies. Phosphatidylserine on surface.
Mitochondria	Increased membrane permeability. Contents released into cytoplasm: Cytochrome C; Apaf1.
Nuclei	Chromatin condensation
DNA degradation	Fragmented. Internucleosomal cleavage, free 3' ends, laddering on electrophoresis. DNA appears in cytoplasm.
Cell degradation	Phagocytosis. No inflammation

Table 1. Specific characteristics of apoptosis**.**

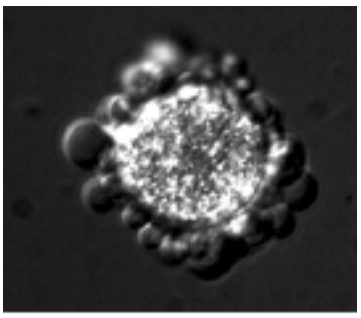

Figure 7. Light microscopy picture of a cell undergoing apoptosis. We can appreciate stereotyped features of programmed cell death such as the membrane blisters on this cell (picture from C. Subauste).

ü *What makes a cell decide to commit suicide?*

The balance between positive signals such as growth factors and the receipt of negative signals such as increase of oxidants, DNA damage (UV light, X-rays, cytotoxic drugs) or exposure to death activators (TNFζ3Lymphotoxin, FasL and TRAIL among others) will determine the fate of the cell (67).

Apoptosis can be triggered by internal signals involving the so-called mitochondrial apoptotic pathway or by external signals mediated by death receptors located in the cell membrane. Both pathways are complementary and connected (as described later) (68).

Á **Intrinsic apoptotic pathway**

ü *Bcl-2 proteins and mitochondrial disruption*

The intrinsic apoptotic pathway is mainly used in response to internal signals such as DNA damage. Bcl-2 family members constitute the main players of this pathway. This family is divided in two subgroups: the pro-survival proteins like Bcl-2 and Bcl-xL and the pro-apoptotic proteins such as Bax, Bak and Bid. The function of these proteins is either to preserve the mitochondrial integrity (anti-apoptotic proteins) or to disturb it (pro-apoptotic proteins) (reviewed by (69, 70)). During cell damage, the pro-apoptotic protein Bid activates Bax and Bak which form channels or interact with channel-forming proteins to increase the permeability of the mitochondrial membrane. The latter results in the release of cytochrome C into the cytosol. Cytochrome c, Apaf-1 (Apoptotic protease activating factor-1), pro-caspase 9 and ATP form a complex called apoptosome. Caspase

9 is activated and triggers the activation of the effector caspases 3 and 7 leading to the digestion of structural proteins, degradation of chromosomal DNA and finally phagocytosis of the cell (Table 2 and Figure 8) (71, 72). The balance between pro- and anti-apoptotic proteins determines the cell's fate.

ü *Potential participation of the endoplasmic reticulum in apoptosis*

It has been recently described that the endoplasmic reticulum (ER) plays a potential role in the intrinsic pathway. The efflux of calcium ions from the ER to the mitochondria seems to be required for mitochondrial disruption. Increasing evidence suggests that the anti-apoptotic protein Bcl-2 can interrupt the cross-talk from the ER to the mitochondria whereas the pro-apoptotic proteins Bax and Bak promote the calcium uptake into the mitochondria as well as the activation of caspase 12, an initiator caspase located on the ER membrane (reviewed by (70)).

Caspases (Cysteine Aspartate Specific Proteases)			
Category	**Name**	**Activators**	**Characteristics**
Initiator (Activator) caspases	Caspase-8 Caspase-9 Caspase-10 Caspase-2 (Caspase 12)	FADD Apaf-1/cyt c FADD DEFCAP, RAIDD TRAF-2, calpain	First to be activated. Cleave and activate effector caspases.
Effector (Executioner) caspases	Caspase-3 Caspase-7 Caspase-6	Caspase 9, 8 Caspase 9, 8 Caspase 3, 7	Cleave and activate cellular substrates.

Table 2. Caspases are highly specific proteases. They cleave proteins exclusively after aspartate residues. They regulate proteolysis during apoptotic cell death. It exists a third category of caspases known as the inflammatory caspases (caspase-1, 4 and 5) which are not represented in the table as their implication in apoptosis is still under discussion (67, 70, 73).

Á **Extrinsic apoptotic pathway**

ü *Structure and activity of death receptors.*

The main players in the extrinsic apoptotic pathway are the members of the tumor necrosis factor receptor (TNFR) superfamily also known as the death receptors. The TNFRs are type I transmembrane proteins and represent a growing family of cell surface receptors including Fas (CD95, APO-1), TNFR1 (p60), CD40, DR3, DR4 (TRAILR1), DR5 (TRAILR2), DR6 etc…. Members of this family contain one to five Cysteine-Rich Domains (CRD) in their extracellular domain, and a death domain in their cytoplasmic tail. The death domain is essential for recruitment of downstream molecules like initiator caspases leading to transduction of the apoptotic signal. (reviewed by (74-77)).

Signaling by the TNFRs is mediated by the binding of the trimeric ligand TNF to three monomeric subunits of the receptor in a 3:3 stoichiometric manner (reviewed by (78)). This binding leads to the activation of the death receptor and recruitment of adaptor proteins and caspases (Figure 8). TNF ligands share a common structural motif, the TNF homology domain (THD), which binds to CRDs of TNF receptors. The ligands are type II proteins that are synthesized as membrane bound proteins. Soluble forms can be generated by proteolysis (reviewed by (79)).

Most of TNF and TNFRs are expressed in the immune system, where they coordinate important processes such as inflammation and cell death which are essential to assure host defense. The capacity to induce cell death is one of the most important properties of this superfamily (reviewed by (78)).

The extrinsic and intrinsic pathway are tightly connected. The best characterized connection between the two pathways is the Bcl-2 family member Bid which translocates to mitochondria after cleavage by caspase-8 causing the activation of the mitochondrial pathway (68, 80).

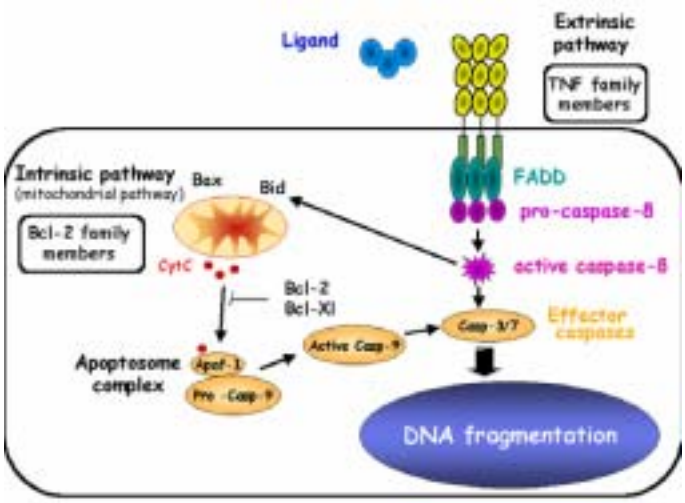

Figure 8. Model representing the extrinsinc and intrinsic apoptotic pathways.

Fas mediated cytotoxicity is the major calcium-independent killing mechanism of $CD8^+$ cytotoxic T cells (reviewed by (78)). Correct functioning of Fas induced apoptosis is essential to prevent autoimmunity and cancers. Indeed, Fas-mediated cell death is required for clonal deletion of autoreactive T cells and for elimination of tumor cells (75, 76). Because Fas is the prototypical member of this superfamily and the major death receptor player in apoptosis and inflammation, the following paragraphs will focus more on the activation and signaling of Fas.

Á **The Fas-FasL death system**

Fas is a type I transmembrane glycoprotein of 319 aa characterized by three extracellular regions, the Cysteine Rich Domain (CRDs), and an intracellular domain, the death domain, critical for apoptosis signaling (Figure 9).

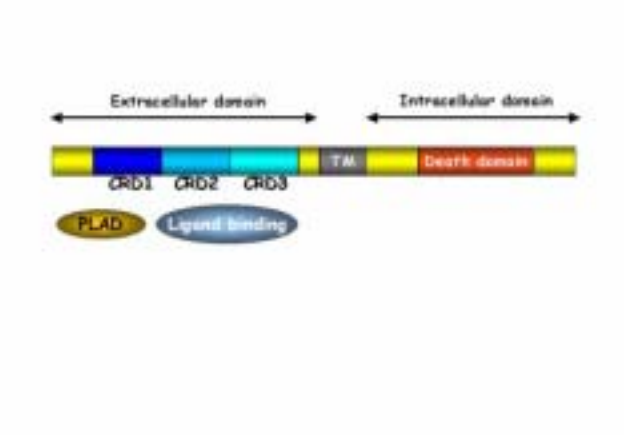

Figure 9. Structure of Fas. Fas contains three cysteine rich domains (CRD1, CRD2, CRD3) at the extracellular domain. The pre-ligand assembly domain (PLAD) described by (81, 82) is located at the N-terminus, followed by the ligand binding domain. The intracellular domain contains the death domain where Fas-associated death domain (FADD) and caspase 8 are recruited.

Fas mediated apoptosis is triggered by its natural ligand, FasL, which is a TNF related type II transmembrane molecule (Figure 10). Trimerization of Fas is required for the transduction of the apoptotic signal. Fas associates via its death domain with two specific proteins, Fas associated death domain (FADD) and caspase-8 (also known as FLICE), to form the Death Inducing Signaling Complex (DISC). FADD binds to Fas via its own death domain. FADD also carries a so-called death effector domain (DED), which recruits the DED containing procaspase-8 into the DISC. Caspase-8 undergoes autocatalytic processing to produce the active caspase 8 protease which is released into the cytoplasm. Active caspase 8 cleaves various proteins in the cell, including Bid and downstream caspases such as procaspase-3, which results in their activation and the execution of programmed cell death (reviewed by (75, 76)).

ü *Post-ligand trimerization of Fas: the conventional model*

The conventional model of Fas signaling proposes that the trimeric ligand FasL recruits three separate monomers of the receptor, thus inducing its trimerization and activation. The resulting complex allows the further recruitment of downstream signaling components (Figure 10 A) (reviewed by (76)).

ü *Receptor preassociation model*

Recently, using crosslinking experiments and fluorescence resonance energy transfer (FRET), a new model of Fas activation has been described (81, 82). In an elegant study with patients suffering from Autoimmune Lymphoproliferative Syndrome (ALPS), a

disease caused by a heterozygous mutation in Fas gene, Siegel and coworkers demonstrate that mutant Fas receptors are unable to bind FasL but can dominantly interfere with wild type Fas and form a complex which cannot signal. These observations allow them to conclude that Fas receptor chains can self-associate before ligand binding. FRET experiments by flow cytometric approach confirm receptor self-interaction in living cells in the absence of ligand (82). They also show that this pre-association is mediated by an amino terminal domain, called the Pre-Ligand Assembly Domain (PLAD), which is located at the CRD1 (Figures 9 and 10 B) (81). The authors provide strong evidence that the receptor is pre-associated and the ligand induces a rearrangement of receptor chains which allows efficient recruitment of downstream components or induces the formation of receptor superclusters (75).

Fas activation

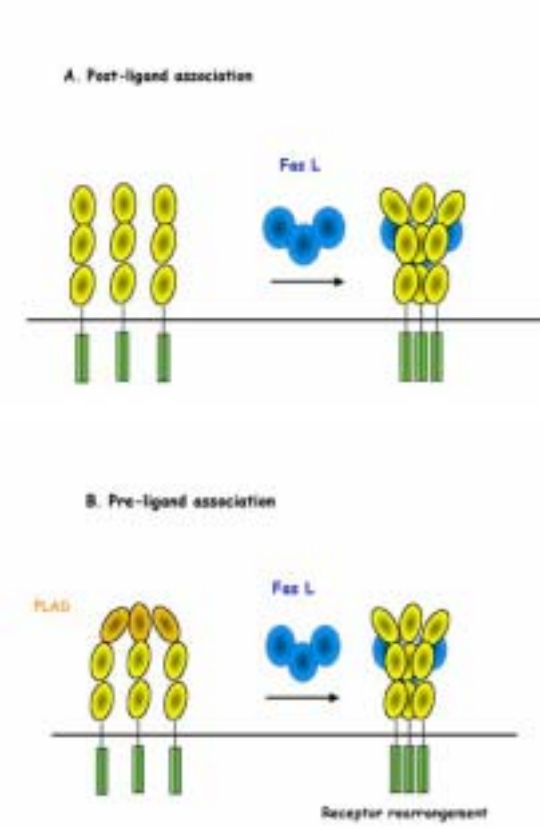

Figure 10.
Models of Fas activation.
A. Post-ligand activation of Fas. This model suggests that Fas exists as monomers and only once it is activated by its ligand FasL it trimerizes.

B. Pre-ligand association. This novel recently described model demonstrates that Fas is already associated through the pre-ligand assembly domain (PLAD) and the binding of FasL triggers a rearrangement of the receptor which leads to its activation.

2. Some techniques frequently used for apoptosis detection

As apoptosis has emerged as an important regulator of tissue homeostasis in multicellular organisms, several methods to quantify and to distinguish it from necrosis have been developed. The determination of whether a cell dies by apoptosis as opposed to necrosis is made on the basis of alterations in the cell membrane and cytoplasm, and changes in the cell's chromatin, both of which occur prior to lysis of the membrane. The changes in the chromatin include extensive condensation as assessed by light or electron microscopy, and DNA fragmentation as assessed by gel electrophoresis, or end-labeling of the nicked DNA fragments. Other specific apoptotic changes include membrane blebbing, which can only be appreciated microscopically, and the display of phosphatidylserine on the cell surface, which can be detected by flow cytometry.

- **Qualitative analysis of internucleosomal DNA fragmentation by agarose gel electrophoresis**

Fragmented DNA released from nuclei of cells undergoing apoptosis fails to sediment with intact chromatin when subjected to centrifugation. Two fractions are generated: the top, which contains the DNA which has been cleaved between nucleosomes, and the bottom (pellet), which contains the high-molecular-weight genomic DNA that has not been apoptotically fragmented. The pellet is discarded and the supernatant is analyzed by agarose gel electrophoresis. This procedure demonstrates the internucleosomal DNA cleavage associated with apoptosis, yielding the typical "ladder" (83).

- **Determination of apoptosis using sub-G_0/G_1 DNA peak**

This method depends on the observation that apoptotic cells stained with DNA binding dyes such as propidium iodide (PI), display an increased sub-G_0/G_1 DNA peak. The latter corresponds to the content of hypodiploid DNA and is caused by apoptotic DNA fragmentation (84).

- **Quantitation of apoptotic cells using TUNEL assay**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) is a method for detecting apoptotic DNA fragmentation. Using the enzyme terminal

deoxynucleotidyl transferase, biotin conjugated dUTP is bound to the nicked ends of the double stranded DNA. This method allows detection of apoptotic cells *in situ* in tissue sections or it can also be used to quantify TUNEL-stained apoptotic cells by flow cytometry (85).

- **Flow cytometric measurement of surface phosphatidylserine exposure as a determinant of programmed cell death**

Annexin V is a protein with a high specificity for binding the membrane lipid phosphatidylserine (PS) in the presence of calcium ions. PS is normally confined to the inner face of the plasma membrane, and this asymmetric distribution is lost as an early event during apoptosis. Thus, exposure of PS on the outside of the cell membrane is a sensitive early marker of apoptosis (86) (Figure 11).

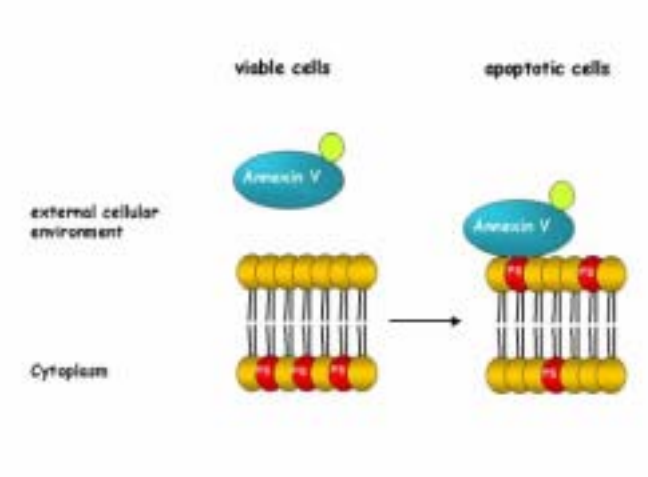

Figure 11. Detection of apoptosis using the AnnexinV method. This method is based on the recognition of phosphatidylserine (shown in red) which are exposed to the outer side of the membrane when cells are undergoing apoptosis. AnnexinV can be labeled with a fluorochrome, allowing quantification of apoptotic cells by flow cytometry.

- **Flow cytometric measurement of PARP cleavage**

During apoptosis, caspase-3 is activated and cleaves PARP (poly-ADP ribose polymerase), an enzyme involved in DNA repair. Detection of the cleaved form of PARP (p85 fragment) in the cytoplasm of cells is a specific characteristic of apoptosis (87).

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Aims of the study

Over the last decade, apoptosis has grown from an obscure process to a complex and interesting scientific field, and many players have been identified. One of the basic features of malignant transformation and autoimmunity is the acquisition of resistance to apoptosis. Many reports suggest that CD44 variant isoforms exhibit an anti-apoptotic effect leading to the progression of cancer and inflammatory diseases. However the molecular mechanisms of CD44s and CD44v actions have so far not been elucidated. Moreover literature about CD44 is controversial, some researchers describe CD44 as a survival molecule, others as a pro-apoptotic protein. Probably, this controversy is due to the presence of several isoforms and it might be possible that isoforms exhibit different and/or even opposite functions.
The aims of this study were to elucidate the following questions:

1) Are CD44 standard and/or variant isoforms regulating Fas-mediated apoptosis and how? (Chapter I and II).

2) Which is the *in vivo* implication of CD44 variant isoforms in autoimmune diseases such as multiple sclerosis and experimental autoimmune encephalomyelitis? (Chapter III).

To find an answer to these questions will improve our understanding of the pathogenesis of life threatening diseases such as cancer and autoimmunity, and may open new treatment strategies.

Chapter I

Chapter I: A novel anti-apoptotic mechanism based on Fas sequestration by CD44 variant isoforms.

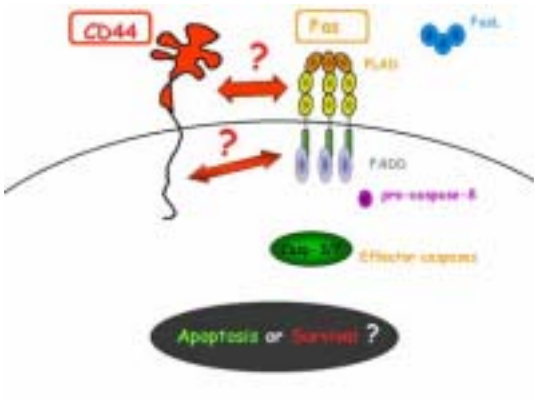
Summary Chapter I

Many reports show that upregulation of CD44v isoforms correlates with a poor prognosis in cancer and inflammatory diseases. However, the molecular basis of this observation has so far not been elucidated. In an experimental colitis model, mice lacking the expression of CD44v6 and v7 showed higher rates of apoptosis implicating CD44 variant isoforms in mediating resistance to apoptosis. To study this hypothesis, establishment of a suitable *in vitro* system was essential. Jurkat cells do not express any CD44 but they constitutively express Fas and can easily be induced to undergo apoptosis with FasL. Thus, these cells provide an excellent cellular system for our study. Stable transfectants expressing different CD44 isoforms were generated and functional apoptosis assays, confocal microscopy and biochemical experiments were performed with the aim to answer the following questions:

1. Are CD44 variant mediating resistance to apoptosis? And is there a specific variant isoform which is more likely to confer resistance to cell death?

2. How do CD44v isoforms mediate this resistance? Do they interact with a key molecule of apoptosis signaling?

3. Can we render the resistant cells again susceptible to apoptosis and how?



Model representing the central questions we aim to answer in this chapter I.

In this report, we are able to answer these biologically important questions. First, we demonstrate that, as hypothesized, CD44v isoforms, but not CD44s, confer resistance to Fas mediated apoptosis. CD44v expressing cells are only protected to apoptosis induced by FasL but not by other apoptotic stimuli. We show that cells expressing a CD44v mutant lacking the cytoplasmic domain are as resistant to apoptosis as cells expressing CD44v full length. The latter suggests that CD44v interferes extracellularly with Fas. In addition, we narrow the region responsible for mediating resistance to apoptosis to the alternatively spliced exons v6 and v9.

Second, we show that CD44v6 and v9 colocalize and interact with Fas. We also demonstrate that during apoptosis CD44v and Fas are both recruited into lipid rafts.

Finally, the anti-apoptotic effect of CD44v6 can be abolished by an antibody targeteting this specific isoform.

Based on these findings we propose a novel anti-apoptotic model to explain how CD44v blocks Fas.

A novel anti-apoptotic mechanism based on Fas sequestration by CD44 variant isoforms

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Abstract

There is growing evidence that one of the central common characteristics of tumor and inflammatory cells is their resistance to programmed cell death. This feature results in the accumulation of harmful cells, which are mostly refractory to Fas (CD95, APO-1) mediated apoptosis. A molecule found on these cells is the transmembrane receptor CD44 with its variant isoforms (CD44v). The establishment of transfectants expressing different CD44v isoforms allowed us to demonstrate that the CD44v6 and CD44v9 isoforms exhibit an anti-apoptotic effect and can block Fas mediated apoptosis. Moreover, we observed that CD44v6 and CD44v9 colocalize and interact with Fas. Importantly, an anti-CD44v6 antibody can abolish the anti-apoptotic effect of CD44v6. These results are the first to show that CD44v isoforms interfere with Fas signaling. Our findings improve the understanding of the pathogenesis of cancer and autoimmunity and open new strategies to treat such disorders.

Introduction

Since its first sequence description (1-3) CD44 has been attributed an ever-growing list of functions, that can be summarized as outside-in signaling events, conveying information from the cell surface to the nucleus. This multifunctional capacity is in part due to the expression of different CD44 isoforms resulting from alternative splicing that affect and change the extracellular domain of CD44 (reviewed by (4, 5)). Further functional variation has been demonstrated in recent reports, namely that CD44 functions as a co-receptor in multi-protein complexes (reviewed by (6)). The association of CD44 with the integrin VLA-4 abrogates the firm adhesion of cells and increases leukocyte extravasation into sites of inflammation (7). Moreover, CD44 variant 6 (CD44v6) forms a multiprotein complex with hepatocyte growth factor (HGF) and its receptor, the tyrosine kinase c-Met, and this interaction is essential for c-Met signaling (8). CD44 can act as a specialized platform for matrix metalloproteinases (MMP), by recruiting MMP9 to the cell surface and thus promoting tumor invasion or MMP7 and ErbB4 to regulate female reproductive organ remodeling (9, 10). The interaction of the CD44v6/v7 isoform with the cytokine-like molecule osteopontin (OPN) promotes inflammation (11, 12) and cell survival of mouse bone-marrow cells (13). Recent studies describe an association between CD44v4-7 and the epithelial cell adhesion molecule EpCAM and demonstrate that the interaction between these two metastasis-associated molecules can influence apoptosis resistance in tumor cell lines (14). In melanoma cells, CD44 can bind the laminin ζ5 chain which results in inhibition of tumor cell migration, invasion, and angiogenesis (15).

While the standard form of CD44 (CD44s) is ubiquitously expressed, the CD44v isoforms are highly restricted to processes like leukocyte activation, inflammation, and malignant transformation (as reviewed by (16-19)).

Since it was discovered that the splice variant CD44v4-7 is involved in metastasis of tumor cells (20), several studies have addressed the relevance of CD44 variant isoforms as diagnostic and prognostic markers for human tumors (reviewed by (16, 21, 22)). In multiple myeloma, CD44v9 expression in the absence of CD44v10 on bone marrow plasma cells is associated with a progressive phase of disease (23), while CD44v9 expression on bone marrow biopsies of myeloma patients is correlated with an adverse prognosis (24, 25).

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High-grade non-Hodgkin's lymphoma (NHL) and acute myeloid leukemia (AML) express CD44v6 isoforms, indicating a prognostic factor for poor prognosis (26, 27).

Earlier studies have indicated a contribution of CD44 in apoptosis blockade of tumor cells without specifying the involved region and the mechanism implicated (28-30).

The relevance of CD44v, but not of CD44s, in inflammation persistence has also been described (as reviewed by (18, 31, 32)). Experimental autoimmune encephalomyelitis and experimental colitis can be efficiently cured by anti-CD44v antibodies (33, 34). The first evidence that the CD44v region might be involved in apoptosis blockade came from studies with mice lacking CD44v6v7. In contrast to the CD44 wildtype mice, these mice were strongly protected against experimental colitis due to increased apoptosis in the inflammatory lesions of the lamina propria (35).

Control and regulation of alternative splicing is not completely understood (36, 37). Expression of CD44v isoforms is under the control of mitogenic signals including the Ras-MAP kinase cascade (38, 39). The mechanism(s) through which CD44v isoforms block Fas mediated apoptosis has so far never been described. Here we show that the presence of CD44v isoforms confers resistance to cell death by interference with Fas, which may have pivotal importance in chronic inflammation and cancer.

Materials and Methods

Cell lines and generation of CD44 transfectants

Jurkat cells were co-transfected by electroporation with 1.5σg of a neomycin resistance plasmid and 15σg of CD44s, CD44v2-10, CD44v3-10, CD44v6-10, CD44v8-10, CD44v3, CD44v6 CD44v9 or CD44v10 encoding DNA. Electroporation was performed by 250V and 960σF with the micropulser (BioRad, Reinach, Switzerland). All constructs contain EGFP (enhanced green fluorescence protein) fused at the CD44 carboxy terminal end (Figure 1A).

Jurkat cells and the plasmacytoma cell lines RPMI-8226, IM-9, NCI-H929 and U266 were obtained from the American Type Culture Collection (ATCC). The IL-6 dependent cell line XG-1 ($XG\text{-}1^{+}$) was a kind gift of Dr. B. Klein, Utrecht, The Netherlands. An IL-6 independent variant ($XG\text{-}1^{-}$) was selected by limiting dilution of $XG\text{-}1^{+}$ cells in the absence

of IL-6. Cells were grown in SF-IMDM with 2.5 % FCS (fetal calf serum) at 37∀C in a 5% CO_2 incubator and selected with 1mg/ml of Geneticin G418 (Gibco, Basel, Switzerland).
For the plasmacytoma cell lines 10σM 94I ƒ#and for the IL-6 dependent XG-1^+ cell line, 1.25 ng/ml IL-6 (Roche, Basel, Switzerland) were added to the culture medium.
Expression of CD44 isoforms and Fas was confirmed by flow cytometry with fluorescently labeled antibodies for panCD44 and CD44v specific isoforms (Table 1). Cells with similar green fluorescence intensities were sorted on a MoFlo high-speed cell sorter (DakoCytomation, Zug, Switzerland). Transfected cells with comparable CD44 expression levels and comparable Fas expression levels were used for the apoptosis assays.

Antibodies and reagents

The antibodies used for Western blotting were: mouse monoclonal anti-human panCD44 (clone Hermes 3 was a kind gift of Dr. E. Butcher, Stanford, CA), rabbit polyclonal anti-human Fas (Immunokontact, Lugano, Switzerland), mouse monoclonal anti-human FADD (clone 1, Transduction Laboratories, Basel, Switzerland), mouse monoclonal anti-human Bcl-2 (clone C-2, St Cruz Biotechnology) and mouse monoclonal anti-tubulin as control for equal loading (clone 236-10501, Molecular Probes, Leiden, The Netherlands), followed by goat anti mouse-horseradish peroxidase (HRP) (Pierce) or goat anti rabbit-HRP (Pierce). The signal was visualized by chemoluminescence with Super Signal Substrate (Pierce).
The blocking antibodies used for interfering with the variant region of CD44 were: mouse monoclonal anti-human CD44v6 (clone BBA 13, R&D Systems, Wiesbaden-Nordenstadt, Germany; clone VFF18, Bender MedSystems, Vienna, Austria) and mouse monoclonal anti-human CD44v9, clone FW11.24 (40).

Apoptosis induction

Neuro-2A cells, producing recombinant mouse FasL, were grown as described (41). Jurkat transfectants (5 x 10^5 cells/ml) were incubated with serial dilutions of Neuro-2A supernatant or with a mouse anti-human Fas IgM mAb (5-200ng/ml) (clone 7C11, Immunotech, Marseille, France) for 1, 3 or 6 hours at 37∀C. For the plasmacytoma cell lines the time of incubation with Fas crosslinking antibody was extended to 24 hours.

The Jurkat transfectants were also induced to undergo non-Fas mediated apoptosis with different stimuli such as: UV light (10,000 J), PMA (0.2σM) /Ionomycin (1σg/ml), heat shock (1h, 43∀C) or ν-irradiation (2.5Gy and 10Gy). Apoptosis was evaluated after 48 hours in culture by flow cytometry with AnnexinV/PI staining.

Evaluation of apoptosis

The cells, either Fas triggered or untreated, were evaluated for apoptosis by five different methods:

1. AnnexinV/PI. Cells were stained with annexinV conjugated to allophycocyanine (APC) (Alexis, Basel, Switzerland) and 5σg/ml propidium iodide (PI) (Sigma, Buchs, Switzerland) for 10 min in the dark at room temperature. Percentage of apoptotic cells was defined as the percentage of cells that were positive for annexinV and propidium iodide staining. The percentage of apoptotic cells measured in the untreated or mouse IgM control cells was subtracted from the percentage of apoptotic cells measured in the induced cells. Apoptosis was evaluated by flow cytometry (FACS Calibur, Becton Dickinson, Basel, Switzerland).

2. p85 PARP. Cells were fixed with 4% paraformaldehyde (PFA in PBS) for 20 min at room temperature, permeabilized for 10 min at room temperature with PBS containing 0.1% saponin and 0.09% sodium azide, and stained with a rabbit anti-p85 PARP antibody (Promega, Wallisellen, Switzerland) followed by a secondary goat anti-rabbit antibody conjugated to PE (Southern Biotechnology, Basel, Switzerland). Percentage of apoptotic cells was evaluated with flow cytometry.

3. Hypodiploid DNA. Cells were incubated overnight, at 4∀C, in the dark in a hypotonic DNA binding buffer containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 σg/ml PI in PBS. Percentages of apoptosis of untreated and FasL treated cells were compared by flow cytometric analysis. Apoptosis was evaluated by measuring loss of PI staining (corresponding to the hypodiploid DNA population, also called subG1 apoptotic population) in the FL3 channel (42).

The data obtained from these three methods were analyzed with the CellQuest program (Becton Dickinson).

4. Caspase-3 activity assay (Roche, Basel, Switzerland). Cells were prepared and processed according to the manufacturer's instructions.

5. SDS-PAGE and Western blotting. Jurkat cells untreated and treated for 30 min or 120 min with 7C11 were collected, lyzed in RIPA buffer (150mM NaCl, 10mM Tris-HCl pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5mM EDTA). Extracts were resuspended in 2x SDS sample buffer (100mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 10%#94I ƒ0, denatured at 96∀C for 5 min and separated on 10% SDS polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Schleicher & Schuell, Dassel, Germany) and blocked for 1 hour at room temperature in PBS with 3% Top Block (Juro, Luzern, Switzerland). PARP cleavage was detected with a mouse anti-PARP antibody (PharMingen, Basel, Switzerland) followed by a horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (Pierce, Basel, Switzerland). Detection was performed by chemoluminescence with Super Signal Substrate (Pierce).

Nitrogen cavitation bomb

According to the method published by T. Harder and coworkers (43) 10^8 cells were collected and incubated for 30 min at 4∀C on a rotating wheel with magnetic beads coated with a secondary goat anti-mouse IgG antibody (Dynal, Hamburg, Germany) which were consecutively coated with a primary anti-Fas antibody (clone ANC 95.1/5E2, Ancell, Sissach, Switzerland) or anti-panCD44 antibody (clone Hermes 3) according to the manufacturer's protocol. The cells coated with beads were then treated with FasL for 10 min at 37∀C or left untreated. For further Western blotting analysis, the extracts were processed as described above and immunoblotted with rabbit polyclonal anti-FAS antibody (Immunokontact) or mouse monoclonal anti-panCD44 antibody (Hermes 3) respectively, followed by goat anti-rabbit-HRP (Pierce) and goat anti-mouse-HRP (Pierce). The signal was visualized by chemoluminescence (Pierce).

Methyl-η cyclodextrin treatment

Cells were exposed to 10mM MηCD (Sigma) for 20 or 40 min before apoptosis induction for 5 hours with FasL. This dose is sufficient to selectively extract the cholesterol from the

plasma membrane and to disrupt lipid rafts. Apoptosis was evaluated by flow cytometry by measuring the sub G1 population (42).

Confocal microscopy

For confocal analysis Jurkat CD44s, Jurkat CD44v2-10, Jurkat CD44v6 and Jurkat CD44v9 cells, untreated or treated with FasL for 1 hour, were stained with mouse monoclonal anti-human panCD44 (Hermes 3), followed by secondary goat anti-mouse IgG conjugated with Cy2 (Amersham). Cells were also stained with an anti-human Fas biotinylated antibody (clone ANC 95.1/5E2 Ancell, Basel, Switzerland), followed by a secondary reaction with streptavidin-TexasRed (SA-TxR) (Southern Biotechnology, Reinach, Switzerland). After staining, the cells were fixed for 20 min at room temperature with 2% PFA, spun onto slides (Cytospin) and mounted in Mowiol. Confocal image stacks were recorded on a Leica TCS 4D operating in the simultaneous acquisition mode. Specimens stained only with one fluorochrome were examined and demonstrated that the setup did not result in crosstalk between channels. Images were analyzed for colocalization using the Imaris software package (Bitplane AG, Zürich, Switzerland) and applying a threshold well above the noise level (44). The data were statistically analyzed with the Wilcoxon signed rank test.

Blocking of apoptosis

Jurkat and XG-1 transfectants were pre-incubated for 2 hours at 37∀C with mouse anti-human specific CD44v antibodies (see description in antibodies and reagents), followed by treatment with FasL for 5 hours. Cells were prepared either for analysis of hypodiploid DNA (42), or intracellular stained with an anti-p85 PARP antibody conjugated with PE. Percentages of apoptosis of untreated, FasL treated and antibody plus FasL treated cells were compared by flow cytometric analysis (Becton Dickinson).

Results

Cells expressing CD44v are resistant to Fas mediated apoptosis

To understand the molecular mechanism(s) by which CD44v confers resistance to programmed cell death, we conducted in vitro functional assays using Jurkat cells, which constitutively express Fas, but not CD44. Jurkat cells can easily undergo apoptosis with Fas

ligand (FasL) or a Fas crosslinking antibody (clone 7C11). The cells were transfected with cDNA constructs for CD44s (Ju CD44s) and CD44v2-10 (Ju CD44v2-10) (Figure 1A).

As a control we used Jurkat cells transfected with the neomycin resistance plasmid alone (Ju neo). The different transfectants were treated with a serial dilution of Fas crosslinking antibody (Figure 1 B), or with a constant amount of antibody in a time course, and the amount of apoptotic cells was evaluated by flow cytometry after annexinV/PI staining (Figure 1 C). We noticed that Jurkat neo (control) and Jurkat CD44s cells quickly underwent apoptosis. Indeed, after 3 hours of treatment with Fas crosslinking antibody we detected about 50% of apoptotic cells and this number increased to 75% after 6 hours of induction. However, Jurkat CD44v2-10 cells showed strong resistance to apoptosis and only 5% and 15% of apoptotic cells were detected after 3 and 6 hours treatment with the Fas crosslinking antibody, respectively. To confirm these results a similar experiment was performed and apoptosis was detected by quantifying cleavage of poly-ADP ribose polymerase (PARP) (Figure 1 D and E). We again observed that Jurkat CD44v2-10 cells were more resistant to Fas mediated cell death than Jurkat CD44s. To confirm that the resistance to apoptosis was due to the presence of CD44v2-10 and not a cloning artifact, transfection was repeated, Jurkat CD44v2-10 (pool II) was generated and the amount of apoptotic cells was determined by measuring hypodiploid DNA (Figure 2 A) (42).

No single clones were isolated; these experiments were performed with “pools” of cells expressing similar levels of CD44. Both transfectants, Ju CD44v2-10 (pool I) and Ju CD44v2-10 (pool II) showed similar resistance to cell death. Before using the “pools”, single clones were isolated and selected according to the levels of CD44 expression and, interestingly, those ones with CD44v high expression were more resistant to Fas mediated apoptosis (data not shown). The difference between CD44s and CD44v concerning resistance to cell death was also confirmed by Western blotting (Figure 2 B).

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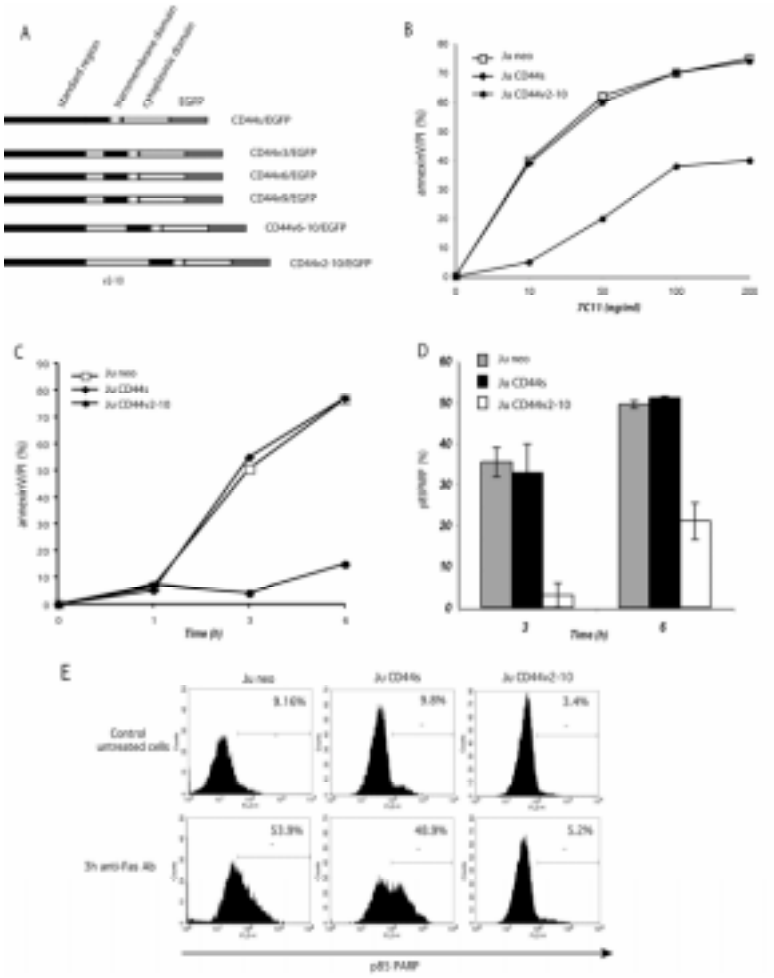

Figure 1. Jurkat CD44v2-10 cells but not Jurkat CD44s cells are protected from Fas mediated apoptosis.
(A) Scheme of the different CD44-EGFP constructs used for transfection of Jurkat and XG-1 cells.
(B) Jurkat neo (negative control), Jurkat CD44s, Jurkat CD44v2-10 cells were treated with a serial dilution (10-200ng/ml) of Fas crosslinking antibody (clone 7C11) for 6 hours. Subsequently, the percentage of apoptotic cells was determined by flow cytometry with annexinV/PI staining and was calculated by subtracting the control values from the values obtained with 7C11 treatment. Values are given for one experiment representative of three.
(C) Jurkat neo, Jurkat CD44s, Jurkat CD44v2-10 cells were treated with Fas crosslinking antibody for 1, 3 or 6 hours. Apoptotic cells were determined with annexinV/PI staining. Values are given for one experiment representative of three.
(D) Jurkat transfectants were treated with recombinant FasL and the percentage of apoptotic cells was determined by flow cytometry with a fluorescently labeled antibody recognizing the cleaved form of PARP, p85 PARP. Data are representative of three independent experiments.
(E) FACS analysis of Jurkat neo, Jurkat CD44s and Jurkat CD44v2-10 untreated and treated with Fas crosslinking antibody. Percentage of p85 PARP positive cells was evaluated by flow cytometry with an anti-p85 PARP PE labeled antibody. The bar is limiting the living cells on the left according to the control. Numbers in the upper right corner represent the percentage of apoptotic cells.

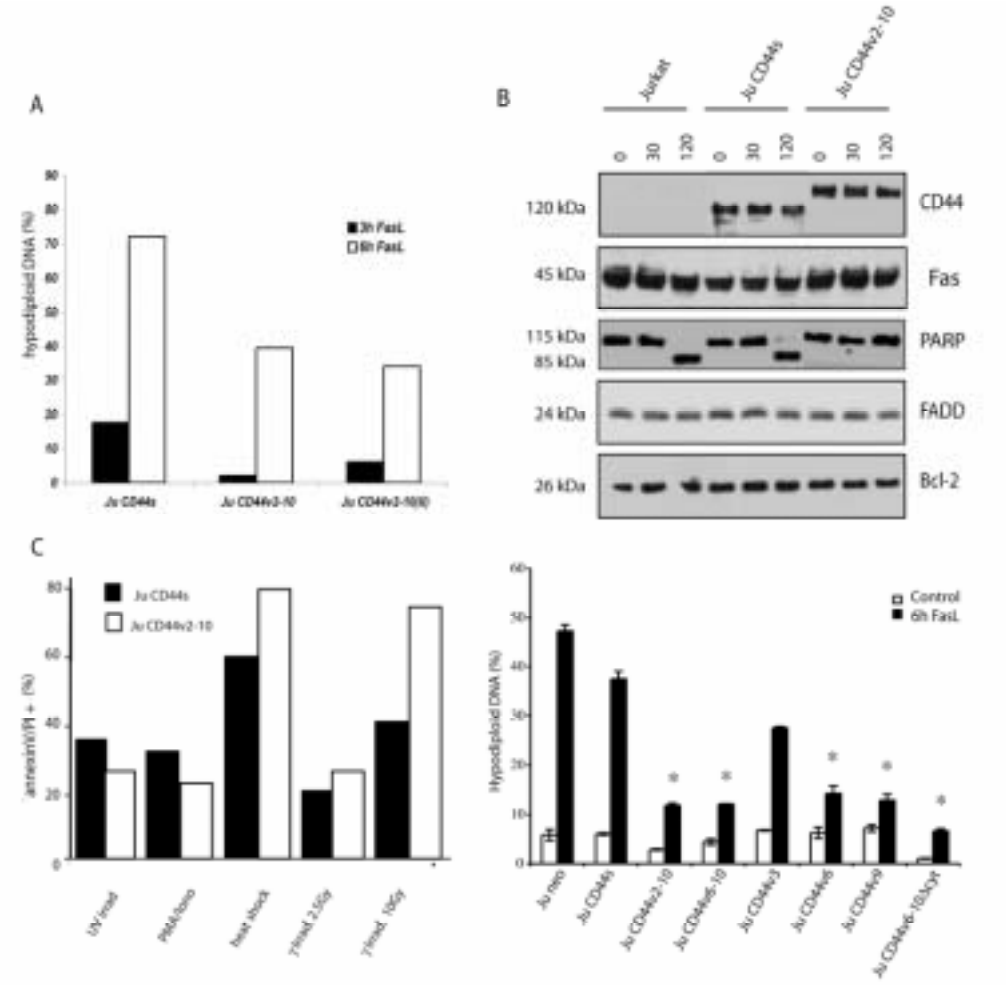

Figure 2. CD44v6 and v9 are sufficient for mediating resistance to Fas mediated apoptosis in Jurkat cells.

(A) A second independent Jurkat CD44v2-10 (pool II) transfectant was established, induced with FasL and the amount of apoptotic cells was determined by measuring hypodiploid DNA by flow cytometry (hypodiploid DNA= subG1 apoptotic population). Values are given for one experiment representative of three.
(B) After treatment with 7C11 for 30 or 120 min, cells were collected, lyzed in Ripa buffer and Western blotted with anti-panCD44, anti-Fas, anti-PARP antibody recognizing both, the uncleaved and the cleaved form of PARP, anti-FADD, and anti-Bcl-2 antibodies.
(C) Jurkat CD44s and Jurkat CD44v2-10 were induced to undergo cell death with UV light, PMA/Ionomycin, heat shock, or ν irradiation. The amount of apoptotic cells was evaluated with annexinV/PI staining by flow cytometry. Values are given for one experiment representative of three.
(D) Jurkat neo, Jurkat CD44s, Jurkat CD44v2-10, Jurkat CD44v6-10, Jurkat CD44v3, Jurkat CD44v6, Jurkat CD44v9 and Jurkat CD44v6-10÷cyt cells were treated with FasL for 6 hours and the percentage of apoptotic cells was determined by measuring hypodiploid DNA (hypodiploid DNA= subG1 apoptotic population). Data are representative of five independent experiments and were analyzed with the Wilcoxon signed rank test (1, p{ 0.05).

All the transfectants showed similar levels of Fas and CD44 upon treatment with Fas crosslinking antibody, except for Jurkat neo cells, which were negative for CD44. Additionally, expression of CD44 was also determined by measuring enhanced green fluorescence protein (EGFP) as well as by use of panCD44 and CD44 variant-specific antibodies by flow cytometry (Table 1). When blots were probed with an anti-PARP antibody recognizing both the uncleaved (115 kDa) and the cleaved (85 kDa) form, we observed cleavage of PARP after treatment with Fas crosslinking antibody, in Jurkat neo and Jurkat CD44s, indicating that those cells underwent apoptosis. In contrast, extracts from Jurkat CD44v2-10 did not show any cleavage of PARP. These results demonstrate that the function of CD44v isoforms is different from CD44s and that cells expressing CD44v2-10 are significantly more resistant to Fas mediated apoptosis. We did not observe any variation in the expression levels of Fas, CD44, Fas-associating protein with death domain (FADD) and Bcl-2 in the different transfectants upon treatment with Fas crosslinking antibody. When cells were treated with UV light, heat shock, PMA/Ionomycin or ν-irradiation, CD44s and CD44v2-10 Jurkat cells showed similar sensitivity to cell death and with higher

doses of ν-irradiation, Ju CD44v2-10 were even more susceptible to apoptosis. These results indicate that CD44v2-10 does not protect the cells from other forms of cell death beside Fas mediated apoptosis (Figure 2 C).

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CD44v6 and v9 are sufficient for mediating resistance to apoptosis

To address which is/are the variant regions responsible for the resistance to programmed cell death we used Jurkat transfectants expressing the variant regions 6 to 10 (Ju CD44v6-10), only variant 3 (Ju CD44v3), variant 6 (Ju CD44v6) or variant 9 (Ju CD44v9) within the context of the standard backbone. To directly examine the behavior of the different variant regions, the different transfectants were treated with FasL for 6 hours and apoptosis was measured by determining hypodiploid DNA. Only cells expressing the variant regions 6 and/or the variant 9 were significantly more resistant to Fas mediated apoptosis (Figure 2 D) than CD44s transfected cells.

Table 1: Expression of CD44 isoforms and Fas in cell transfectants.

Cell lines	pan CD44	CD44 v3	CD44 v6	CD44 v7	CD44 v9	CD44 v10	Fas
RPMI-8226	-	-	-	-	-	-	+
IM-9	+	-	-	-	-	-	+
U266	+	+	-	-	+	-	+
NCI-H929	+	+	-	-	+	-	+
XG-1 $^{+}$	+	+	+	-	+	-	+
XG-1 $^{-}$	+	-	-	-	-	-	+
XG-1 neo	+	-	-	-	-	-	+
XG-1 v6	+	-	+	-	-	-	+
XG-1 v9	+	-	-	-	+	-	+
Ju neo	-	-	-	-	-	-	+
Ju CD44s	+	-	-	-	-	-	+
Ju CD44v2-10	-	+	+	+	+	+	+
Ju CD44v6-10	-	-	+	+	+	+	+
Ju CD44v3	-	+	-	-	-	-	+
Ju CD44v6	-	-	+	-	-	-	+
Ju CD44v9	-	-	-	-	+	-	+

Expression of CD44s, v3, v6, v7, v9, v10 and Fas on plasmacytoma cell lines, CD44 transfected XG-1 plasmacytoma cell lines and CD44 transfected Jurkat (Ju) cell lines, measured by FACS. Plasmacytoma cell lines U266 and XG-1^{+} are IL-6 dependent.

The Ju CD44v3 was slightly more resistant to apoptosis compared to Ju CD44s. Nevertheless, this difference was not statistically relevant. This suggests that the resistance to apoptosis observed in the variant positive cells is not due to the proliferation stimulus caused by growth factors binding to the heparan sulphate chains located at the variant 3 region (45). Moreover, Jurkat cells expressing CD44v6-10 (or CD44v2-10, not shown), but lacking the cytoplasmic domain were also strongly protected from cell death. This observation clearly points out the significance of the extracellular domain of CD44v for apoptosis resistance (Figure 2 D).

Previous analyses demonstrated a significant correlation between CD44v9 expression and a poor prognosis for patients with multiple myeloma (23-25). To investigate a possible

relationship between CD44v expression and apoptosis resistance with more potential clinical relevance, we chose plasmacytoma cell lines. Various myeloma cell lines were assayed for their susceptibility to Fas mediated apoptosis and correlation to CD44v9 expression. CD44 and Fas expression levels of six plasmacytoma cell lines analyzed by flow cytometry are listed in Table 1. When different cell lines were induced to undergo apoptosis, we observed that CD44v9$^+$ cells were clearly more resistant to apoptosis than cells lacking v9 expression (Figure 3 A). XG-1$^-$ was selected as an Il-6 independently growing variant of XG-1$^+$. Compared to XG-1$^+$, XG-1$^-$ had lost the expression of CD44v3, v6 and v9, but still expressed CD44s. To substantiate these observations, the XG-1$^-$ cells were transfected with CD44v6, CD44v9, CD44v10, CD44v8-10, and CD44v3-10 constructs. The different transfectants were treated with the Fas crosslinking antibody and apoptosis was measured by detection of active caspase 3 (Figure 3 B). We observed that all XG-1 cells expressing the CD44v9 region were resistant to cell death induction. Further the cells were treated with Fas crosslinking antibody and apoptosis was measured by detecting PARP cleavage by flow cytometry (Figure 3 C) or by annexinV/PI staining (Figure 3 D). We again observed that only the cells expressing CD44v9 showed lower levels of p85 PARP and annexinV/PI staining. In this system the expression of CD44v9 could protect the cells from undergoing cell death whereas the expression of CD44v6 or CD44v10 did not give such a protection. This difference concerning apoptosis resistance conferred by CD44v6 in Jurkat, but not in XG-1 cells could be due to post-translational modifications (e.g. alterations in glycosylation) that are cell-type and growth-condition specific. These data demonstrate that the CD44 variant 6 and 9 regions, depending on the cell type, can play essential roles in preventing the cells from undergoing programmed cell death.

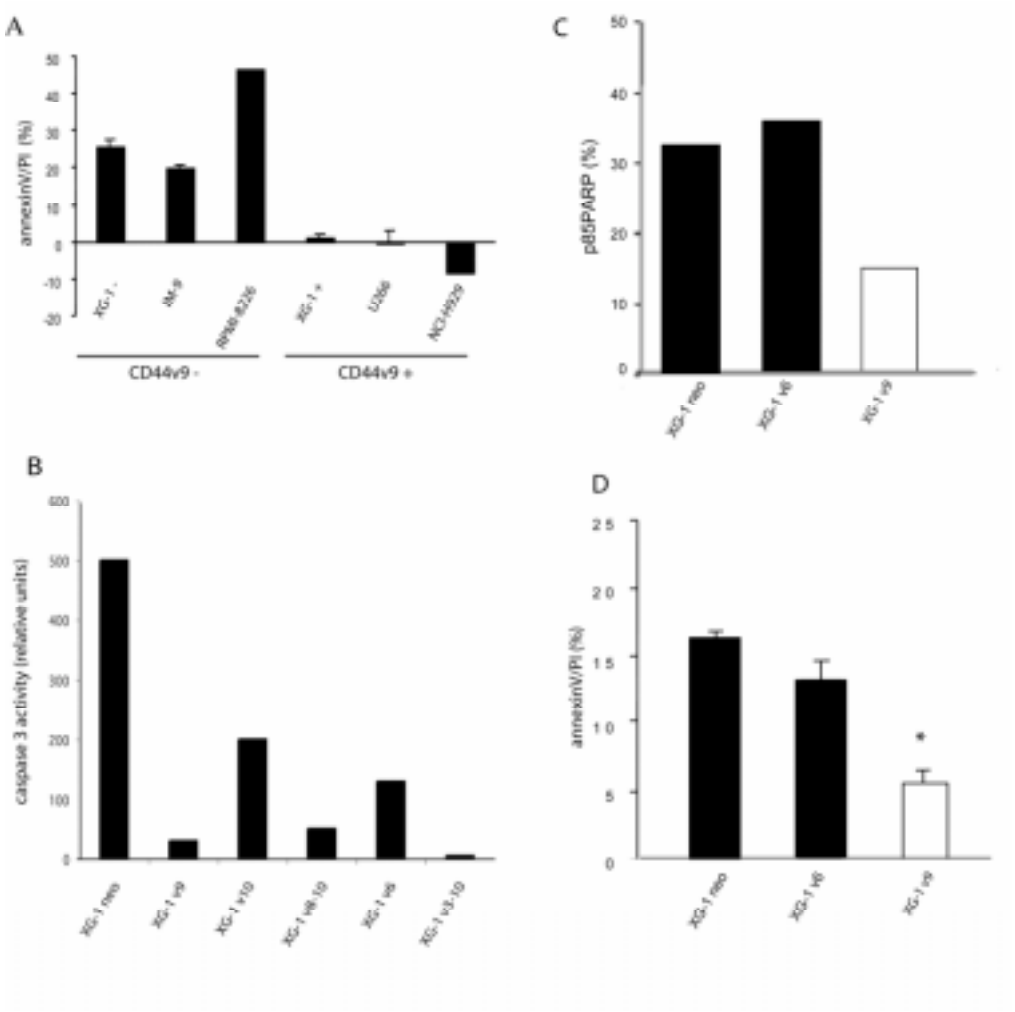

Figure 3. CD44v9 is sufficient for mediating resistance to Fas mediated apoptosis in XG-1 plasmacytoma cells.

(A) Plasmacytoma cell lines XG-1$^-$, IM-9, RPMI-8226, XG-1$^+$, U266 and NCI-H929 were treated for 24 hours with the Fas crosslinking antibody (7C11) or with control mouse IgM. Apoptosis was determined by annexinV/PI staining. Values are representative of three independent experiments.
(B) XG-1$^-$ transfectants were treated with the Fas crosslinking antibody (7C11) or control IgM for 24 hours and the percentage of apoptotic cells was determined by flow cytometry with a fluorescently labeled antibody recognizing the cleaved form of PARP. Values are given for one experiment representative of three.
(C) XG-1$^-$ transfectants were incubated for 24 hours with 7C11 or control IgM and the percentage of apoptotic cells was detected by measuring active caspase-3. Values are given for one experiment representative of three.
(D) XG-1$^-$ transfectants were incubated for 24 hours with 7C11 or control IgM and the percentage of apoptotic cells was detected by measuring annexinV/PI positive cells. Data are representative of three independent experiments and were analyzed with a two sided unpaired Student t-test (1, p{ 0.05).
The percentage of apoptotic cells was calculated by subtracting the control values (IgM treatment) from the values obtained with anti-Fas treatment.

Colocalization of CD44v and Fas

We have shown that Jurkat and XG-1 cells expressing CD44v6 and/or v9 are strongly protected from Fas mediated apoptosis. Consequently, we were interested in understanding the molecular mechanism(s) of protection. Therefore we explored by confocal microscopy if CD44 and Fas colocalize. The results showed that CD44v2-10, CD44v6 and CD44v9 strongly colocalized with Fas (Figure 4B, C, D), whereas colocalization between CD44s and Fas was significantly less intense (Figure 4 A). To quantify the percentage of colocalization, confocal image stacks were recorded and analyzed with the Imaris software. In cells not induced to undergo apoptosis, the percentage of colocalization between Fas and CD44v was significantly higher (36%) than between Fas and CD44s (13%) (Figure 4 E). When the cells were treated with the Fas crosslinking antibody or with FasL, the colocalization between CD44v and Fas strongly increased and was significantly higher than the colocalization observed between CD44s and Fas. These results strongly suggest a possible molecular interaction between CD44v and Fas.

Cells expressing CD44s or CD44v show reduced Fas mediated apoptosis after disruption of lipid rafts.

CD44 and Fas have both been described to be recruited into lipid rafts (46, 47). To understand the relevance of this reaction for CD44 and Fas colocalization during apoptosis induction in vitro, we performed functional assays using the Jurkat CD44s and CD44v2-10 transfected cells. Both transfectants were treated with methyl-η cyclodextrin (MηCD), followed by treatment with FasL. Controls of untreated cells and cells treated only with FasL were performed. The amount of apoptotic cells was determined by measuring hypodiploid DNA by flow cytometry (42) (Figure 5 A). We could again observe that after induction of apoptosis with FasL, cells expressing CD44v2-10 were significantly more resistant to apoptosis than cells expressing the CD44s isoform. Furthermore, we observed that treatment with MηCD (a disrupter of lipid rafts) caused a significant reduction of hypodiploid DNA in both transfectants (37% to 12-10% for the Ju CD44s and 12% to 6-7% for the Ju CD44v2-10). These results support the notion that integrity of lipid rafts is required for Fas mediated apoptosis, irrespective whether the cells express CD44s or CD44v.

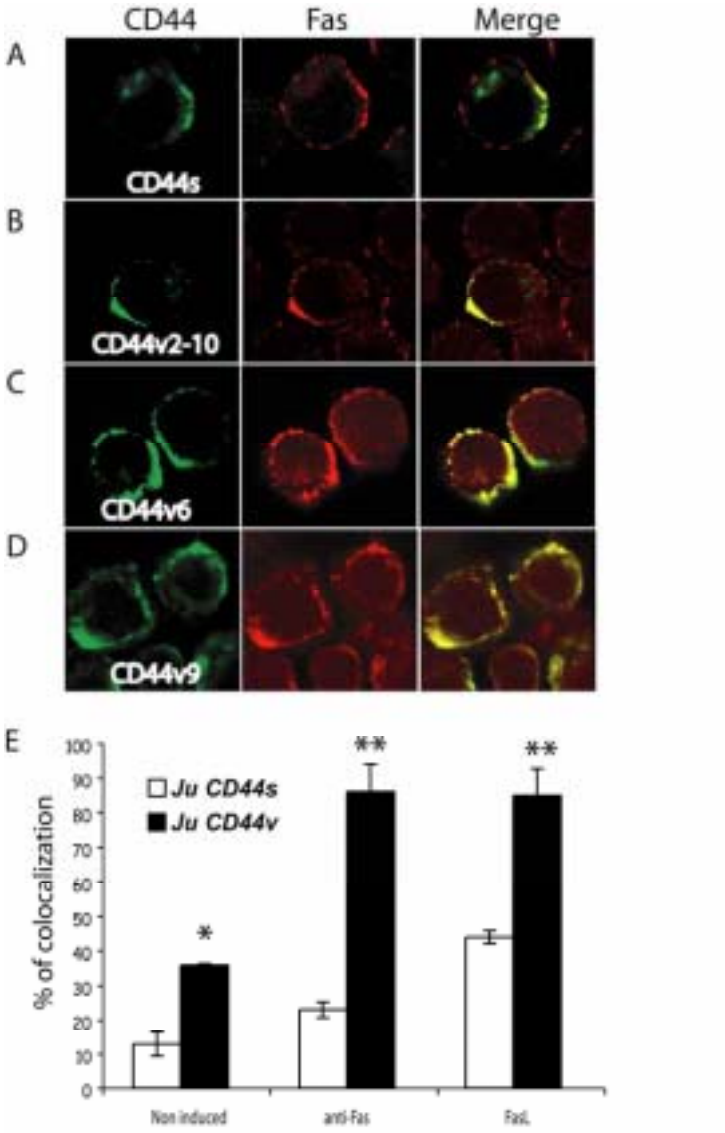

Figure 4. The CD44 variant region and Fas specifically colocalize
Jurkat cells transfected with CD44s (A), CD44v2-10 (B), CD44v6 (C) and CD44v9 (D) were treated for 1 hour with FasL and then stained with the anti-panCD44 antibody Hermes-3, followed by Cy2 labeled goat anti-mouse antibody (green fluorescence) and with an anti-Fas biotinylated antibody, followed by SA-TxR.
(E) Histogram representing the percentages of CD44 and Fas colocalization under non- apoptosis inducing conditions, induced with Fas crosslinking antibody (clone 7C11) and induced with FasL. White bars represent the percentage of colocalization between CD44s and Fas, black bars the percentage of colocalization between CD44v and Fas. Data are representative of 5 independent experiments and were analyzed with the Wilcoxon signed rank test (1, p{0,05; 11, p{0,01).

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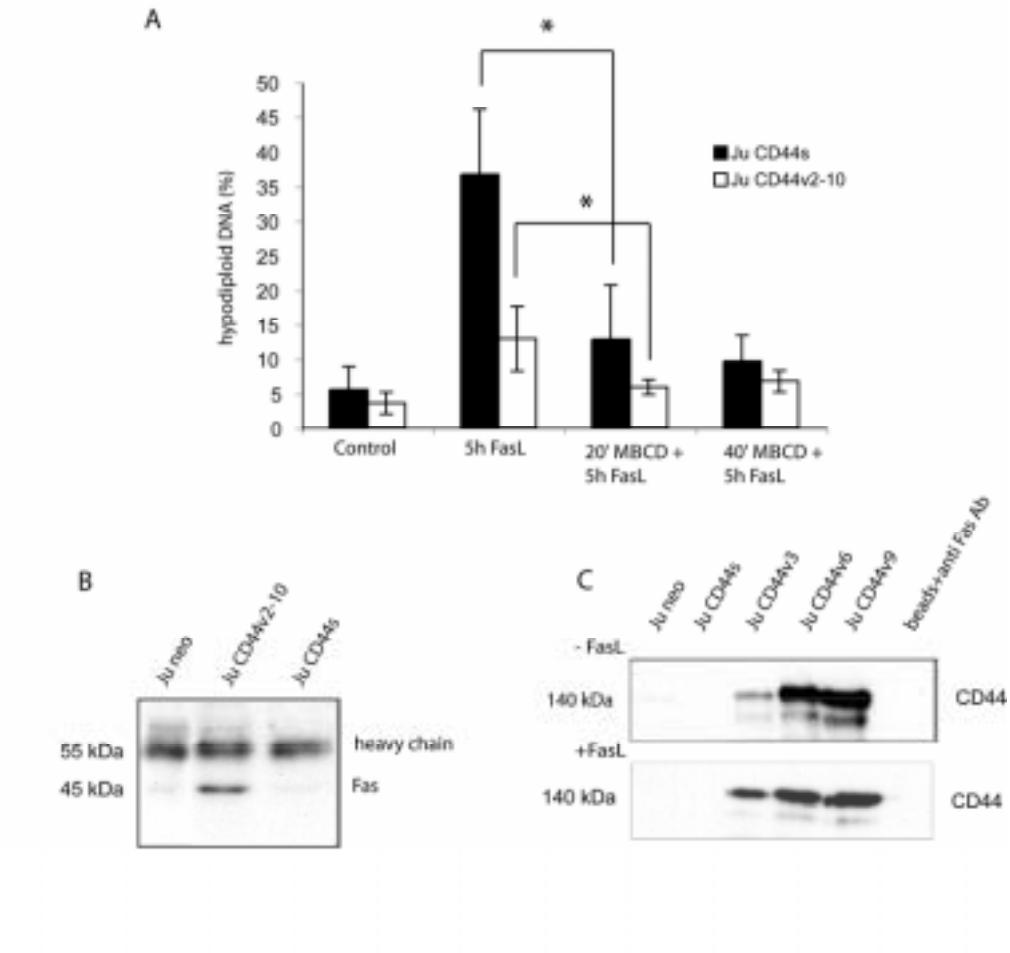

Figure 5. Interaction between Fas and CD44v isoforms in lipid rafts.
(A) Jurkat CD44s and Jurkat CD44v2-10 cells were pre-treated for 20 or 40 min with MηCD, then induced with FasL for 5 hours. Subsequently, the percentage of apoptotic cells was determined by measuring hypodiploid DNA (hypodiploid DNA= subG1 apoptotic population). Data are representative of three independent experiments.
(B) Western blotting for Fas on CD44 cavitation bomb extractions from Jurkat neo, Jurkat CD44v2-10 and Jurkat CD44s cells. Cells were incubated with magnetic beads coated with mouse anti-human CD44, treated for 10 min with FasL, before nitrogen cavitation bomb extraction. 10% SDS-PAGE was performed under reducing conditions, followed by Western blotting with polyclonal rabbit anti-Fas antibody and HRP-conjugated goat anti-rabbit secondary antibody.
(C) *Upper panel*. Western blotting for panCD44 on Fas cavitation bomb extractions from Jurkat neo, Jurkat CD44s, Jurkat CD44v3, Jurkat CD44v6, Jurkat CD44v9 cells. Cells were incubated with magnetic beads coated with mouse anti-human Fas antibodies before nitrogen cavitation bomb extraction. 10% SDS-PAGE was performed under reducing conditions followed by Western blotting with mouse anti-human CD44 antibody and HRP-conjugated goat anti mouse secondary antibody.
Lower panel. An equivalent experiment, as shown in the upper panel, was performed with cells treated with FasL for 10 min.

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The CD44 variant region interacts with Fas

To find out whether a physical association exists between CD44v and Fas, we performed co-immunoprecipitations using the nitrogen cavitation bomb method. This is an alternative procedure to the standard co-immunoprecipitations for examining interactions between membrane proteins (43). The advantage of this procedure is that it is detergent free and therefore highly suitable to study interactions between membrane proteins, which can be recruited into lipid rafts and are very difficult to extract with conventional lysis buffers without destroying the natural interactions existing between the candidate molecules.

To examine the association between CD44 and Fas, we first used CD44 antibody-decorated cavitation bomb extractions from Jurkat neo, Jurkat CD44s and Jurkat CD44v2-10 cells and performed Western blotting detection with anti-Fas antibody. Before extraction, the samples were surface cross linked with 3,3'-Dithiobis-(sulfosuccinimidylpropionate) (DTSSP) (cross linker cleavable under reducing conditions). Only anti-CD44 extractions from Jurkat CD44v2-10 cells probed with anti-Fas antibody gave rise under reducing conditions to a strong 45kDa band corresponding to Fas (Figure 5 B). When cavitation bomb extractions were performed without cross linker, we observed similar results, but the 45kDa band was less intense (data not shown). To confirm these results, Western blotting for CD44 on Fas decorated cavitation bomb extractions from the different transfectants was performed. This experiment was reproduced under two conditions: cells untreated (Figure 5 C, upper panel) and cells treated with FasL (Figure 5 C, lower panel). In the untreated cells we detected a strong band at 140 kDa, corresponding to CD44 fused to EGFP, in the extracts obtained from Jurkat CD44v6 and Jurkat CD44v9 transfected cells (upper panel). A weaker band appeared in the extracts from Jurkat CD44v3. No bands were observed in the extracts from the control Jurkat neo cells or Jurkat CD44s transfected cells. When the cells were treated with FasL similar results were observed: no bands for Jurkat neo and Jurkat CD44s, a strong band at 140 kDa for Jurkat CD44v6 and v9 and a weaker band for Jurkat CD44v3 extracts (lower panel). Western blotting was also performed under non-reducing conditions and high molecular aggregates in the Ju CD44v were observed (data not shown). These data confirm that only CD44v, but not CD44s, interacted with Fas and that CD44v6 and v9 strongly interacted with Fas, whereas CD44v3 did so at lesser intensity.

Blocking the variant 6 region of CD44 restores the apoptotic potential

We have demonstrated that CD44v positive cells were more resistant to Fas mediated apoptosis and that CD44v colocalizes and interacts physically with Fas. With the knowledge that resistance to Fas mediated apoptosis is a major caveat for treatment of tumor cells and cells in chronic inflammatory lesions, we were interested in preventing the interaction of CD44v/Fas and therefore make the cells susceptible to Fas mediated cell death. For this purpose we tried to block the variant region of CD44 with different antibodies. Blocking the CD44v6 region with anti-CD44v6 antibodies (clones VFF18 and BBA13) successfully worked in Jurkat cells. However, the anti-v9 antibody (clone FW11.24) was unable to block the CD44v9 region, neither in Jurkat nor in XG-1 cells. This antibody could be used for FACS staining of the cells, but is not functioning as a blocking antibody. Jurkat CD44s and Jurkat CD44v6 transfected cells were incubated with anti-CD44v6 antibody (BBA 13), followed by apoptosis induction with FasL and determining PARP cleavage (Figure 6A). When cells were only treated with FasL we observed that 47.4 % of cells were apoptotic in the Jurkat CD44s sample and only 5.5% in Jurkat CD44v6 (Figure 6 A middle panels). When cells were pre-incubated with BBA13 and then induced with FasL, the percentage of apoptotic cells in the Jurkat CD44v6 sample significantly increased to 37.7%, whereas the apoptotic ratio for the Jurkat CD44s was unchanged (lower panels). When in a similar set-up apoptosis was measured by determining hypodiploid DNA, we obtained equivalent data (Figure 6 B). These data confirm v6 as one of the variant regions involved in preventing Fas mediated apoptosis and that it is possible to regain susceptibility to apoptosis by blocking this variant region.

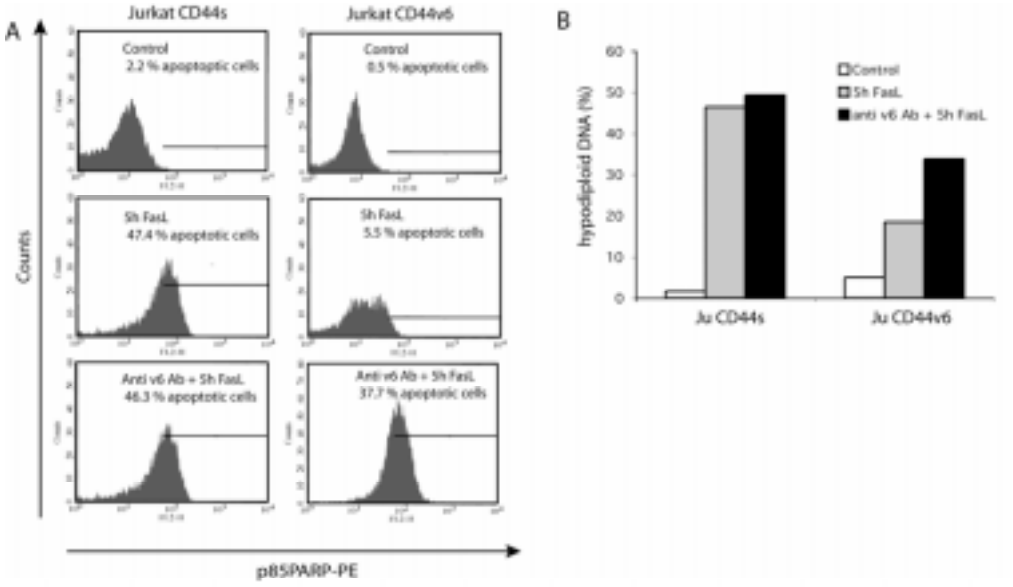

Figure 6. Interference with CD44v6 could induce susceptibility to apoptosis of CD44v6$^+$ cells.
(A) *Upper panels*: Jurkat CD44s and Jurkat CD44v6 untreated cells. *Middle panels*: Jurkat CD44s and Jurkat CD44v6 treated with FasL for 5 hours. *Lower panels:* Jurkat CD44s and Jurkat CD44v6 pre-incubated for 2 hours with anti-CD44v6 antibody, followed by 5 hours incubation with FasL.
The numbers in the quadrants represent the percentage of apoptotic cells obtained in one experiment representative of three. The percentage of apoptotic cells was evaluated by measuring PARP cleavage. The bar is limiting the living cells on the left according to the control.
(B) Jurkat CD44s and Jurkat CD44v6 untreated, treated with FasL or 5 hours or pre-incubated for 2 hours with anti-CD44v6 antibody, then treated with FasL for 5 hours were analyzed in hypotonic buffer. The percentage of apoptotic cells was evaluated by measuring loss of PI staining (hypodiploid DNA= subG1 apoptotic population). Values are given for one experiment representative of three.

Discussion

Programmed cell death is not only essential for normal development and regeneration, but also to eliminate cells that represent a threat to the integrity of the organism. Defects in the apoptotic machinery are associated with the development of autoimmune and neoplastic diseases. Fas is a major trigger for apoptosis, especially in activated immune cells. Thus, the blockade of Fas inhibits an important apoptotic pathway which is essential to maintain a proliferation-apoptosis equilibrium and immune homeostasis (48, 49). It is well known that cell adhesion molecules such as the hyaluronan receptor CD44 play a critical role in tumor progression (reviewed by (21, 50)).

We were interested to find out whether the correlation between poor prognosis in leukemia and lymphoma and presence of CD44v may be due to resistance to apoptosis. In a colitis model it was observed that mice lacking CD44v6/v7 showed increased apoptosis and consequently recovered from the life-threatening disease (35). In the present study we demonstrate that CD44v isoforms, which differ from CD44s only in the extracellular domain, confer resistance to apoptosis by colocalization and interaction with the death receptor Fas. Importantly, we have been able to narrow down the variant region responsible for this resistance to apoptosis to CD44v6 and v9, which were sufficient to protect T leukemia and plasmacytoma cells from programmed cell death. Based on these findings, we propose a model in which CD44v interacts extracellularly with Fas, preventing FasL binding and consequently Fas death signaling (Figure 7).

More specifically, we suggest that the extracellular variant region of CD44 sequesters Fas and hence prevents its trimerization, which is a necessary initial step to FasL binding (51). This may concern binding of CD44v to the first contact sites for Fas trimerization, the pre-ligand assembly domain (PLAD) (52). Therefore, these CD44v isoforms could conceivably function as pro-survival molecules by binding to and sequestering the Fas death receptor.

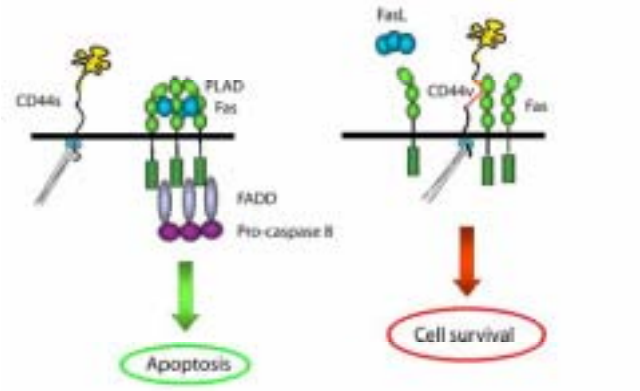

Figure 7. A model for Fas sequestration by CD44v
Molecular mechanism postulating how CD44v prevents apoptosis. We suggest that the variant region of CD44 may interact with the PLAD (pre-ligand assembly domain) of Fas and may thus prevent transiently its trimerization, which is a prerequisite for FasL binding (53). Hence, Fas remains inactivated and the death-signaling cascade does not take place.

We are postulating this mechanism to be responsible for the apoptosis resistance, as seen in many studies on autoimmune diseases or human cancer, in which CD44v expression is prevalent (6, 31, 35). Thus, when CD44v isoforms are aberrantly upregulated, Fas and CD44v remain associated and inhibit activation of Fas mediated apoptosis.The structural features of CD44v isoforms may provide clues to their function with regard to Fas sequestration.

A similar mechanism of Fas sequestration, employing the tyrosine kinase receptor c-Met, has recently been described (54). In this study the authors show that the interaction of the ζ chain of c-Met with Fas prevents its trimerization and binding to FasL and its further clustering. Thus, the association of c-Met and Fas provides a new mechanism of inhibition of death receptor-induced apoptosis. Interestingly, it has also been shown that c-Met and its substrate HGF interact with CD44v6 and this association is required for c-Met survival signaling.

The CD44v isoforms arise by alternative splicing of its mRNA (reviewed by (6)). There is evidence that tumor promoters might control the choice of alternative splice sites, and such factors are upregulated during tumorigenesis (39, 55). There are several indications that the transcripts of most genes encoding apoptotic regulators are subject to alternative splicing, which can result in the production of anti- or pro-apoptotic protein isoforms (reviewed by (55, 56)). This strategy may allow tumor and inflammatory cells to escape cell death and evade immune surveillance. Interestingly, our data demonstrate that anti-CD44v specific antibodies can interfere with the CD44v mediated apoptosis blockade and restore the potential of a cell to undergo apoptosis. Hence, interfering with the CD44 variant region by using specific tools could restore cell death capacity and resolve neoplastic and inflammatory lesions. This finding opens new strategies of therapeutic intervention in human cancers and autoimmune diseases that over-express CD44v isoforms.

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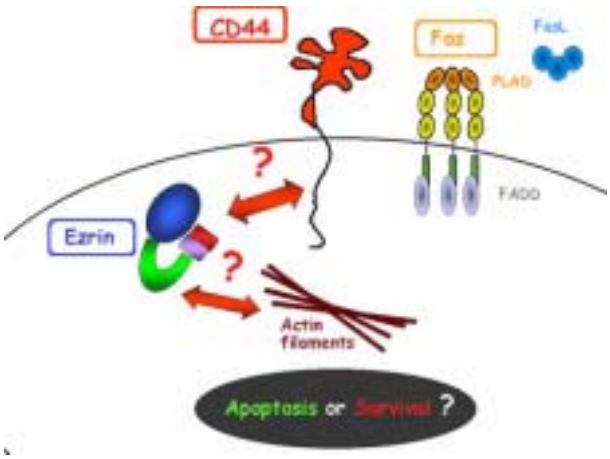
Chapter II

Chapter II: The complex CD44 standard/ezrin regulates Fas-mediated apoptosis.

Summary Chapter II

The membrane-cytoskeleton crosslinker protein ezrin has been identified as an intracellular interacting partner of CD44. However, CD44 is subjected to alternative splicing giving rise to numerous isoforms, which differ only extracellularly. It has often been reported that these isoforms can play different functions. Indeed, we have already described in chapter I that CD44v isoforms exhibit an anti-apoptotic activity by sequestering Fas and therefore abolishing the death signal transduction. In this second part of the thesis, we aim to determine if the complex CD44/ezrin is also implicated in regulation of cell death. For this purpose we use stable transfectants expressing different CD44 isoforms and perform confocal microscopy, biochemistry experiments and apoptosis assays. An advantage of this *in vitro* system is that CD44s and CD44v isoforms are not-co-expressed in the same cell. This allows us to study independently the behavior of the different isoforms. In this report, we try to answer the following questions:

1. Does ezrin interact with all CD44 isoforms?
2. Is this interaction implicated in Fas-mediated apoptosis?
3. How is the complex ezrin/CD44 regulating cell death?



Model representing the questions we aim to solve in this chapter II.

The experiments performed in this report allow us to answer many of these questions. In fact, we show that ezrin only colocalizes and interacts with CD44s but not with CD44v isoforms. Interestingly, we also demonstrate that the complex CD44s-ezrin-actin regulates Fas-mediated apoptosis. We provide evidence that CD44s enhances programmed cell death by indirect interaction with the actin cytoskeleton. In addition, we show that we can reduce this regulatory effect of CD44s by disrupting the actin cytoskeleton. Hence, we demonstrate that the association of CD44s with ezrin contributes to the modulation of downstream signaling events in which rearrangement of actin cytoskeleton is involved. We suggest that the interaction of CD44 with ezrin leads to a reorganization of the actin cytoskeleton which is required for apoptosis induction. These results together with the study described in chapter I provide an explanation to the controversial literature existing about the pro- and anti-apoptotic activities of CD44. We finally propose a model explaining the antagonistic activities of CD44 isoforms in the context of cell death.

The complex CD44 standard/ezrin regulates Fas-mediated apoptosis.

Abstract

The transmembrane receptor CD44 performs an important function in communicating across the membrane, conveying extracellular signals to the cytoplasm, a phenomen known as outside-in signaling. CD44 has been identified as a membrane binding partner for the membrane-cytoskeleton crosslinker protein ezrin. However, CD44 exists in different isoforms resulting from alternative splicing which differ only in the extracellular domain and exhibit different activities. In this study, using established transfectants expressing different CD44 isoforms, we demonstrate that only CD44 standard (CD44s) colocalizes and interacts with the membrane-actin crosslinker ezrin. Importantly, we also show that the association CD44s-ezrin-actin modulates Fas-mediated apoptosis. This report provides evidence that extracellular differences regulate intracellular signaling activities involved in programmed cell death.

Introduction

CD44, a cell adhesion transmembrane receptor ubiquitously expressed, exists in different isoforms. All isoforms retain a common transmembrane and intra-cytoplasmic domain. The large number of isoforms result from alternative splicing of the extracellular domain. Post-translational modifications (such as glycosylation) of the extracellular domain and alternative splicing influence CD44 functions. Nevertheless, these do not completely explain the multifunctional activity of CD44.

Many studies have reported the ability of CD44 cytoplasmic domain in co-ordinating signaling events. Indeed, the carboxy terminal cytoplasmic domain of CD44 supports the binding of proteins with crucial functions in cytoskeletal organization and signaling (reviewed by (1)). These proteins include ankyrin (2), annexin II (3) and members of the ERM (Ezrin, Radixin, Moesin) family (4-7). ERM proteins are crosslinkers between actin cytoskeleton and membrane receptors (8). ERM proteins become activated either by phospholipid binding by their N-terminal domain or by phosphorylation of their C-terminal threonine residue (9-12). Once they are in their open active conformation, the ERM C-terminal domain can bind actin cytoskeleton and the N-terminal domain is able to associate with transmembrane receptors like CD44 among others (reviewed by (9)). These linker proteins have been shown to localize to membrane ruffles, microvilli, filopodia and cleavage furrow (8, 13). In addition to their structural function, they have been implicated in various cellular functions that involve actin cytoskeleton rearrangement such as cell migration, cell shape, tumorigenesis and apoptosis (8, 14-17). The prototype member of the ERM protein family is ezrin. Ezrin and CD44 are ubiquitously expressed and can associate together. The interaction between ezrin and CD44 is located in the cytoplasmic domain of CD44, at the proximity of the transmembrane domain (5, 18, 19). This association may function to regulate its subsequent interactions with downstream signaling pathways (reviewed by (4)). Phosphorylation of serine 291 by protein kinase C (PKC) and dephosphorylation of serine 325 in the cytoplasmic tail of CD44 results in dissociation of ezrin and promotes cell migration (20). The interaction CD44-ezrin is also regulated by the GTP binding protein Rho and phosphatidyl inositol diphosphate (PIP_2) (21).

Beside these observations, many reports also suggest that ERM proteins regulate the initial phase of apoptosis through their effects on the actin cytoskeleton rearrangement. Indeed, it has been demonstrated that formation of blebs during apoptosis requires neopolymerization of actin and that actin accumulates at the basis of the apoptotic bodies (16, 17, 22). Hence, it is clear that actin crosslinker proteins such as ezrin interact with CD44 and that cytoskeletal linker proteins are required for the regulation of cell death. However, the impact of the interaction between CD44 and ezrin in the context of apoptosis has not yet been described. In a recent study we have shown that CD44 variant (CD44v) isoforms can block Fas mediated apoptosis (Mielgo et al, submitted) whereas the shorter standard isoform (CD44s) does not confer any protection against cell death. In this report we investigate if there is a differential interaction between ezrin and different CD44 isoforms and the impact of this interaction in the context of cell death.

Material and methods

Cell lines and generation of CD44 transfectants

Jurkat cells (human T cell leukemia line) obtained from the American Type Culture Collection (ATCC) were co-transfected by electroporation with 1.5σg of a neomycin expression plasmid and 15σg of CD44s or CD44s lacking the cytoplasmic domain (CD44s÷cyt) encoding DNA. The constructs contain EGFP (enhanced green fluorescence protein) fused at the CD44 carboxy terminal end (Figure 1 A).

The transfectants were grown in SF-IMDM with 2.5 % FCS (fetal calf serum) at 37∀C in a 5% CO^2 incubator and selected with 1mg/ml of Geneticin G418 (Gibco, Basel, Switzerland).

Expression of CD44 isoforms was confirmed by flow cytometry with antibodies anti-CD44s and anti-CD44v specific isoforms, fluorescently labeled. Cells with similar green fluorescence intensity were sorted on a MoFlo high speed cell sorter (DakoCytomation, Zug, Switzerland). Expression of Ezrin and Fas was also confirmed the same way and transfected cells with similar CD44, Fas and ezrin expression levels were used for all the experiments.

Cytochalasin D treatment

Cells were exposed to 0.5σg/ml of cytochalasin D (CD, Sigma) for 90 minutes before apoptosis induction for 5 hours with FasL. The CD dose used in this study inhibits actin polymerization without being cytotoxic, as assessed by analytical cytology analyses (23). Apoptosis was evaluated by flow cytometry by measuring the sub G1 population.

Induction and evaluation of apoptosis

FasL and anti-human Fas treatment

Neuro-2a cells producing recombinant FasL were kindly provided by A. Fontana (University Hospital, Zürich, Switzerland). The cells were grown in SF-IMDM with 2.5% FCS and selected with 800σg/ml G418. Jurkat transfectants (5 x 10^5 cells/ml) were incubated with serial dilutions of FasL or with a Fas crosslinking monoclonal mouse anti-human antibody (100ng/ml) (clone 7C11, Immunotech, Marseille, France) for 1, 3 or 6 hours at 37°C.

The cells were collected, washed twice in FACS buffer (PBS with 2% FCS, 0.02% sodium azide) and analyzed for apoptosis. Treated and untreated Jurkat cells were evaluated for apoptosis with different methods.

1. PARP p85. Jurkat cells were fixed with 4% paraformaldehyde (PFA in PBS) for 20 min at room tmeperature, washed twice with FACS buffer, permeabilized for 10 minutes at room temperature with PBS containing 0.1% saponin and 0.09% sodium azide, washed and stained with a rabbit polyclonal anti-p85 PARP specific antibody (Promega, Wallisellen, Switzerland), followed by a secondary goat anti-rabbit labeled with phycoerythrine (PE) (Southern Biotechnology, Basel, Switzerland) for 30 min on ice and in the dark. Percentage of apoptotic cells was evaluated by flow cytometry by measuring the PE positive cells.

2. Hypodiploid DNA. Cells were incubated overnight, at 4°C, in the dark in a hypotonic DNA binding buffer containing 0.1% sodium citrate, 0.1% Triton X-100 and 50σg/ml PI in PBS. Apoptotic rates of untreated and FasL treated cells were compared by flow cytometry. Apoptosis was evaluated by measuring loss of PI staining (corresponding to the subdiploid DNA population also called subG1 population which represents the apoptotic population) (Nicoletti et al,. 1991).

3. AnnexinV/PI. Cells were stained with annexinV labeled with allophycocyanine (APC) (Alexis, Basel, Switzerland) and 5σg/ml propidium iodide (PI) (Sigma, Buchs, Switzerland) for 10 min in the dark at room temperature. Percentage of apoptotic cells was evaluated by flow cytometry (FACSCalibur, Becton Dickinson, Basel, Switzerland) by measuring the AnnexinV and PI double positive cells.
The data obtained from these three methods were analyzed with the CellQuest programm (Becton Dickinson).

Confocal microscopy

For confocal analysis Jurkat CD44s and Jurkat CD44s÷cyt cells untreated and treated with FasL for 1 hour were washed in PBS, fixed 20 minutes at room temperature with 2% PFA, washed twice with PBS, permeabilized 10 minutes on ice with 1% triton, then blocked 1 hour on ice with mouse serum. Staining was performed with mouse monoclonal anti-human pan-CD44 (Hermes 3) followed by secondary goat anti-mouse labeled with Cy2 (Amersham). Cells were washed and double stained with rabbit polyclonal anti-human ezrin antibody (Cell signaling, Allschwill, Switzerland) followed by secondary goat anti-rabbit labeled with TxR fluorochrome (Southern Biotechnology, Reinach, Switzerland). Incubations with the antibodies were performed on ice, in the dark, during 1 hour and the cells were extensively washed with PBS between the different steps. All antibodies were diluted in PBS with 1% BSA. After staining, the cells were spun onto slides for 5 minutes at 800 rpm (Cytospin) and mounted in Mowiol. Confocal image stacks were recorded on a Leica TCS 4D operating in the simultaneous acquisition mode. Examination of specimens stained with one fluorochrome only, demonstrated that the setup did not result in crosstalk between channels. Images have been analyzed for colocalization using the Imaris software package (Bitplane AG, Zürich) and applying a threshold well above the noise level (24).

Nitrogen cavitation bomb

10^8 cells were collected, washed with PBS, centrifuged 5 minutes at 1300 rpm and incubated for 30 minutes at 4°C on a rotating wheal with magnetic beads (Dynal,

Hamburg, Germany) which were previously coated with anti-CD44 antibody (clone Hermes 3) according to the manufacturer's protocol. The cells coated with beads were then treated with FasL for 10 minutes at 37∀C, washed with ice cold H-Buffer (250mM sucrose, 10mM Na-Hepes pH 7.2, 2mM $MgCl_2$, 10mM NaF, 1mM sodium vanadate) and retrieved with a magnetic device (Dynal) several times. The same procedure was followed for untreated cells. Cells were intensively washed in H-Buffer, resuspended in H-Buffer+ (H-Buffer with 6.6σM Pervanadate and 1 mini tablet of a protease inhibitor cocktail (Roche, Penzberg, Germany) for 10 ml of solution. The number of cells coated with beads was counted under the microscope and approximately 40.10^5 cells per sample were disrupted in a nitrogen cavitation bomb at -196°C and 600 psi (pounds per square inch) (25). After disruption of the membranes, the samples were washed and retrieved four times and finally resuspended in 2X SDS sample buffer. For further western blotting analysis, the extracts were processed as described above and immunoblotted with mouse monoclonal anti-ezrin antibody (Transduction Laboratories) followed by goat anti mouse-HRP (Pierce). The signal was visualized by chemoluminescence (Pierce).

Antibodies

Expression of CD44, Ezrin, Fas and tubulin in Jurkat transfectants was also confirmed by western blotting. The antibodies used were: mouse monoclonal anti-human CD44 (clone Hermes 3), mouse monoclonal anti-human ezrin (clone, Transduction Laboratories, Basel, Switzerland), rabbit polyclonal anti-human Fas (Immunokontact, Lugano, Switzerland) mouse monoclonal anti-tubulin (clone 236-10501, Molecular Probes, Leiden, The Netherlands), followed by goat anti mouse-HRP (Pierce). The signal was visualized by chemoluminescence with Super Signal Substrate (Pierce).

Results

CD44s and ezrin colocalize and interact

The cytoplasmic domain of CD44 has been described to play a role in co-ordinating signaling events. In fact it has been shown that the CD44 cytoplasmic tail can interact with the membrane-cytoskeleton crosslinker protein ezrin (4-6, 20). Here we are interested in determining if ezrin interacts with any isoform of CD44 in the context of Fas mediated apoptosis. For this purpose we used Jurkat cells which can easily be induced to undergo apoptosis with FasL or a Fas crosslinking antibody (clone 7C11) and express constitutively Fas but not CD44. Jurkat cells were transfected with cDNA constructs for CD44s (Ju CD44s), CD44v2-10 (Ju CD44v2-10) and CD44s÷cyt (Ju CD44s÷cyt) (Figure 1 A). As a control we used Jurkat cells transfected with the neomycin resistance plasmid alone (Ju neo). CD44, Fas and ezrin expression levels of the transfectants were analyzed by flow cytometry and Western blotting (Figure 1 B). Transfected cells with comparable CD44, Fas and ezrin levels were used for performing the experiments.

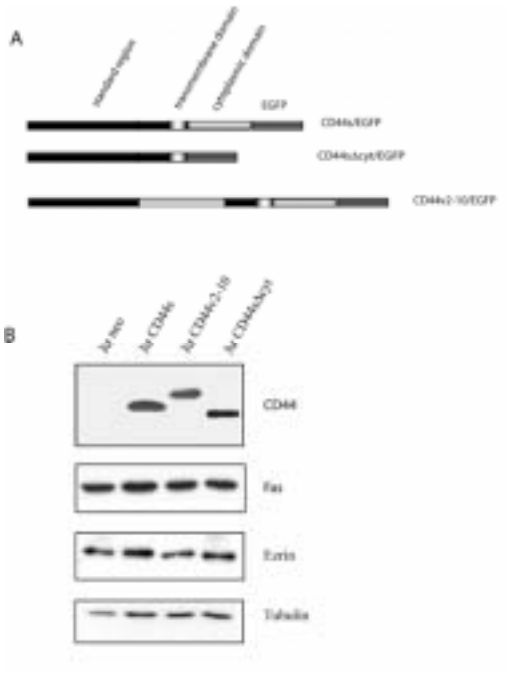


Figure 1. Jurkat transfected cells.
(A) Scheme of the different CD44-EGFP constructs used for transfection of Jurkat cells.

(B) Western blots showing the expression of panCD44, Fas, ezrin and tubulin in the different transfectants.

An advantage of this *in vitro* system is that CD44s and CD44v isoforms are not-co-expressed in the same cell. This allows us to study independently the behavior of the different isoforms.

Confocal experiments were conducted with the different transfectants under two conditions: cells untreated and treated with FasL. Interestingly, we could observe that in untreated as well as in treated cells CD44s colocalized with ezrin (yellow merge) (Figure 2 A and B), whereas no colocalization between CD44v2-10 and ezrin was detected (Figure 2 C). These results suggest a possible molecular interaction between ezrin and the cytoplasmic domain of CD44s but not CD44v.

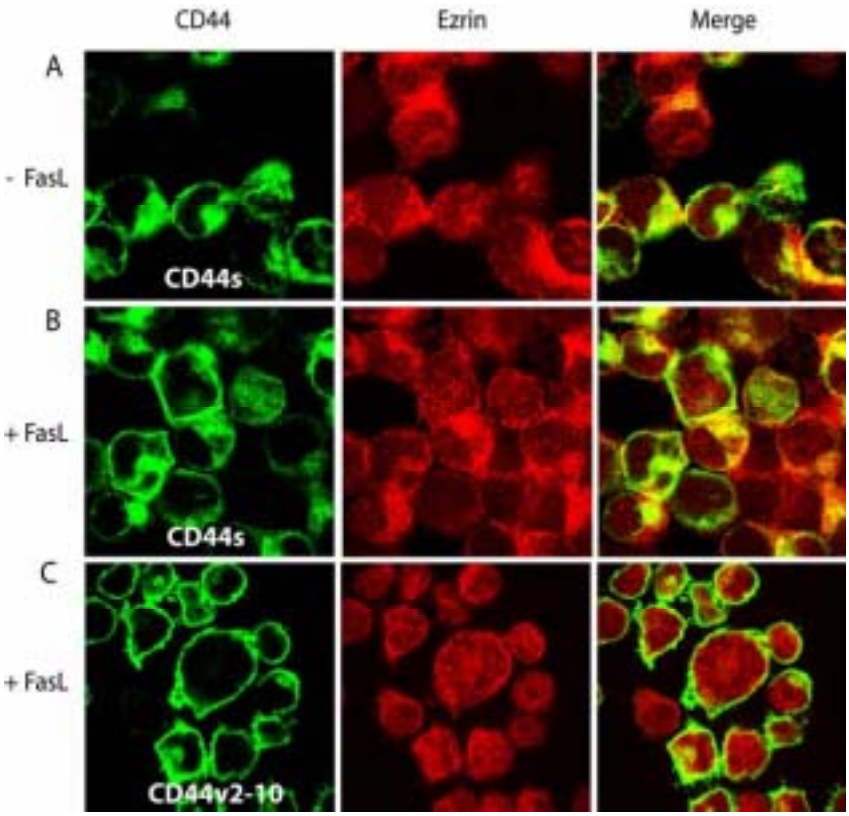


Figure 2. CD44s cytoplasmic domain and ezrin specifically colocalize.
Jurkat cells transfected with CD44s untreated (A) or treated for 1 hour with FasL(B). Ju CD44v2-10 treated for 1 hour with FasL (C). Cells were stained with anti-panCD44 antibody (Hermes-3), followed by Cy2 labeled goat anti-mouse antibody (green fluorescence) and with anti-ezrin antibody followed by TxR labeled goat anti-rabbit antibody. The merge in yellow represents the colocalization between CD44s and ezrin.

To determine whether a physical association exists exclusively between the standard isoform of CD44 and ezrin, we performed coimmunoprecipitations using the nitrogen cavitation bomb method. This is an alternative procedure to the standard coimmunoprecipitations for examining interactions between membrane proteins (25). The advantage of this procedure is that it is detergent free and therefore an excellent method to study interactions between membrane proteins which can be recruited into lipid rafts and are very difficult to extract with conventional lysis buffers without destroying the natural interactions existing between the candidate molecules. To study the association between CD44 and ezrin, we used cavitation bomb extractions from Jurkat neo, Jurkat CD44s, Jurkat CD44v2-10 and Jurkat CD44s÷cyt targeting CD44 and performed Western blotting detection with anti-ezrin antibody. Anti-CD44 extractions from Jurkat neo, Jurkat CD44s and Jurkat CD44v2-10 were reproduced under two different conditions: cells untreated and cells treated with FasL for 15 min (Figure 3). A band at 80kDa corresponding to ezrin appeared only in the extracts from Ju CD44s cells untreated and treated with FasL. No bands consistent with ezrin were observed neither in the extracts from Ju neo (negative control) nor the extracts from Ju CD44v2-10. A band at 50 kDa corresponding to the heavy chain of the antibody coupled to the magnetic beads was observed in all the samples and could be considered as loading control.

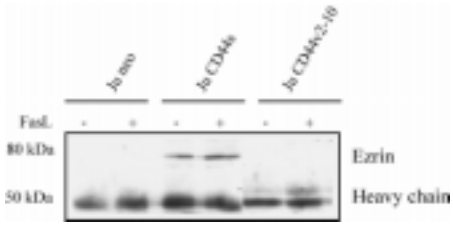


Figure 3. Interaction between CD44s and ezrin.
Western blotting for ezrin on CD44 cavitation bomb extractions from Jurkat neo, Jurkat CD44s and Jurkat CD44v2-10. Cells were incubated with magnetic beads coated with mouse anti-human CD44 antibody, untreated or treated for 15 min with FasL, before nitrogen cavitation bomb extraction. 10% SDS-PAGE was performed under reducing conditions, followed by Western blotting with mouse anti-ezrin antibody and HRP-conjugated goat anti-mouse secondary antibody.

These data confirm that only CD44s full length interacts with ezrin. Interestingly, even if all CD44 isoforms share an identical cytoplasmic domain, no interaction between CD44v isoforms and ezrin was observed.

Cells expressing CD44s÷cyt are more resistant to Fas mediated apoptosis.

To understand the role of CD44 cytoplasmic domain in apoptosis signaling we conducted *in vitro* functional assays using the different Jurkat transfected cells. For this purpose, we used different methods for apoptosis detection. The different transfectants were treated with FasL or a Fas crosslinking antibody for 3 or 6 hours and the amount of apoptotic cells was detected by quantifying PARP cleavage. We observed that Ju CD44v2-10 were highly protected from Fas-mediated apoptosis (Mielgo et al. submitted). Interestingly, Ju CD44s÷cyt were also significantly more resistant to apotosis than Ju CD44s. In fact, after 3 and 6 hours treatment with FasL, Ju CD44s÷cyt showed only 15 and 30% of apoptotic cells whereas Ju CD44s exhibited 32 and 51% respectively (Figure 4 A). We obtained similar results when the cells were treated for 3 hours with Fas crosslinking antibody (Figure 4 B). Similar results were obtained by measuring hypodiplod DNA after treating the cells for 3 and 6 hours with FasL (Figure 4 C). Indeed after 6 hours treatment we observed 38% of apoptotic cells in the Ju CD44s but only 15% in the Ju CD44s÷cyt cells. To confirm these results a similar experiment was performed and apoptosis was detected by annexinV/PI staining. The different transfectants were treated for 1, 3 and 6 hours with 200ng/ml of Fas crosslinking antibody. We could confirm that cells expressing CD44s÷cyt were more resistant to cell death than cells expressing CD44s full length (data not shown). These results suggest that the cytoplasmic tail of CD44s is required for Fas-mediated apoptosis. In the absence of the cytoplasmic domain of CD44s, no link via ezrin to the cytoskeleton is generated and this influences susceptibility to cell death.

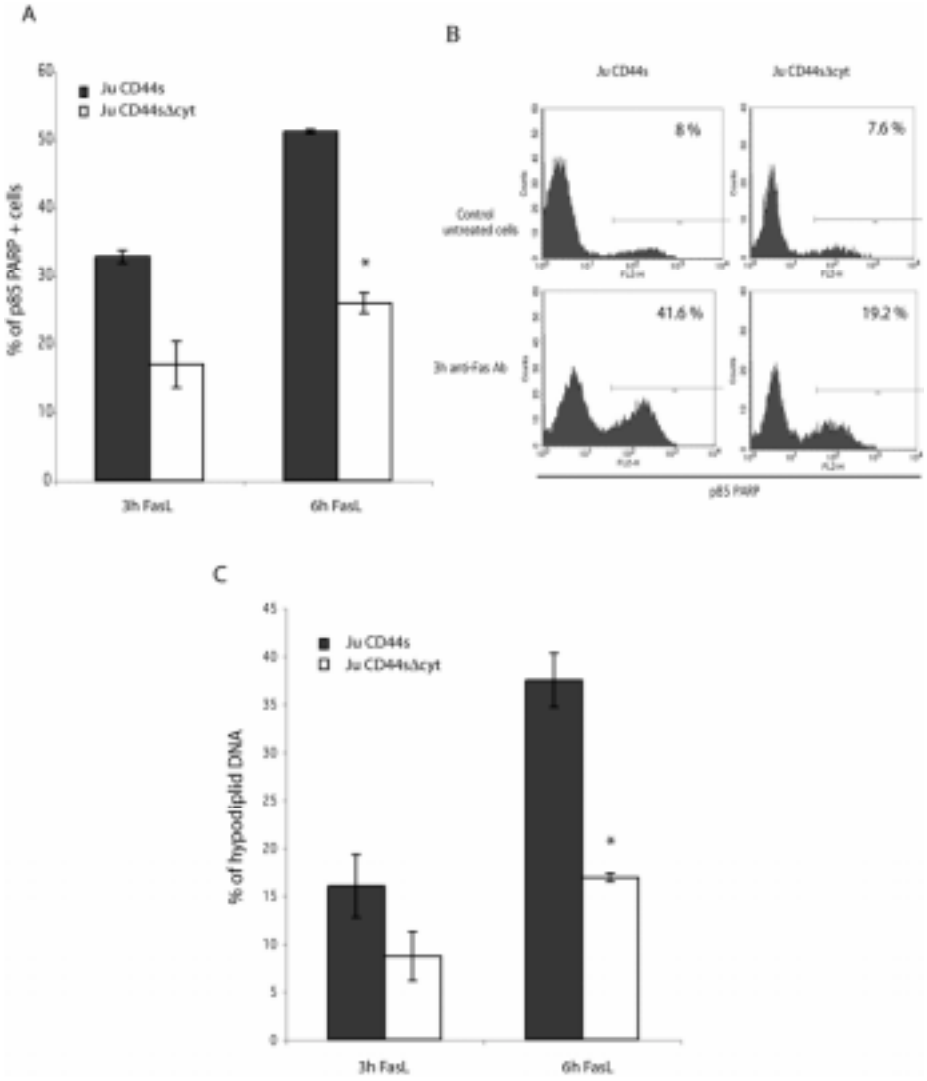


Figure 4. Jurkat CD44s÷cyt cells are more protected from Fas mediated apoptosis than Jurkat CD44s.

(A) Jurkat CD44s and Jurkat CD44s÷cyt were treated with recombinant FasL for 3 or 6 hours. The percentage of apoptotic cells was determined by flow cytometry with an antibody recognizing the cleaved form of PARP followed by a secondary antibody labeled with PE. Apoptosis was calculated by subtracting the control values (between 2% and 4%) from the values obtained with FasL treatment. Data are representative of three independent experiments and were analyzed with the Wilcoxon signed rank test (1, p{ 0.05).

(B) FACS analysis of Jurkat CD44s and Jurkat CD44s÷cyt untreated and treated for 3 hours with Fas crosslinking antibody. Percentage of p85 PARP positive cells was evaluated by flow cytometry with an anti-p85 PARP antibody followed by a secondary antibody labeled with PE. The bar is limiting the living cells on the left according to the control. Numbers in the upper right corner represent the percentage of apoptotic cells.

(C) Jurkat transfectants were treated with recombinant FasL for 3 or 6 hours. The amount of apoptotic cells was determined by measuring hypodiplod DNA (hypodiploid DNA= subG1 apoptotic population). Data are representative of three independent experiments and were analyzed with the Wilcoxon signed rank test (1, p{ 0.05).

Cells expressing CD44s show reduced apoptosis after treatment with cytochalasin D

We have given evidence that: (i) the cytoplasmic domain of CD44s but not CD44v interacts with the actin cytoskeleton associated protein ezrin and (ii) the cytoplasmic domain of CD44s is required for apoptosis induction via Fas. Many reports describe that the cytoplasmic domain of CD44 can interact with actin cytoskeleton (2-4). We were then interested in understanding the relevance of the connection CD44s-actin cytoskeleton during apoptosis induction via Fas. For this purpose, the cells CD44s, CD44s#÷cyt and CD44v2-10 were either treated for 5 hours only with FasL or pre-treated with the inhibitor of actin polymerization Cytochalasin D (CD) for 90 min and then induced to undergo apoptosis for 5 hours with FasL. The amount of apoptotic cells was evaluated by measuring hypodiploid DNA using flow cytometry. We observed similar amounts of apoptotic cells in the Ju CD44v2-10 and Ju CD44s÷cyt before and after treatment with CD meaning that in those transfectants apoptosis signaling is not dependent on actin cytoskeleton (Figure 5 A). A negative control Ju neo which expresses Fas but does not express any isoform of CD44 did not exhibit any change in susceptibility to Fas-mediated apoptosis after treatment with CD (data not shown). However, Ju CD44s showed a significant decrease in apoptosis from 35% to 18%, when they were pre-treated with CD (Figure 5 A). Cells expressing CD44s exhibit after treatment with FasL a 10 fold increase in apoptosis induction whereas cells expressing truncated CD44s (CD44s÷cyt) or CD44v2-10 only exhibit a 2 fold increase of cell death (Figure 5 B). When the cytoskeleton was disrupted with cytochalasin D previous induction to apoptosis, Ju CD44s showed much lower increase in apoptosis. No changes were observed for Ju CD44s÷cyt and Ju CD44v2-10 (Figure 5 B and C). Together, these results suggest that CD44s-ezrin linkage and, in turn, the CD44s-actin connection are crucial requirements for rendering a cell prone to Fas-mediated apoptosis.

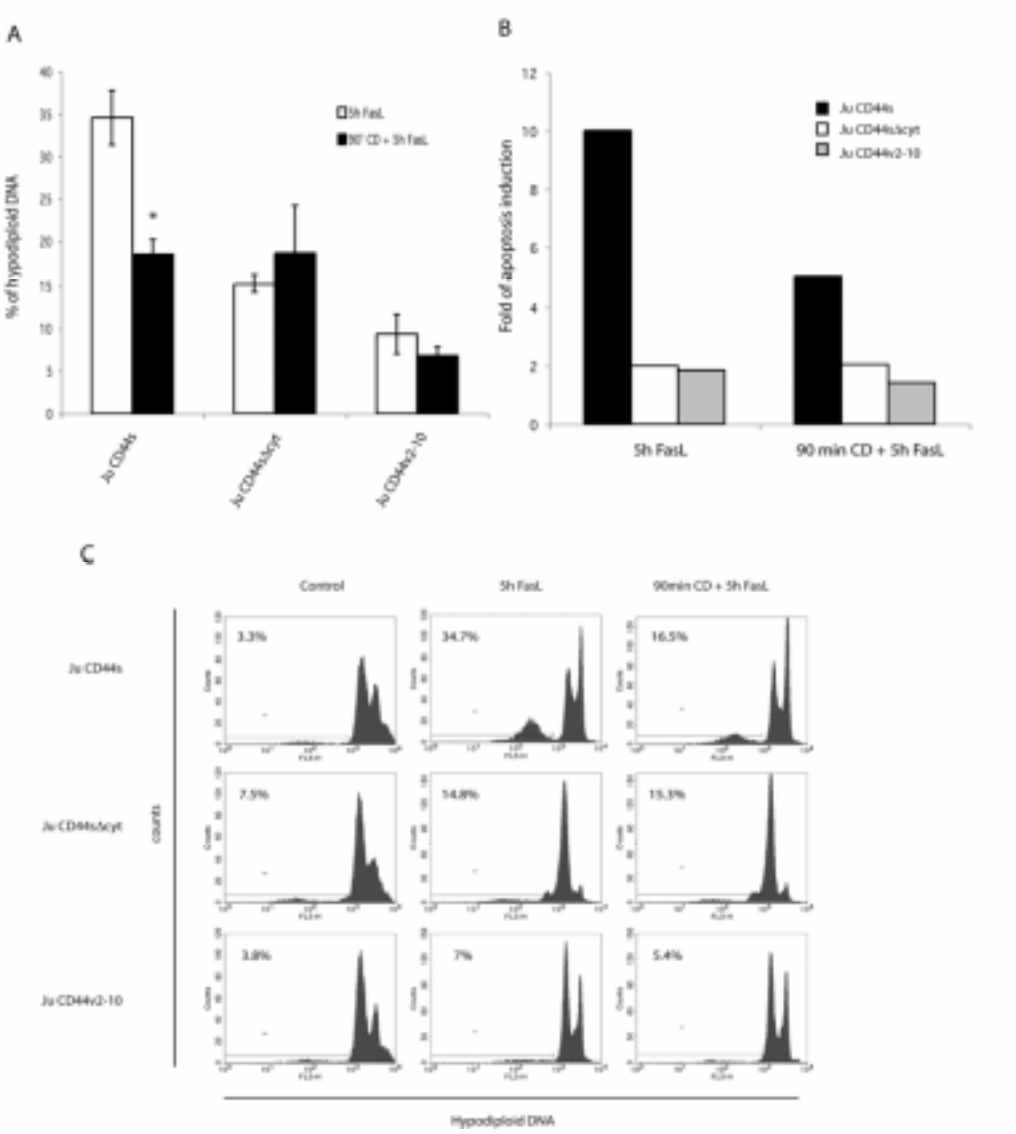


Figure 5. Pre-treatment with cytochalasin D protects Jurkat CD44s cells from Fas mediated apoptosis
(A) Jurkat transfectants were either only treated for 5 hours with recombinant FasL or pre-treated for 90 min with Cytochalasin D (CD) followed by 5 hours treatment with FasL. Apoptotic cells were evaluated by measuring hypodiploid DNA. Data are representative of three independent experiments and were analyzed with the Wilcoxon signed rank test (1, p{ 0.05).
(B) Histogramm representing the fold of apoptosis induction when Jurkat CD44s, Jurkat CD44s÷cyt and Jurkat CD44v2-10 were treated for 5 hours with recombinant FasL or pre-treated for 90 min with Cytochalasin D (CD) followed by 5 hours treatment with FasL. The relative numbers were obtained by calculating the ratio between the percentage of apoptosis in the treated cells and the one in the control cells.
(C) FACS analysis of Jurkat CD44s, Jurkat CD44s÷cyt and Jurkat CD44v2-10 untreated cells, treated with FasL for 5 hours or pre-treated with Cytochalasin D (CD) for 90 minutes followed by incubation for 5 hours with FasL. The numbers in the quadrants represent the percentage of apoptotic cells obtained in one experiment representative of three. The percentage of apoptotic cells was evaluated by measuring hypodiploid DNA (hypodiploid DNA= subG1 apoptotic population). The bar is limiting the living cells on the right according to the control.

Discussion

CD44s-actin connection is required for Fas-mediated apoptosis

Many of the direct interactions between ERM proteins and transmembrane proteins involve adhesion receptors. ERM proteins were first found to interact with the cell adhesion receptor CD44 (4, 5), through a positively charged juxtamembrane region in the CD44 cytoplasmic domain (6). The interaction between ezrin and CD44 is tightly regulated by Rho GTPases (3) and PKC (20). The ability of ezrin to interact with CD44 in a highly regulated fashion strongly indicates that ezrin might have a role in organizing signaling complexes that regulate cytoskeleton rearrangement. In this report we demonstrate that ezrin binds differentially to CD44 isoforms. Indeed, here we show that ezrin only colocalizes and interacts with CD44s but not with CD44v. CD44s and CD44v isoforms only differ in their extracellular domain, therefore we suggest that specific extracellular partners influence the intracellular events. A similar behavior has been observed in other cell adhesion molecules such as integrins. Indeed η1 and η3 integrins which differ extracellularly transduce the stimuli in different ways. η1 integrin regulates Rac/JNK activity and lamellipodia formation, whereas η3 integrin promotes Rho activity and stress fibers formation. A mutant of η1 integrin expressing the extracellular binding ligand motif of η3 also enhances Rho activity (26). Thus, differences in the extracellular domain of integrins require different ligand candidates which trigger divers intracellular signals and modulate cellular behavior. This process is known as outside-in signaling (27). Interestingly, our data also confirm this concept and provide evidence that isoforms of CD44 regulate specific intracellular signaling events. As for the integrins, this could be explained by different ligands or extracellular partners.

This study shows that the multimeric complex actin-ezrin-CD44s is implicated in Fas-mediated apoptosis. In fact, in the absence of CD44s cytoplasmic domain, interaction between CD44s and ezrin does not exist, and cells are significantly more resistant to FasL induced cell death. Disruption of actin cytoskeleton in CD44s expressing cells also strongly reduces the ability of the cells to undergo apoptosis. Together these data demonstrate that the association between CD44s, ezrin and actin is required for rendering a cell susceptible to Fas-mediated apoptosis. In another cellular system, the interaction of

the death membrane receptor Fas with ezrin has also been reported to be required for apoptosis induction (28). However, in Jurkat cells we did not observe any interaction between Fas and Ezrin. We suggest that Ezrin has a high ability to bind many membrane receptors such as Fas, CD44 among others. The specificity of such interactions are probably cell type dependent. The association of ezrin to membrane receptors leads to its activation and subsequent actin cytoskeleton organization required for induction of cell death.

CD44 regulates apoptosis.

One way to explain these observations could be that different ligand binding or extracellular partner(s) may change the conformation of CD44, thus affecting the recruitment of signaling molecules and the anchorage of the cytoskeleton. Therefore, further experiments to determine the extracellular partner(s) will help to clarify these events. In a previous report, we have demonstrated that CD44v isoforms but not CD44s interact with the death receptor Fas, thus inhibiting the death signaling cascade (Mielgo et al. submitted). Hence, it is very likely that CD44v isoforms have a higher affinity for Fas and the association with Fas might prevent the interaction with ezrin. Interestingly, this hypothesis could explain the controversial literature existing about CD44. In fact, CD44 has often been described as a pro-apoptotic molecule (29-32) and as an anti-apoptotic and pro-metastatic protein (33-36). Probably, this controversy is in part due to the presence of several isoforms of this multifunctional adhesion molecule. The present study together with our previous data (Mielgo et al., submitted) provide an explanation to this controversy. We suggest, on one hand that CD44v isoforms, which are upregulated only under special conditions such as chronic inflammations and cancer, exhibit an anti-apoptotic effect by sequestaring Fas (Mielgo et al., submitted). On the other hand we have shown *in vitro* that only the standard form of CD44 interacts with ezrin and actin cytoskeleton forming a multimeric complex which is required for rendering a cell susceptible to Fas-mediated apoptosis. Hence, the isoforms of CD44 can exhibit different and even opposite functions concerning regulation of cell death. This is a common phenomenon, and many molecules involved in apoptosis are subjected to alternative splicing giving rise to isoforms with different and/or opposite functions. In fact, the

inhibitor of caspase 8, FLIP, can exist in two isoforms with opposite functions. While $FLIP_L$ exhibit an anti-apoptotic function, FLIPs exerts a pro-apoptotic effect (37, 38). Here, we provide a new model of regulation of cell death by the isoforms of a transmembrane receptor, which have a high ability to interact with divers signaling molecules, thus exhibiting multiple functions.

These findings contribute to understand the molecular behavior of the different CD44 isoforms in the context of apoptosis. Armed with this knowledge we might be able to develop new therapeutic strategies for the treatment of life threatening diseases such as cancer and autoimmunity.

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Chapter III

Chapter III. Involvement of specific CD44 variant isoforms in multiple sclerosis and experimental autoimmune encephalomyelitis.

Summary Chapter III

CD44 has been described to play an essential role in experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS). CD44 expression on astrocytes of active lesions is also associated with the pathogenesis of these diseases. However the functional role of CD44 variant isoforms in these disorders is not yet understood. Therefore, the goal of our study is to analyze the implication of CD44v in MS and EAE. For this purpose, *post-mortem* MS brains have been tested for expression of CD44v isoforms by immunohistochemistry. In addition, the role of CD44v7 and v10 have been studied in transgenic mice. Our aim in this study was to answer the following questions:

1. Are CD44v isoforms over-expressed in MS patients and EAE mice? And is there a specific variant isoform implicated in these disorders?

2. Which immune cells upregulate CD44v isoforms? And how do they participate in the development of the disease?

In this chapter, we first show by immunohistochemistry that MS patients over-express CD44v3 and v10 and that genetic deletion of CD44v7 or v10 in mice protects them from EAE. The latter observation also correlates with a reduction of the inflammatory infiltrates in the spinal cord. Second, adoptive transfer experiments confirm that expression of v7 on both T cells and antigen presenting cells (APC), participates in the development of EAE. Indeed, v7 deficient cells did not induce EAE after transfer in wild type (WT) recipients, and neither injection of WT cells in v7 deficient recipient mice did. In addition, pronounced reduction of EAE was observed when both, donor and recipient cells were deficient for CD44v7. Hence, from these experiments we conclude that v7 and v10 are strongly involved in the pathogenesis of EAE and MS. Development of specific therapies based on targeting CD44v7 and v10 could be suitable for treatment of these central nervous system diseases.

Involvement of specific CD44 variant isoforms in MS and EAE

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Abstract

CD44 variant isoforms (CD44v) are transmembrane molecules that have been suggested to play an important role in the development of various autoimmune disorders. Ten CD44 variant exons (v1-v10) can be alternatively spliced and inserted into the CD44 standard (CD44s) backbone.

Here we determined the functional role of CD44v isoforms in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Both, CD44v3 and CD44v10 were expressed on glial cells and on perivascular infiltrating cells in *post-mortem* analysed brain lesions of MS patients. In the EAE model, brain infiltrating $CD44v10^+$ leukocytes preceded the onset of EAE and paralleled EAE disease scores. Genetic deletion of CD44v7 or CD44v10 isoforms reduced clinical EAE burden, and the number of inflammatory infiltrates in the spinal cord. Furthermore, adoptive transfer experiments showed that CD44v7 expression on both, effector T cells and antigen presenting cells participates in the development of EAE. CD44v7/v10 expression may contribute to the development of MS and EAE by increasing the longevity of autoantigen specific $CD4^+$ T cells.

In conclusion, we here show that the specific CD44v isoforms v7 and v10 are critically involved in the pathogenesis of EAE and MS. Targeting these CD44v isoforms might therefore reduce inflammatory processes and clinical symptoms in MS patients.

Introduction

Multiple sclerosis (MS) is a common chronic and disabling autoimmune disease of the CNS and often affects young adults. No efficient and long lasting therapies are available yet. Therefore, there is an urgent need for further elucidation of the mechanisms underlying the pathogenesis of MS. So far, current evidence suggests that MS is mediated by inflammatory attacks against components of the myelin sheath of nerve axons in the CNS.

Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS and greatly advanced our knowledge on the pathogenesis of autoimmunity affecting the CNS. Together with clinical observations, data from animal experiments suggest that an initial $CD4^+$ mediated autoreactive T cell response initiates a cascade of inflammatory events including complement activation, production of reactive oxygen and nitrogen species and digestion of myelin by macrophages (MA) that finally result in impaired nerve conduction, demyelination, axonal loss and neurological impairment[1-3]. The formation of mononuclear cell infiltrates in the CNS as well as the activation of T and B cell mediated effector mechanisms require intercellular communication between antigen-presenting and effector cells. Thus, various chemokines and cytokines, as well as specific interactions through accessory/activation molecules on the cell surfaces regulate the initiation and progression of autoimmune responses.

Several adhesion molecule interactions, i.e. VLA-4/VCAM and LFA-1/ICAM-1, have been implicated to play a crucial role in the development of MS and EAE[4-6]. CD44 and its variant isoforms (CD44v) are also important adhesion molecules. Ten CD44v isoform exons (v1-v10) can be alternatively spliced and inserted into the CD44s backbone, potentially forming over a thousand different CD44v isoforms. Interestingly, CD44 and its isoforms are involved in various biological processes such as lymphocyte activation, migration, adhesion, extravasation into inflammatory sites and protection against apoptosis[7-11]. Furthermore, MMP-9, involved in the degradation of type IV collagen, can bind specific glycans of CD44 and in addition may activate latent TGF-η[12]. Additionally, osteopontin, a pro-inflammatory cytokine, can bind to CD44v6/v7 isoforms[13]. Both of these ligands have been shown to promote the progression of MS and EAE[14-16]. CD44 plays not only an essential role in EAE[17,18] but also in other autoimmune disease models such as rheumatoid arthritis[19,20], and

diabetes [21]. In these models, treatment with an anti-panCD44 mAb, which recognizes all CD44 molecules, completely abrogated disease development.

Physiologically, CD44s is ubiquitously expressed on various cells, while the expression of CD44v isoforms is restricted to activated leukocytes and epithelial cells[22]. Recently CD44 has been shown to associate with VLA-4 on T cells extravasating to inflammatory lesions[9]. Targeting CD44v isoforms, which are specifically upregulated on activated leukocytes, may therefore reduce inflammatory responses and refine therapeutic approaches. In fact, blocking a selected number of CD44v isoforms reduces or prevents autoimmune disease development. In TNBS-induced colitis, an animal model for inflammatory bowel disease, treatment with mAb anti-CD44v7, but not with mAb anti-CD44v10 prevents disease[23]. Accordingly, mice with a targeted deletion for CD44v7 develop only transient and minimal disease[8,24,25]. Corresponding lesions contain high numbers of apoptotic cells, indicating that absence of CD44v7 might protect from autoimmunity by mediating apoptosis of inflammatory cells.

So far, very little is known about the role of different variant isoforms of CD44 in MS and its animal model EAE. Increased expression of CD44 on astrocytes in active lesions in MS brain could be associated with the pathogenesis of the disease[26]. In an adoptive transfer EAE mouse model, it has been shown that CNS infiltrated MBP-specific $CD4^+$ T cells, representing the effector/memory phenotype, specifically express CD44 and CD45RB(low)[27]. Previously, we have shown panCD44 and CD44v10 expression on mononuclear cells infiltrating the CNS of mice with EAE, whereas CD44v6 was not expressed.

In order to determine the functional involvement of selected CD44v isoforms in EAE and MS we assessed the expression of CD44v3-v10 in *post-mortem* MS brain tissue. Furthermore, we specifically addressed the role of CD44v7 and CD44v10 in EAE development using mice with single deletions for these specific CD44v isoforms.

Materials and methods

Patients and brain tissue samples

Human *post mortem* brain tissue was provided by the Netherlands Brain Bank (Coordinator: Dr. R. Ravid, Amsterdam). Information regarding MS patients and non-demented control subjects is shown in Tables 1a and b, respectively.

Histological staging of MS brain lesions

Brain lesions were quantified by immunohistology. To determine the stage of the MS lesions, a generally accepted staging system was used[28]. An additional stage, the so called pre-active lesion had been described in combination with MRI measurements[29]. Despite the fact that it is not proven that pre-active lesions progress into active lesions, they reflect abnormalities in the white matter, that might preceed the development of active lesions and were therefore included in our study.

Pre-active lesions include the presence of HLA-II-expressing clusters of activated microglia cells, few perivascular inflammatory cells, but no phagocytic cells containing myelin breakdown products or areas of demyelination. Active lesions are represented by the additional presence of demyelinated areas. Phagocytic cells in these areas contain myelin breakdown products, which can be visualized by oil red O, a histochemical staining for neutral lipids. In these lesions, HLA-II is strongly expressed by perivascular and parenchymal MA. To evaluate cell numbers in lesions we used a previously published 5-point scale system[6].

Immunohistological staining for CD44v isoforms in human brain tissue

Immunohistological staining was performed as described[30,31]. In addition, the Tyramide Signal Amplification (TSA) Biotin System (Perkin Elmer, Boston, MA) was used, according to the manufacturer's instructions. Antibodies of the isotype IgG2a (Table 2) were applied with 25% normal human serum to prevent binding to Fc-receptors. Horseradish peroxidase conversed 3-amino-9-ethyl-carbazole into a bright red precipitate. Nuclei were counterstained by hematoxylin. Reactive human tonsil sections were included in each staining procedure as positive control tissue.

Generation of CD44v10 deficient mice

The mouse CD44 variant region was isolated from a 129SV genomic library. Two 34 bp loxP sites were inserted in direct repeats into a single BstEII site 5' of exon v10 and at the 3' end of the neo^r cassette, which was then inserted into the single BstXI site 3' of exon v10. For electroporation the targeting vector was linearized with PvuI and 20 σg were transfected into 10^7 R1 embryonic stem cells. Embryonic stem cells were maintained on a feeder layer of embryonic fibroblasts in the presence of leukemia inhibitory factor. After selection with G418 (300 σg/mL) 391 clones were analyzed by Southern blotting using a 3' external probe (HindII-BamHI). BamHI digests revealed that 2 clones showed homologous recombination. Positive clones were injected into C57Bl/6 blastocysts and chimaeric male offspring was mated with C57Bl/6 cre deleter females. Offspring was genotyped by PCR using exon v10 flanking oligos and analysing for deletion of the loxP targeted region.

Mice

Female $CD44v7^{-/-}$ and $CD44v10^{-/-}$ mice were bred in the mouse facility at the Institute of Medical Microbiology, Basel or at the Erasmus MC, Rotterdam and backcrossed for ∅10 generations on the appropriate C57Bl/6 or SJL/J background depending on the experiment. All mouse experiments were performed in accordance with Swiss and Dutch law and were approved by institutional ethics comittees and the local authorities. Mice were housed under specified pathogen free conditions and received water and food *ad libitum*. Paralyzed mice with EAE scores over 2.5 were afforded easier access to food and water.

Induction and clinical evaluation of EAE

C57Bl/6 mice were immunized s.c., at four sites (axillar and inguinal), with 200 σg MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) in 0.1 ml PBS emulsified in an equal volume of CFA containing 100 σg *Mycobacterium tuberculosis* (H37/Ra; Difco Laboratories, Detroit, MI). All mice were injected i.p. with 200 ng of *Bordetella pertussis* toxin in 0.2 ml PBS on days 0 and 2 (Sigma-Aldrich). SJL/J mice were immunized with 50 or 100 σg $PLP_{139-151}$ (HSLGKWLGHPDKF) in 0.1 ml PBS emulsified in 0.11 ml of CFA containing 110 σg *Mycobacterium tuberculosis*. For adoptive transfer, lymph nodes (SJL/J) or lymph nodes and

spleens (C57Bl/6) were isolated 10 days after immunization. Crude cell suspensions ($4x10^6$/ml) were cultured at 37∀C and 5% CO_2 in the presence of 10 σg/ml MOG_{35-55} (C57Bl/6) or 10 σg/ml $PLP_{139-151}$ (SJL/J) for 4 days, washed and then injected into recipient mice. Mice were weighed and scored for clinical signs of EAE daily according to the following internationally accepted scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE. Cumulative EAE scores were calculated by adding up all the scores per animal over the indicated time points after EAE induction, representing a total disease load.

Histopathology of mouse CNS

Mice were euthanized and brain or spinal cord tissues were snap frozen in liquid nitrogen. Frozen sections were fixed in acetone and stained with hematoxylin.

Isolation of mononuclear brain cells

Mononuclear cells were isolated from brain tissues as described[32]. Briefly, brains were removed and single cell suspensions were prepared by passage through a wire mesh. After washing, cells were resuspended in 7 ml of 80% Percoll (Pharmacia, Uppsala, Sweden), and then overlaid with 8 ml of 40% Percoll to form a discontinuous gradient in a 15-ml centrifuge tube. The gradient was centrifuged at 400 *g* for 40 min at 21ºC, and the cells at the 40–80% interface were harvested.

Flow cytometry

Immunofluorescence analysis of cells was performed on a FACScan™ (Becton Dickinson, Mountain View, CA). $PLP_{139-151}$ restimulated lymph node cells and mononuclear brain cells were incubated with the specific mAbs for 30 min at 4°C in staining buffer (PBS with 2% FCS and 0.02% sodium azide). The following antibodies were used: CD4 (clone GK1.5, rat IgG2b), CD8 (Ly-2)(clone 53-6.7, Rat IgG2a), CD45R (B220)(clone RA3-6B2, rat IgG2a) and CD11b (Mac-1)(clone M1/70, rat IgG2b), isotype control (clone R35-95, rat IgG2a), all purchased from PharMingen (San Diego, CA) and CD44v10 (clone K926, rat IgG2a)[33]. The

goat anti-rat IgG secondary Ab used to detect CD44v10-expressing cells was obtained from Southern Biotechnology, Birmingham, AL. As a negative control for CD44v10 an isotype matched control antibody of irrelevant specificity was used. Dead cells and debris were excluded using propidium iodide.

MOG_{35-55} and $PLP_{139-151}$ specific T cell proliferation assay

Lymph node cells ($4x10^5$/well) were cultured in 96-well plates in 200 σl of RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 100 U/ml penicillin and 100 σg/ml streptomycin (Bio Whitaker Inc.) in the presence or absence of MOG_{35-55} or $PLP_{139-151}$. After 72 hours of culture, 1 σCi of ^{3}H-thymidine (Amersham Biosciences, Buckinghamshire, England) was added for 16 hours. Incorporation of ^{3}H-thymidine was measured in triplicate using a filtermat harvester and a beta-plate counter (Perkin Elmer, Wellesley, MA). Alternatively, supernatants from MOG_{35-55} proliferation assays were harvested 96 h after culture, centrifuged and stored for further cytokine analysis at -20∀C.

Establishment of auto-antigen specific cell lines

After the first *in vitro* restimulation with 10 σg/ml MOG_{35-55} (C57Bl/6) or $PLP_{139-151}$ (SJL/J) for 4 days**,** lymph node cells were washed and diluted 1:1 in complete RPMI. After a resting period of 10 days, cells were restimulated ($1x10^6$ cells/ml) with 1#σg/ml peptide for 4 days. Conditions of each following restimulation cycle were identical to the second restimulation.

Cytokine measurement

IL-4, IL-10, IFN-ν and TNF-ζ levels were measured using OptEIA ELISA kits (BD Biosciences) according to the manufacturer's instructions. Small volumes of supernatants were tested with the Cytometric Bead Array Th1/Th2 Cytokine kit (BD Biosciences) for the content of IL-2, IL-4, IL-5, IFN-ν and TNF-ζ by FACS analysis according to the manufacturer's instructions.

Statistical evaluation

Statistical evaluation was performed using SPSS 11 software. The Mann-Whitney U test was used for the evaluation of differences in the onset of EAE, maximum and cumulative EAE

severity scores. For analyzing differences in EAE incidences a θ^2 test was used. For analyzing statistical differences in proliferation, the stimulation index was used (cpm of antigen-stimulated cells divided by cpm of non-stimulated cells). Proliferation responses and cytokine levels were compared using ANOVA and the t-test. A p value of < 0.05 was considered statistically significant.

Results

CD44v3 and v10 are expressed in MS brain lesions

To determine whether CD44v isoforms are expressed in MS and non-demented control brain tissues, we assessed the presence of CD44v3, v4, v5, v6, v7, v7/v8, v9 and v10 in *post-mortem* frozen brain sections by immunohistochemistry (Table 2). Frozen sections of human tonsil were used as positive control tissue for the expression of CD44v isoforms.

We did not observe specific staining for CD44v7 and CD44v7/v8 in reactive human tonsil and brain tissue. In accordance with this finding, it has been shown that immuno-staining with both Mab anti-CD44v7 (VFF-9) and anti-CD44v7/v8 (VFF-17), by flow cytometry and immunohistochemistry resulted in false negative results[34]. By using transfected cell lines it was shown that mAb anti-CD44v7 (VFF-9) does not detect unglycosylated CD44v7 molecules. Glycosaminoglycan side chains can modulate the conformation of CD44 molecules[35], and can expose the CD44v7 epitope for Mab binding. Most likely, CD44v7 molecules are expressed in MS and reactive human tonsil, since this epitope is specifically upregulated at sites of chronic inflammation, in patients with Crohn's disease and rheumatoid arthritis[36,37]. Therefore, we have functionally assessed the role of the CD44v7 molecule in the development of mouse EAE, as described below.

In contrast to epithelial cells in human tonsil tissue, we could not detect CD44v4, v5, v6 and v9 expressing cells in MS and non-demented control brain tissue. CD44v3 and v10, on the other hand, were expressed in MS brain tissue samples (n=9 patients) containing different lesion stages (Table 1a and Table 3). CD44v3-expressing cells were sporadically observed in perivascular infiltrates of pre-active lesions (Table 3). In active lesions, CD44v3 was expressed by many of the foamy MA. CD44v10 was expressed on endothelial cells in brain tissue from MS patients and non-demented controls (Fig. 1C). CD44v10 was also expressed

on cells with a glial morphology in pre-active (Fig. 1B, C) and active MS lesions, as well as in areas also containing HLA-II-expressing parenchymal cells from six out of seven non-demented control brain tissues (Table 1b). The number of v10-expressing cells was higher compared to the number of HLA-II-expressing cells (Figures 1 A, B). In 78% of pre-active perivascular infiltrates (*n*=72 infiltrates), few leukocytes expressed CD44v10 (Fig. 1C). In addition, CD44v10 was also expressed by occasional to moderate numbers of foamy MA (Table 3 and Fig. 1C, D).

Table 1a. Clinical and neuropathological data of MS patients

Case	Sex	Age (yrs)	Disease duration (yrs)	Number of lesions	Lesion stages
93/089	F	71	23	3	pre-active
93/319	F	39	3	1	chronic active
95/148	F	56	6	4	pre-active
96/102*	F	74	24	1	pre-active
96/232[a]	F	40	4	4	3 pre-active and 1 active demyelinating
				3	pre-active, active demyelinating and chronic inactive
96/234	F	81	49	0	
96/352	F	53	13	2	active demyelinating and chronic inactive
97/024#	F	62	25	1	pre-active
97/189	F	82	15	3	pre-active
97/202	M	50	17	2	pre-active* and chronic active

* Not analysed for CD44v10 expression
\# Not analysed for CD44v3 expression
[a] Two different tissue samples

Table 1b. Clinical and neuropathological data of non-demented controls

Case	Sex	Age (yrs)	Neuropathology
94/104	M	79	Many HLA-II-expressing cells in one area of the tissue.
94/110	M	83	No abnormalities.
96/016	M	63	Individual HLA-II-expressing cells throughout the tissue.
96/030	F	68	Some clusters and individual HLA-II-expressing cells throughout the tissue.
96/132	F	90	Small clusters of HLA-II-expressing cells in one area of the tissue.
96/238	F	87	Many HLA-II-expressing cell clusters throughout the tissue.
96/249	F	78	Many HLA-II-expressing cell clusters throughout the tissue.

Table 2. Antibodies against human CD44v isoforms

Marker	Isotype-label	Clone designation	Supplier
CD44v3	IgG2b	3G5	R&D Systems, Oxon, England
CD44v4	IgG2a-biotin	FW11.10	[22]
CD44v5	IgG1-biotin	VFF-8	Bender Medsystems, Vienna, Austria
CD44v6	IgG2a-biotin	FW11.9	[22]
CD44v7	IgG1	VFF-9	Bender Medsystems
CD44v7	polyclonal	n.a.	Chemicon International, Hampshire, England
CD44v7/v8	IgG1-biotin	VFF-17	Bender Medsystems
CD44v9	IgG1	FW11.24	[22]
CD44v10	IgG1	VVF-14	Bender Medsystems
Isotype control	IgG1	11711.11	R&D Systems
Isotype control	IgG2b	MPC-11	BD Biosciences, San Jose, CA
Isotype control	IgG2a	20102.1	R&D Systems
Isotype control	IgG2a-biotin	20102.1	R&D Systems

(n.a.) not applicable

Table 3. Expression of CD44v3 and v10 in MS brain lesions

CD44v isoform		v3	v10[#]
lesion type	area		
pre-active	parenchymal	-	+++[*]
	perivascular	∂	+
active demyelinating		++/+++	+
chronic active	hypercellular border	++	++
	hypocellular rim	∂	∂
chronic inactive		∂	∂

[*] glial cells
[#] endothelial cell expression
positive cell number:
- none
∂ occasional
\+ few
++ moderate, compare to perivascular CD44v10-expressing cells in Fig. 1C
+++ abundant, compare to parenchymal CD44v10-expressing cells in Fig. 1B

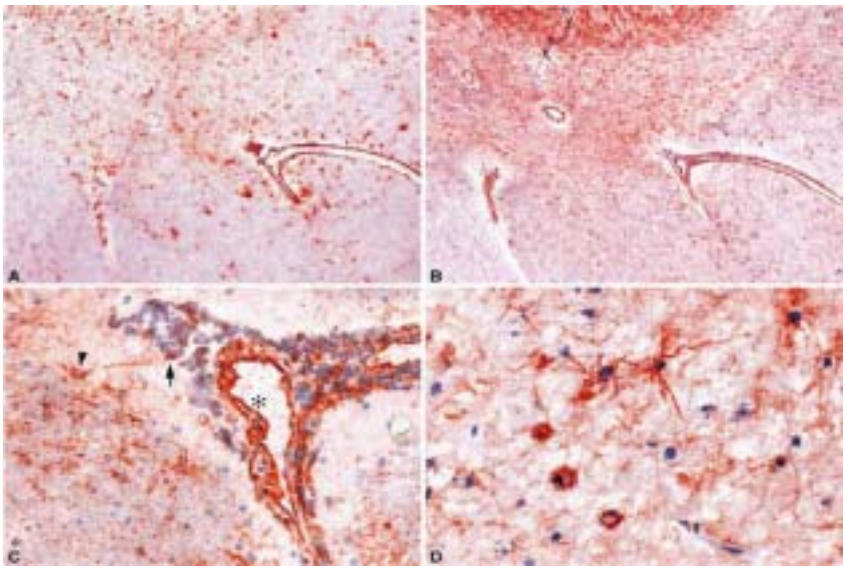

Figure 1. CD44v10 is expressed in MS brain tissue.
Pre-active lesions are characterized by HLA-DP/DQ/DR-expressing activated MA/microglia (A), which are located in perivascular infiltrates or in clusters in the brain parenchyma (A). An abundant cell number expressed CD44v10 in the parenchyma within pre-active lesions (B). These parenchymal v10-expressing cells had a fibrillary glial morphology (arrowhead) (C). Besides glial cells also few perivascular cells (arrow) and endothelial cells (asteriks) expressed CD44v10 (C). In active lesions a moderate to an abundant number of foamy MA expressed CD44v10 (D). Bar = 100 σm.

In conclusion, CD44v isoforms were differentially expressed in MS and non-demented control white matter brain tissue. In normal white matter, glial cells did neither express CD44v3 nor CD44v10. However, in activated (HLA-II-expressing) areas, in both MS and non-demented control brain, fibrillary glial cells expressed CD44v10. In pre-active and active MS lesions, but neither in normal white matter from MS or non-demented controls, perivascular infiltrating leukocytes and foamy MA expressed both, CD44v3 and CD44v10.

CD44v10 expression correlates with EAE development

In accordance with our findings in MS lesions, we have previously shown that CD44v10 is also expressed by infiltrating cells in mouse EAE spinal cord tissue. Moreover, it is known that the combined treatment with mAb against the CD44v6, v7 and v10 isoforms reduces EAE severity[38]. To more specifically assess the functional role of CD44v10, we asked whether EAE development in C57Bl/6 mice correlates with CD44v10 expression on specific brain cell subsets.

Brain cells were isolated and evaluated for the expression of CD44v10 on different cell subsets by flow cytometry at different time points after immunization. Analysis of mononuclear brain cell suspensions from both, CFA and CFA/MOG_{35-55} immunized mice, revealed CD44v10 expression on $CD4^+$ and $CD8^+$ T cells, $CD11b^+$ MA and $B220^+$ B cells (Fig. 2A-D). Thus, the presence of inflammatory cells is not dependent on encephalitogenic immune responses but rather correlates with the non-specific inflammatory response. However, the maximum number of all $CD44v10^+$-expressing cells peaks earlier in CFA/MOG_{35-55} immunized mice than in CFA immunized controls (Fig. 2A-E). Furthermore, the number of $CD4^+$ CD44v10-expressing cells was 2-fold higher in mice immunized with CFA/MOG_{35-55} compared with CFA immunized mice at days 11 and 15 after immunization (Fig. 2A). In addition, CD44v10-expressing $CD4^+$ T cells persisted in low numbers in CFA/MOG_{35-55} immunized mice, whereas a decrease back to baseline levels at day 54 after immunization was found in CFA immunized mice (Fig. 2A).

In relation to the EAE course, elevated numbers of CD44v10-expressing cells were found in CFA/MOG_{35-55} immunized mice as early as 5 days after immunization (Fig. 2E). Maximum numbers of CD44v10-expressing cells were detectable at day 11 after immunization, and

preceded the peak of disease between days 17-22 after immunization. During the remission phase (days 29 and 54 after immunization) CD44v10 cell numbers also decreased.

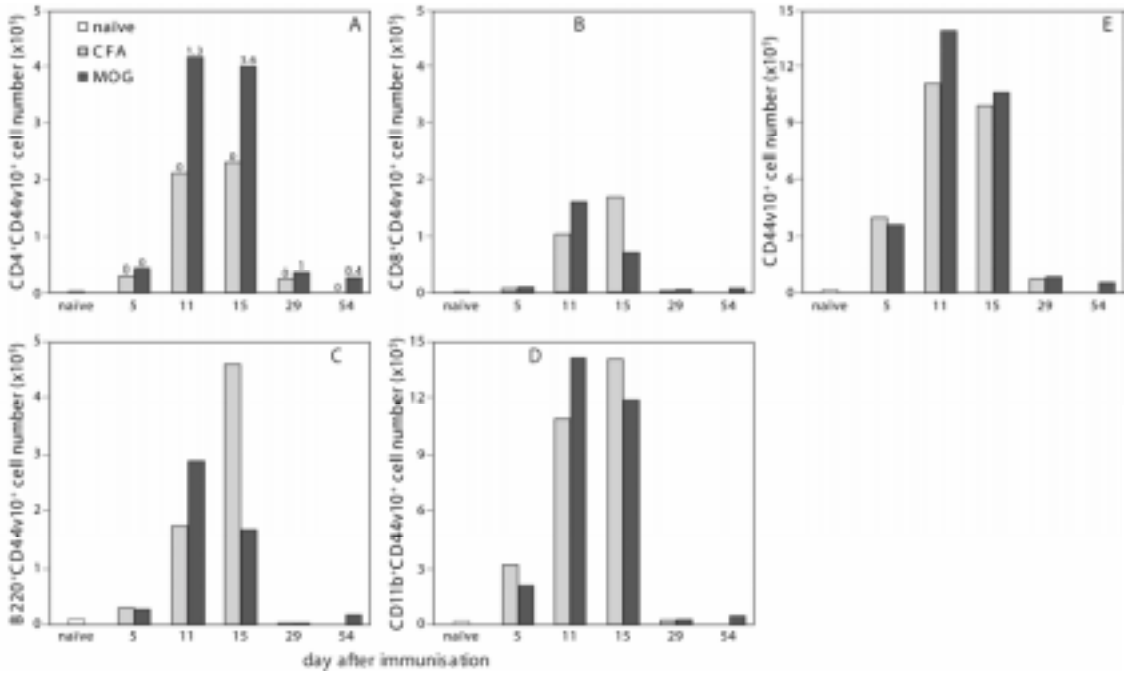

Figure 2. Increase in brain infiltrating CD44v10$^+$ cells upon CFA immunization.
C57Bl/6 mice were naïve (open bars) or immunized with CFA alone (gray bars) or MOG_{35-55} with CFA (black bars). Brain cells were isolated at different time points after immunization. Bars represent the absolute number of CD44v10-expressing CD4$^+$ T cells (A), CD8$^+$ T cells (B), CD11b$^+$ MA (C) B220$^+$ B cells (D) and total brain mononuclear cells (E). Numbers above the bars indicate mean EAE scores (n=4 mice). Note the higher scales for D and E, reflecting higher number of MA.

Taken together, induction of non-specific inflammation results in the accumulation of CD44v10-expressing T cells, B cells and MA in the brain. Development of EAE is associated with a significant increase of infiltrating CD44v10$^+$CD4$^+$ T cells in CFA/MOG_{35-55} immunized mice. These CD44v10-expressing cells persisted in the brain of EAE mice for up to 54 days after immunization.

Autoreactive CD4$^+$ T cells express CD44v10

Next, we addressed the capacity of CD44v10$^+$ CD4$^+$ T cells to mount autoreactive, encephalitogenic responses. First we determined the numbers of CD44v10 expressing CD4$^+$ T cells in the draining lymph nodes of C57Bl/6 mice after immunization with MOG_{35-55}/CFA and in SJL/J mice immunized with $PLP_{139-151}$ at day 10 after immunization. Whereas CD44v10$^+$ CD4$^+$ T cells were present in lymph nodes of immunized C57Bl/6 mice, they were detectable in SJL/J mice only after *in vitro* restimulation with $PLP_{139-151}$. After two rounds of restimulation with $PLP_{139-151}$, CD4$^+$ T cells proliferated dose-dependently (Fig. 3A) accompanied by a dose-dependent upregulation of CD44v10 and CD25 (IL-2R, T cell activation marker) (Fig. 3B). After three rounds of *in vitro* restimulation CD44v10 was upregulated on 86-97% of the CD4$^+$ T cells.

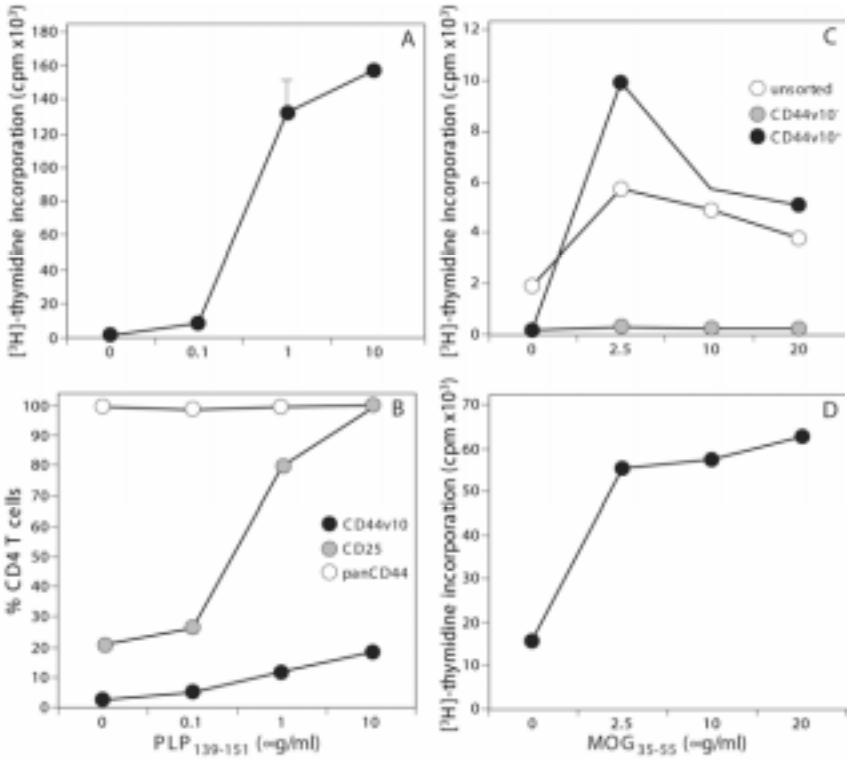

Figure 3. CD44v10$^+$ CD4$^+$ T cells respond specifically to auto-antigen.
CD4$^+$ T cells were restimulated and proliferated dose-dependently to $PLP_{139-151}$ (A). Upon restimulation, CD44v10 was dose-dependently upregulated on $PLP_{139-151}$-specific CD4$^+$ T cells (B). Ten days after MOG_{35-55}/CFA immunization CD4$^+$ T cells were sorted for CD44v10 expression. CD4$^+$CD44v10$^+$ T cells responded specifically to MOG_{35-55} (C) and survived thirteen rounds of restimulation (D).

To determine whether CD44v10 is important for antigen-specific T cell proliferation, lymph node cells were isolated 11 days after immunization of C57Bl/6 mice with MOG_{35-55}/CFA and $CD4^+$ T cells were sorted for CD44v10 expression. As shown in Figure 3C, $CD4^+CD44v10^+$ T cells expanded *in vitro* on MOG_{35-55} pulsed irradiated antigen-presenting cells. In contrast, $CD4^+CD44v10^-$ T cells did not proliferate. An intrinsic defect of $CD4^+CD44v10^-$ T cells to respond to activation was excluded by the observation that these cells showed strong proliferation upon ConA stimulation (data not shown). In addition, $CD4^+CD44v10^+$ T cells survived thirteen rounds of *in vitro* restimulation with MOG_{35-55} (Fig. 3D).

Taken together, these data show that antigen-specific in vitro expansion of $CD4^+$ T cells is restricted to the CD44v10-expressing population.

Genetic deletion of CD44v7 and CD44v10 protects from EAE

To test the hypothesis that CD44v7 and v10 are crucial for the development of EAE, we determined how genetic deletion of CD44v7 or CD44v10 affects EAE susceptibility.

As shown in Tables 4, 5, and Figure 4, disease severity was significantly reduced by deletion of CD44v7 in both SJL/J and C57Bl/6 mice compared to wild type mice. Genetic deletion of one CD44v7 allele ($CD44v7^{+/-}$) did not affect EAE (Table 5). Histopathological analysis of SJL/J spinal cord tissues obtained at different time points after immunization revealed a delayed development of infiltrates in CD44v7 deficient mice (Fig. 5). As expected, a correlation was found between delayed infiltration and delayed onset of EAE development (Table 5, experiment 1). This delay in onset of EAE between $CD44v7^{-/-}$ and wild type mice was observed in 2 out of 6 experiments, suggesting that deletion of CD44v7 does not per se lead to a delayed influx of inflammatory cells into the spinal cord.

Fifteen and 20 days after immunization, spinal cord infiltrates were reduced in number and size in six out of eight $CD44v7^{-/-}$ mice compared to wild type mice. These findings correlated with the absence of EAE development or reduced EAE severity in CD44v7-/- mice (Fig. 5). In contrast, no differences in the number and size of infiltrates were observed in brain tissue derived from wild type and $CD44v7^{-/-}$ mice 5, 10, 15 and 20 days after immunization (data not shown).

Table 4a. Clinical parameters of actively-induced EAE in C57Bl/6 mice

Genotype	Number of mice	Disease incidence (%)	Disease onset[#] (day)	Maximum score[#]	Cumulative score[#] (day)
wild type	6	100	11.7 ± 0.5	3.1 ± 0.3	26.3 ± 9.3 (29)
CD44v7$^{-/-}$	7	71	10.4 ± 1.9	1.9 ± 1.2	12.5 ± 8.2* (29)
wild type	16[a]	100	11.7 ± 1.4	3.4 ± 0.6	44.0 ± 7.8 (28)
CD44v7$^{-/-}$	13[a]	92	14.7 ± 2.8**	3.1 ± 1.0	31.4 ± 13.7* (28)
wild type	13	77	10.3 ± 1.3	1.7 ± 1.1	23.0 ± 24.4 (55)
CD44v7$^{-/-}$	12	67	18.0 ± 10.3	1.5 ± 1.4	14.5 ± 17.0 (55)
CD44v10$^{-/-}$	14	43	12.5 ± 1.4*	0.4 ± 0.5**	4.6 ± 7.7* (55)
wild type	6	100	10.2 ± 1.3	2.0 ± 0.5	4.8 ± 2.4 (12)
CD44v7$^{-/-}$	6	33*	10.5 ± 0.7	1.5 ± 0.7*	1.1 ± 2.0* (12)
CD44v10$^{-/-}$	6	0.0**		0.0**	0.0** (12)

[#] mean values
[a] 1 mouse died due to EAE (score 5), and was excluded from calculations after that time point
* $p < 0.05$ compared to wild type mice
** $p < 0.005$ compared to wild type mice

Table 4b. Histological analysis of CNS infiltrates in C57Bl/6 mice

Genotype	Cumulative score	Infiltrate number		Infiltrate size	
		brain	sc	brain	sc
wild type	4.5	10	6	++	++
wild type	6.0	7	8	+	++
CD44v7$^{-/-}$	5	4	10	+	++
CD44v7$^{-/-}$	0	2	1	∂	∂
CD44v10$^{-/-}$	0	7	0	++	-
CD44v10$^{-/-}$	0	7	0	++	-

sc spinal cord

Infiltrate size

-	no infiltrating cells	
∂	1-50 cells	small
+	51-500 cells	medium
++	> 500 cells	large

Similar to CD44v7$^{-/-}$ mice, CD44v10$^{-/-}$ C57Bl/6 mice also showed significantly reduced cumulative EAE scores compared to wild type controls after MOG_{35-55}/CFA immunization (Table 4a). Histological analysis of brain and spinal cord tissues 12 days after immunization revealed a similar extent of infiltrates in the brain tissue of CD44v10$^{-/-}$ and wild-type mice (Table 4b). However, analysis of spinal cord tissues revealed no infiltrates in CD44v10$^{-/-}$ mice compared to wild type controls. Accordingly, CD44v10$^{-/-}$ mice did not develop clinical signs of EAE, 12 days after immunization.

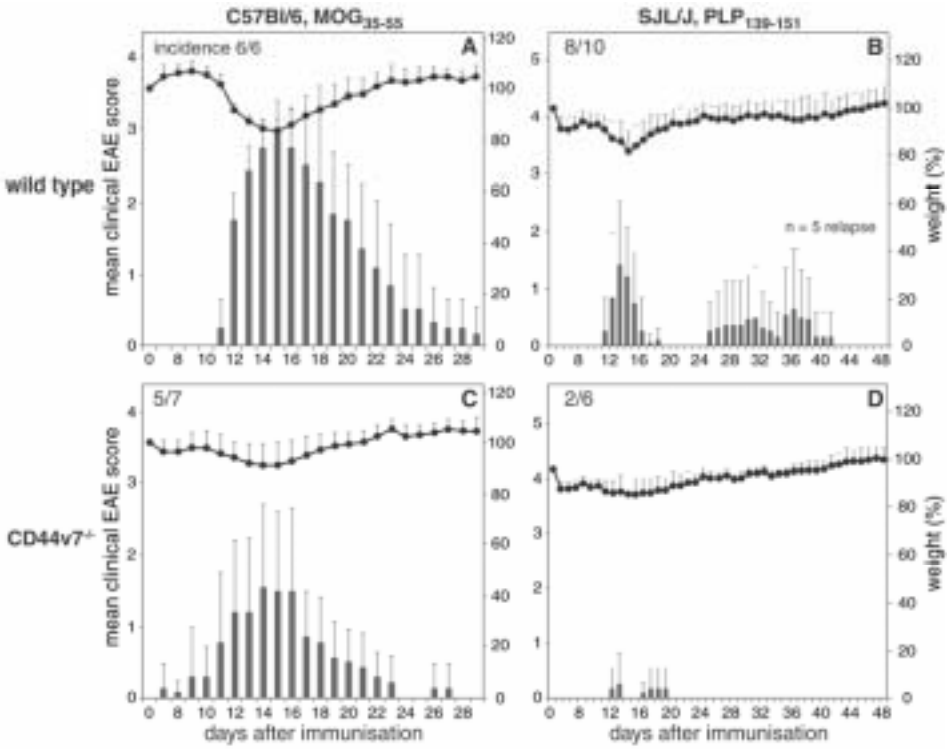

Figure 4. CD44v7 deletion in two different mouse strains results in reduced EAE burden, induced by active immunization.
C57Bl/6 were immunized with MOG_{35-55} (A, C) and SJL/J mice were immunized with $PLP_{139-151}$ (B, D). Cumulative scores of wild type mice (A, B) were significantly higher ($p<0.05$) compared to CD44v7$^{-/-}$ mice (C, D). Mice were weighed and scored for clinical signs of EAE daily. Bars represent the mean clinical scores, lines the mean weight. (d.i.) disease incidence.

Table 5. Clinical parameters of actively-induced EAE induced in SJL/J mice

Genotype	Number of mice	Disease incidence (%)	Disease onset[#] (day)	Maximum score[#]	Cumulative score[#] (day)
wild type	10	70	11.1 ± 0.7	1.6 ± 1.5	7.4 ± 5.4 (20)
CD44v7$^{-/-}$	9	33	13.0 ± 1.0[*]	2.0 ± 1.4	1.8 ± 3.9[*] (20)
wild type	10	80	12.1 ± 0.8	1.6 ± 1.1	10.7 ± 13.2 (48)
CD44v7$^{+/-}$	5	80	12.5 ± 1.7	1.6 ± 1.2	12.3 ± 17.0 (48)
CD44v7$^{-/-}$	6	33	13.5 ± 1.5	0.4 ± 0.2[*]	1.0 ± 1.4[*] (48)
wild type	10	70	11.7 ± 0.8	0.8 ± 1.1	10.2 ± 10.6 (35)
CD44v7$^{+/-}$	10	40	13.0 ± 1.4	0.6 ± 0.8	6.5 ± 12.2 (35)
CD44v7$^{-/-}$	10	20[*]	12.5 ± 0.7	0.3 ± 0.7	1.1 ± 2.3[*] (35)

[#] mean values

[*] $p < 0.05$ compared to wild type mice

In summary, both CD44v7 and CD44v10 deficient mice develop reduced clinical EAE scores compared to wild type mice. Strikingly, this observation is paralleled by a reduced inflammatory infiltrates in the spinal cord, but not in the brain tissue of CD44v7 and CD44v10 deficient mice.

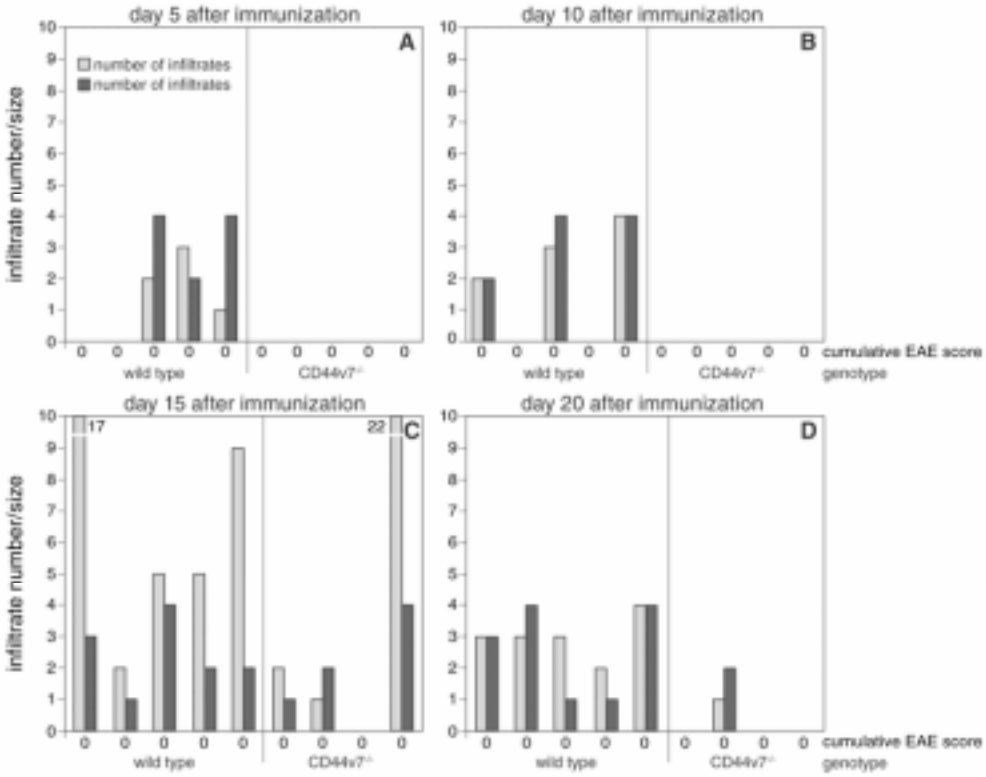

Figure 5. Reduced infiltration in spinal cord tissue from CD44v7$^{-/-}$ SJL/J mice.
Wild type and CD44v7$^{-/-}$ SJL/J mice were immunized with $PLP_{139-151}$ in CFA. The number and size of infiltrates was determined in spinal cord tissues, which were isolated at the indicated time points. Bars indicate the number and size of infiltrates in individual mice.
size of infiltrates
0 no infiltrating cells
1 1-20 cells
2 21-50 cells
3 51-101 cells
4 >101 cells

Distinct roles for CD44v7 and CD44v10 isoforms expressing antigen specific T cells

As previously described, CD44v isoforms are involved in APC-T cell interactions and modulation of T cell proliferation and cytokine production[33,39-41]. In order to address the mechanisms explaining the reduced EAE severity in CD44v7$^{-/-}$ and CD44v10$^{-/-}$ mice, we asked whether auto-antigen specific lymph node cell proliferation and cytokine production is altered.

Results from three independent experiments showed that in SJL/J and C57Bl/6 mice, CD44v7 deficiency resulted in moderate time-dependent effects on auto-antigen specific T

cell proliferation (Fig 6A-C). At day 5 after immunization, lymph node cells from CD44v7 deficient SJL/J mice (*n*=5 mice) showed a variable proliferative response against $PLP_{139-151}$, which was significantly increased ($p<0.05$) compared with wild type cell proliferation (Fig. 6A).

Twelve days after immunization, C57Bl/6 $CD44v7^{-/-}$ and $CD44v10^{-/-}$ lymph node cells proliferated equally well as wild type cells upon *in vitro* restimulation with MOG_{35-55} (data not shown). Similar results were found with SJL/J mice at days 10 and 15, respectively (Fig. 6A). No overt differences were observed in IFN-ν levels between C57Bl/6 $CD44v10^{-/-}$ and wild-type lymph node cells (wild type: 2413 ± 84 pg/ml, $CD44v10^{-/-}$: 2806 ± 78 pg/ml) with 10 σg/ml MOG_{35-55}. Upon restimulation with 10 σg/ml MOG_{35-55}, $CD44v10^{-/-}$ cells produced higher levels of IL-10 (356 ± 2 pg/ml) compared to wild type cells (114 ± 6 pg/ml). In both groups the levels of TNF-ζ and IL-4 were below the detection limit of the ELISA. In SJL/J mice IFN-ν, IL-4 and IL-10 levels did not differ between wild type and CD44v7-/- mice (data not shown).

To assess whether differences were present in the T cell or APC compartment, we purified $CD4^+$ T cells from wild type and $CD44v7^{-/-}$ mice at day 10 after immunization. $CD44v7^{-/-}$ and wild type $CD4^+$ T cells were co-cultured with $CD44v7^{-/-}$ and wild type irradiated spleen cells as APC and restimulated with different concentrations of MOG_{35-55}. In all combinations, T cells proliferated equally well in a dose-dependent response upon restimulation with MOG_{35-55} (data not shown). Furthermore, levels of IFN-ν, TNF-ζ, IL-2, IL-4 and IL-5 did not differ (data not shown).

In the remission phase of EAE, 20 days after immunization, a modest decrease in T cell proliferation of SJL/J $CD44v7^{-/-}$ lymph node cells compared to wild type cells was observed with 20 σg/ml $PLP_{139-151}$ (Fig 6A). Also at day 52 after immunization, $CD44v7^{-/-}$, but not $CD44v7^{+/-}$, lymph node cells (*n*=10 mice per group) proliferated significantly less ($p<0.05$) upon restimulation compared to wild type cells. More pronounced effects of CD44v7 deletion were observed in C57Bl/6 MOG_{35-55} specific T cell proliferation responses at day 43 after immunization. While auto-antigen specific T cell proliferation was observed with cells from all wild type mice (*n*=4), $CD44v7^{-/-}$ cells did not respond to MOG_{35-55} restimulation, with the exception of 1 animal (Fig. 6C). Addition of IL-2 only partially restored

proliferation, since a significant decrease ($p<0.05$) in T cell proliferation compared to wild type cells was still observed in CD44v7$^{-/-}$ mice (n=4).

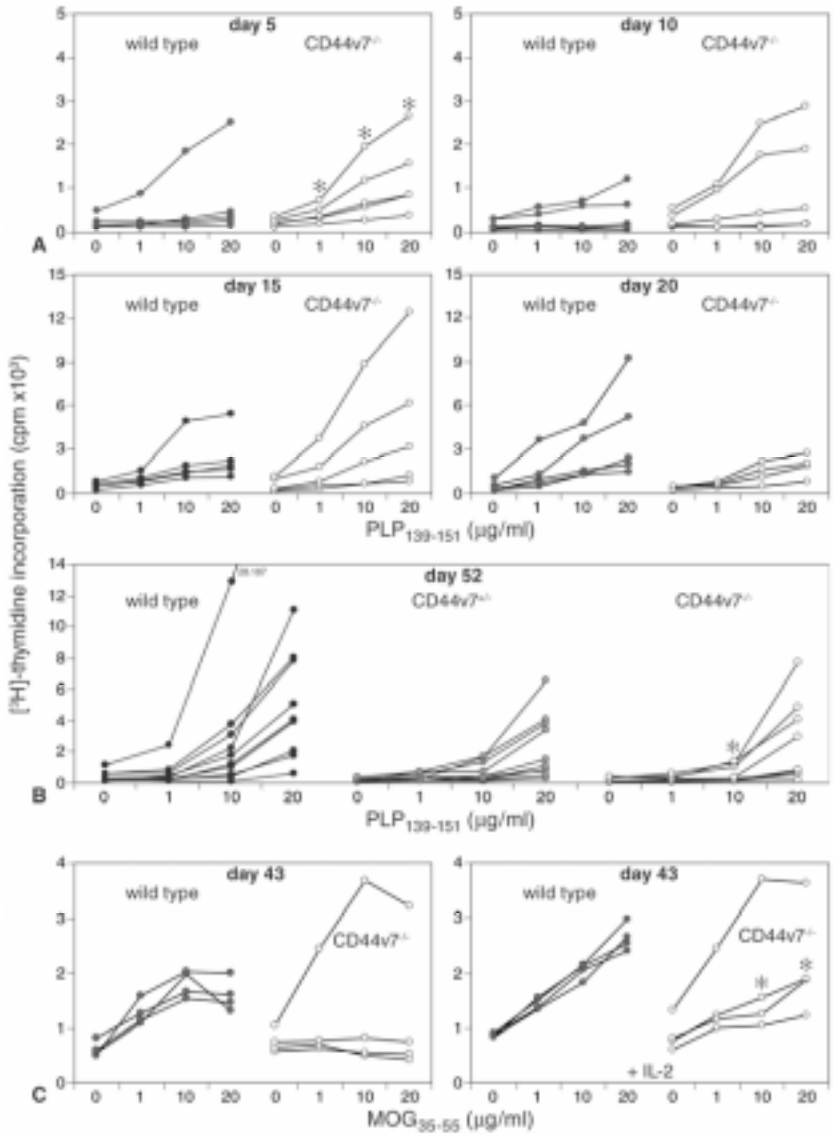

Figure 6. Time-related effects of CD44v7 deletion on auto-antigen specific T cell proliferation.
At different time points after immunization with $PLP_{139-151}$ (A, B) or MOG_{35-55} (C) in CFA, draining lymph node cells were isolated and restimulated *in vitro* with the respective antigens for 4 days. Auto-antigen specific T cell proliferation of CD44v7$^{-/-}$ or CD44v7$^{+/-}$ cells was compared to wild type cells. Lines represent cpm values of individual mice. (*) $p<0.05$

These data indicate that CD44v7$^{-/-}$ lymph node cells were capable to proliferate in an auto-antigen specific manner, however the capacity to expand upon antigen challenge is reduced during the remission phase of EAE (day 20-52).

CD44v7 deficiency affects both autoreactive T cells and the non-T cell compartment

To determine how CD44v7 affects the capacity of T cells to transfer EAE in recipient mice, C57Bl/6 wild type mice were injected with MOG_{35-55} specific, either wild-type or CD44v7 deficient CD4^{+} T cells (Table 6). Compared to wild-type cells, MOG_{35-55} specific CD44v7$^{-/-}$ cells did not induce EAE after transfer in C57Bl/6 wild type recipients. CD44v7 deletion in recipient mice also resulted in a significantly reduced severity of EAE compared to wild type mice, upon injection with wild type cells.

Table 6. Clinical parameters of EAE induced by adoptive transfer

Strain cell number[a]	Genotype cells – mice	Number of mice	Disease incidence (%)	Disease onset[#] (day)	Maximum score[#]	Cumulative score[#] (day)
C57Bl/6	wt – wt	5	100	8.2 ± 1.6	2.5 ± 0.3	17.8 ± 5.9 (33)
4x10^6	wt – v7$^{-/-}$	5	60	7.0 ± 0.0	1.8 ± 0.5*	6.3 ± 5.7* (33)
	v7$^{-/-}$ – wt	5	0***		0.0**	0.0** (33)
SJL/J	wt – wt	4	100	7.5 ± 0.9	3.1 ± 0.4	42.3 ± 19.0 (37)
5x10^6	wt – v7$^{-/-}$	4[b]	100	6.5 ± 0.5	3.9 ± 0.7	65.0 ± 17.9 (37)
	v7$^{-/-}$ – wt	4	100	12.8 ± 3.9*	2.5 ± 0.8	25.3 ± 16.9 (37)
	v7$^{-/-}$ – v7$^{-/-}$	4	100	10.8 ± 2.5	2.5 ± 0.6	17.6 ± 8.5 (37)
SJL/J	wt – wt	6	100	7.2 ± 1.0	2.5 ± 0.6	41.1 ± 3.6 (30)
15x10^6	wt – v7$^{-/-}$	6	83	9.8 ± 3.1	1.4 ± 0.9	7.3 ± 4.4*** (30)
	v7$^{-/-}$ – wt	6	83	7.0 ± 0.9	2.5 ± 0.4	33.4 ± 13.1 (30)
	v7$^{-/-}$ – v7$^{-/-}$	6	100	7.8 ± 1.9	2.1 ± 1.1	16.6 ± 8.7*** (30)

wt wild type
v7$^{-/-}$ CD44v7$^{-/-}$
[#] mean values
[a] number of injected *in vitro* reactivated auto-antigen specific cells
[b] 1 mouse died due to EAE, and was excluded from calculations after that time point
mice – cells donor cells injected into recipient mice
* $p < 0.05$ compared to wt - wt
** $p < 0.01$ compared to wt - wt
*** $p < 0.005$ compared to wt - wt

In SJL/J mice, both wild type and CD44v7 deficient effector cells induced disease in wild type mice (Table 6). Two individual adoptive transfer experiments in SJL/J mice were performed in separate laboratories (Table 6, Fig. 7). Injection of 5 or 15 million wild type cells into wild type recipients showed similar cumulative disease scores, however in contrast to our expectation, the maximum EAE score was higher in the experiment where 5 million cells were injected. In both SJL/J experiments wild type and CD44v7 deficient effector cells could induce disease efficiently in wild type mice (Table 6). Disease onset was delayed upon injection of 5 million, but not 15 million CD44v7$^{-/-}$ cells compared to wild type cells. A significant reduction in cumulative EAE scores occurred upon injection of 15 million cells into CD44v7$^{-/-}$ recipient mice, but not in the experiment where 5 million donor cells were injected (Fig. 7). However, using 5 million cells, a pronounced reduction in total EAE burden was also found when both, donor and recipient cells were deficient for CD44v7 (Fig. 7A).
Taken together, these data show in two different mouse strains that CD44v7 deficiency affects both the autoreactivity of CD4^{+} T cells and susceptibility of the recipient.

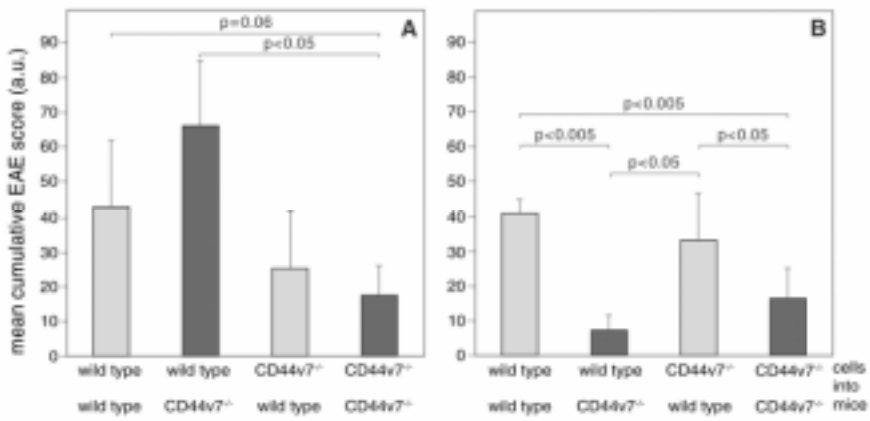

Figure 7. CD44v7 deficiency reduces EAE severity, induced by adoptive transfer.
CD44v7$^{-/-}$ or wild type donor mice were immunized with $PLP_{139-151}$ in CFA. Draining lymph node cells were isolated at day 10 after immunization and restimulated for 4 days with 10 σg/ml $PLP_{139-151}$. Wild type or CD44v7$^{-/-}$ recipient mice were injected with 5 or 15x10^{6} wild type or CD44v7 deficient $PLP_{139-151}$ reactivated lymph node cells (A and B, respectively). Mice were weighed and scored for clinical signs of EAE daily. Bars represent mean cumulative EAE scores. The cumulative EAE score is a measure for the severity of the disease. This was calculated by adding up all the scores per animal over the indicated time points after EAE induction, representing the total disease load.

Discussion

This study shows that CD44v3 and CD44v10 are expressed in inflammatory brain lesions in MS patients. In agreement with previous findings, where combined mAb treatment against CD44v6, v7 and v10 reduced EAE disease burden[38], we here moreover show that absence of CD44v7 or CD44v10 by genetic deletion reduced the severity of EAE, which was associated with reduced infiltration of the spinal cord.

A role for CD44v3 and v10 in MS

It has been shown previously that panCD44 is expressed constitutively by normal white matter astrocytes under normal conditions and expression is upregulated in MS brain lesions[26,42-45]. In contrast, CD44v isoforms are not expressed by glial cells under normal conditions[45]. However, upon *in vitro* stimulation, mouse astrocytes do express CD44v6/v7 and CD44v10-containing isoforms[46]. In agreement with these findings we describe here that glial expression of CD44v10 isoforms is restricted to activated areas (HLA-II-positive) of the brain, in non-demented controls and in MS patients. CD44v3-v6 and CD44v9 were not expressed in these activated regions. We could not assess the expression of CD44v7 and CD44v7/v8 isoforms, which may partly be explained by the incapacity of these mAbs to stain unglycosylated CD44v isoforms, as previously described[34].

Furthermore, CD44v10 was expressed by endothelium and perivascular infiltrating cells in MS brain tissue and may well participate in the migration and adhesion of cells, as such functions of CD44v10 have been described previously[47,48]. Additionally, CD44v3 is involved in extravasation of leukocytes as described in mouse delayed-type hypersensitivity[49], and may as well participate in leukocyte extravasation in MS. Under inflammatory conditions, such as TNF-ζ stimulation, human monocytes differentiate into dendritic cells and express CD44v3[50]. This finding may explain why CD44v3 is expressed only occasionally by perivascular infiltrating cells (including monocytes) in pre-active lesions, but by many foamy MA in active MS brain lesions. CD44 can be post-translationally modified by the attachment of sulfate groups[51,52]. Sulfated proteoglycans are binding sites for growth factors, chemoattractants and cytokines[53,54]. CD44v3- and CD44v10-expressing cells in MS brain can potentially carry heparin and chondroitin sulfate side chains and as such may function as a collector of inflammatory mediators. Additionally, CD44v3-expression may stimulate the

production of pro-inflammatory cytokines, since blocking CD44v3 in Th cell and APC interactions by mAb treatment reduced the production of IL-2, IL-12 and IFN-ν[49].

How might CD44v10 deletion impair the development of EAE?

A minority of mouse lymph node cells expresses CD44v10 under normal conditions[55]. However, upon *in vitro* or *in vivo* stimulation CD44v10 expression is upregulated by T cells, B cells and MA[49,55]. In agreement with these findings, we here show that CD44v10 expression is specifically upregulated on *in vitro* restimulated encephalitogenic $CD4^+$ T cells. CD44v10 is also expressed by brain infiltrating leukocytes and CD44v10 expression parallels the development of EAE. Extravasation of CD44v10-expressing leukocytes to brain tissue is auto-antigen independent, since these cells were also detected in brain tissue of CFA immunized control mice. This observation is not surprising, because it is well known that activated or resting memory T cells with irrelevant antigen specificity can migrate to the CNS[27,56-58]. MOG_{35-55} specific $CD4^+CD44v10^+$ sorted T cells proliferate upon MOG_{35-55} restimulation, while $CD4^+CD44v10^-$ cells do not respond. As described previously, treatment with mAb anti-CD44v10 did not affect the proliferation of ConA stimulated spleen cells[55]. According with these findings, we demonstrate here that $CD44v10^{-/-}$ and wild type lymph node cells proliferate equally well upon restimulation with MOG_{35-55}. Therefore it can be excluded that the reduced EAE burden in $CD44v10^{-/-}$ mice is the result of a lack of proliferation.

Whereas proliferation was similar comparing wild type and $CD44v10^{-/-}$ cells, $CD44v10^{-/-}$ lymph node cells produced IFN-ν and elevated levels of IL-10 upon *in vitro* restimulation with MOG_{35-55}. It has been described very recently that antigen specific regulatory T cells producing IL-10 and IFN-ν develop during Th1 polarized responses[59]. The fact that MOG_{35-55} restimulated $CD44v10^{-/-}$ lymph node cells produced elevated levels of IL-10 compared with wild type cells may indicate that regulatory T cells are elevated or induced in $CD44v10^{-/-}$ mice and may explain the reduced EAE burden. Indeed, it was recently demonstrated that upon stimulation more $CD4^+CD25^+$CTLA-4^+ T regulatory cells are present in $CD44v7^{-/-}$ mice[60]. Further evaluation of MOG_{35-55} restimulated $CD44v10^{-/-}$ cells may demonstrate expression of the transcription factor Foxp3, which is known to be specifically expressed by regulatory T cells[61].

How might CD44v7 deletion impair the development of EAE?

Here we show in two mouse strains that the severity of EAE, induced by active immunization or adoptive transfer, was significantly reduced in CD44v7 deficient mice compared to wild type controls. Similar, but more pronounced effects of CD44v7 deficiency were observed in adoptive transfer EAE and T cell proliferation of C57Bl/6 mice, compared with SJL/J mice. CD44v7 deficiency reduced EAE burden by modulating APC and/or T cell effector functions, since CD44v7 deficiency affected both compartments in adoptive transfer EAE. Proliferation of auto-antigen specific cells was gradually regulated by CD44v7, since modest differences were observed in proliferation of CD44v7$^{-/-}$ cells before the onset and in the remission phase of EAE, compared to wild type cells. IL-2 could partially restore T cell proliferation defects of CD44v7$^{-/-}$ C57Bl/6 cells, which is in agreement with the fact that CD44 can enhance T cell proliferation and IL-2 production, as described[62,63]. In conclusion, these findings may imply that early after sensitization CD44v7 deficient cells proliferate faster, but eventually also exhaust faster, i.e. by a higher susceptibility to apoptosis, than wild type cells.

Interestingly, reduced EAE burden in CD44v7$^{-/-}$ and CD44v10$^{-/-}$ mice is associated with a reduced number and size of infiltrates in spinal cord, but not in brain tissue. This finding may imply an impaired migration into the spinal cord or an increase in apoptosis of infiltrated cells. Accordingly, it was previously suggested that CD44v6 and CD44v10 may participate in the development of HAM/TSP, a chronic inflammatory disease of the spinal cord[64]. Alternatively, CD44v7/v10 deleted cells may infiltrate the spinal cord, but are more susceptible to apoptosis. Constitutive CD44v7-expression prevents T cells from going into apoptosis, as was shown by *in vitro* anti-CD3 stimulation of *in vivo* pre-activated mesenteric lymph node cells. CD44v7 deletion resulted in opposite results[60]. Also *in vivo*, reduced disease development in TNBS-induced colitis in CD44v7$^{-/-}$ mice was associated with high numbers of apoptotic cells in inflammatory lesions of the gut[8,24,25]. Taken together, absence of CD44v7 might protect from EAE by an impaired adhesion to spinal cord tissue. Additionally, induction of apoptosis of inflammatory cells or induction of regulatory T cells could also reduce inflammation in CD44v7$^{-/-}$ mice.

Concluding remarks

Here we show that CD44v3 and CD44v10 were expressed in inflammatory lesions in the CNS of MS patients. In the development of mouse EAE, the number of CD44v10-expressing brain infiltrating cells correlated with disease activity. Furthermore, we provide functional evidence that CD44v7 and CD44v10-deficiency reduced the clinical severity of mouse EAE. We have demonstrated a reduced number of inflammatory cells in the spinal cord tissue of CD44v7$^{-/-}$ and CD44v10$^{-/-}$ mice compared to wild type mice. CD44v10$^{-/-}$ mice produced elevated levels of IL-10 upon *in vitro* restimulation of lymph node cells with MOG_{35-55}. In addition, a reduced proliferation of CD44v7$^{-/-}$ lymph node cells was found in the remission phase of EAE, upon *in vitro* auto-antigen restimulation. The contribution of CD44v7 and CD44v10 in the development of EAE is likely dependent on cell migration, adhesion, apoptosis and T cell regulation, functions already attributed to these variant isoforms of CD44. These data indicate that modulating CD44v isoform expression and function might be used as a directed therapeutical approach for MS.

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Concluding remarks

Concluding remarks

Many cancers and inflammatory diseases exhibit two common aspects: an up-regulation of CD44v isoforms and a defective apoptosis (reviewed by (1, 2)). Here, we elucidate the implication of CD44 in programmed cell death and we explain the molecular actions of this membrane receptor in the main apoptotic pathway involved in these disorders: the Fas mediated cell death. The correct functioning of this pathway is crucial for life because defective Fas-mediated apoptosis can cause severe disorders like autoimmunity and tumorigenesis (reviewed by (3, 4)).

In chapter I, we first demonstrate *in vitro* that expression of CD44v isoforms strongly protects the cells from Fas-mediated apoptosis by interacting with Fas receptor. We provide evidence that CD44v isoforms, notably v6 and v9 function as anti-apoptotic molecules by sequestering Fas in the presence and absence of FasL. We show that the extracellular domain of CD44v isoforms is sufficient to confer resistance to cell death. Indeed, a CD44v mutant lacking the cytoplasmic tail protects the cells from apoptosis as efficiently as CD44v full length. We propose a model in which CD44v extracellular domain interacts with Fas receptor, and thus prevents FasL binding and Fas death signaling. We suggest that CD44v isoforms associate with the pre-ligand binding domain (PLAD) which is essential for Fas trimerization (reviewed by (4)). A similar mechanism of Fas sequestration by the tyrosine kinase receptor c-met has been observed (5). Importantly, we also demonstrate that it is possible to revert this anti-apoptotic mechanism by targeting v6 isoform with a specific anti-v6 antibody (Figure 1). The latter observation strongly encourages immunotherapeutic strategies using anti-CD44v antibodies for the treatment of severe disorders such as cancers and inflammatory diseases.

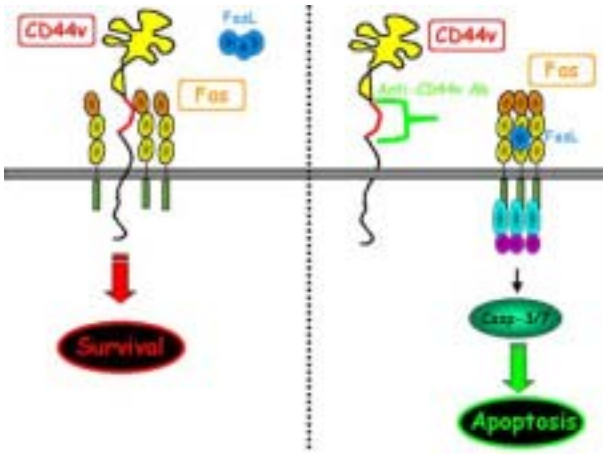

Figure 1. Model representing the blockade of the anti-apoptotic effect of CD44v by using anti-CD44v specific antibodies. In the presence of anti-CD44v specific antibodies, CD44v is unable to confer resistance to Fas-mediated apoptosis, probably due to the incapacity to interact with Fas.

In the second chapter of this thesis we further investigate the role of the intracellular domain of CD44 in apoptosis. It is known that CD44 cytoplasmic domain can interact with various membrane-actin cytoskeleton crosslinker proteins such as ankyrin, annexin II or ezrin (6-10). It has been described that the interaction of CD44 with ezrin is tightly regulated by PKC. CD44 only interacts with ezrin when the serine 325 at the cytoplasmic domain of CD44 is phosphorylated. Dephosphorylation of serine 325 and phosphorylation of serine 291 by PKC results in ezrin dissociation and promotes cell migration (11). The advantage of our *in vitro* system is that CD44s and CD44v isoforms are not-co-expressed in the same cell. This allows us to study independently the behavior of the different isoforms. We demonstrate that CD44s and CD44v isoforms which share a common cytoplasmic domain, differ for intracellular partners. In fact, we show that ezrin interacts exclusively with CD44s but not with CD44v isoforms. We further demonstrate that disruption of actin cytoskeleton with cytochalasin D decreases Fas-mediated apoptosis only in CD44s expressing cells. These results suggest that the complex CD44s-ezrin-actin cytoskeleton modulates Fas-mediated apoptosis. Further

experiments using small interference RNA to downregulate ezrin could provide more information about the function of this multimeric complex in apoptosis regulation.

Together, these two studies could explain the controversial literature concerning the pro- (12-15) and anti-apoptotic activities of CD44 (2, 16-18). In fact, we demonstrate that CD44 is implicated in cell death and we describe the molecular mechanisms of apoptosis regulation by CD44. We suggest that CD44v and CD44s have different affinities for divers extracellular partners, and that interaction with their specific partners outside the cell, modulates intracellular events. Indeed, other membrane receptors such as integrins also exhibit a similar effect. η1 and η3 integrins which differ in their extracellular domain transduce the stimuli in different ways. η3 integrin regulates Rho activity and stress fibers formation whereas η1 integrin promotes Rac activity and lamellipodia formation. A mutant of η1 integrin expressing the extracellular ligand binding domain of η3 integrin also increases Rho activity (19). These observations demonstrate that the extracellular domains of membrane receptors play a crucial role in transducing the extracellular information into specific intracellular signaling events.

Our data contribute to explain that CD44 is involved in more than cell adhesion events. CD44 is a family of transmembrane receptors and this report adds evidence that the different CD44 isoforms exhibit very diverse and even opposite functions. This is most probably associated to the different extra and intracellular partners. We propose that while CD44v isoforms interact extracellularly with Fas receptor, preventing apoptosis, CD44s associates with ezrin and actin cytoskeleton, and the resulting complex modulates cell death (Figure 2).

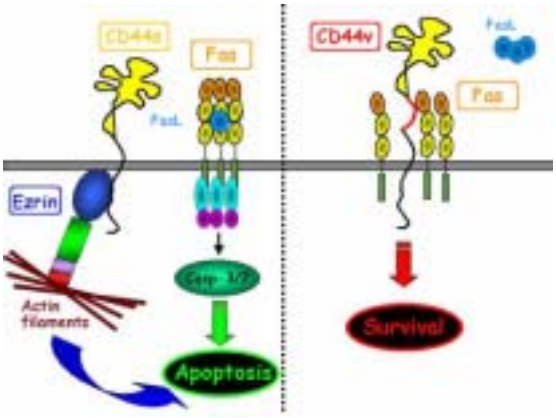

Figure 2. Model representing the mechanisms of apoptosis regulation by CD44 described above.

These findings open new interesting questions. Indeed, we suggest that CD44s and CD44v isoforms must have affinities for different extracellular ligands (Figure 3) or membrane receptors (Figure 4). The extracellular interaction(s) would lead to a conformational change or to phosphorylation(s)/dephosphorylation(s) processes which would regulate the intracellular events. This hypothesis would explain why CD44s and CD44v which only differ extracellularly have different intracellular partners and subsequently exhibit different functions.

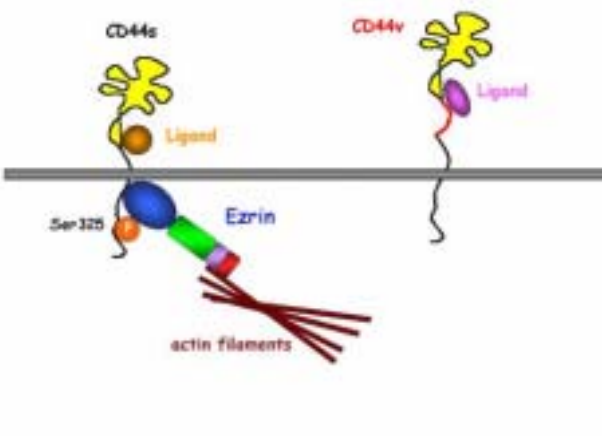

Figure 3. Model representing how different extracellular ligands might regulate the intracellular signaling events. The P in orange represents the Serine 325 which has been shown to be phosphorylated when ezrin interacts with CD44 (11).

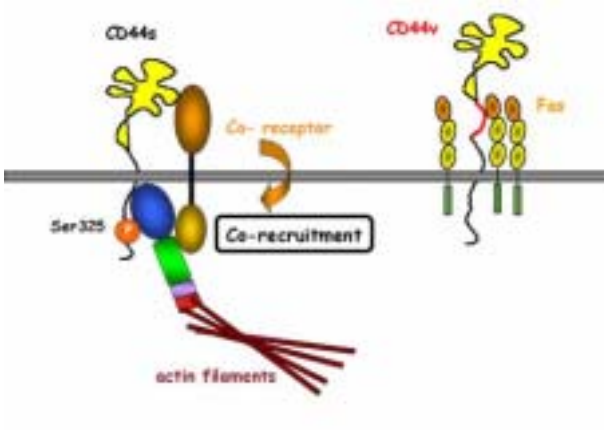

Figure 4. Model representing how a co-receptor might interact extracellularly and specifically with CD44s and participate to the co-recruitment of ezrin, forming a multimeric complex which organizes actin cytoskeleton. In contrast, CD44v isoforms interact specifically with Fas and exhibit the anti-apoptotic effect described above.

In this report we have described a novel anti-apoptotic mechanism of CD44v *in vitro*. The next interesting step for a future therapeutic approach is to confirm this observation *in vivo*.

For this purpose, we have performed *ex vivo* experiments with transgenic mice (see annexe after Chapter II). As a first approach, lymph node cells from wild type mice, which express all isoforms of CD44, and CD44v6v7 deficient mice were stimulated either with Phytohemagglutinin (PHA) or anti-CD3 antibody and analyzed for apoptosis induction. Interestingly, we could observe that T cells from CD44v6v7 deficient mice exhibit significantly higher rates of apoptosis (unpublished observations). These preliminary results nicely correlate with our *in vitro* data and demonstrate that T cells lacking expression of CD44v6v7 undergo apoptosis more readily. Further *in vivo* experiments would help to establish new therapeutic strategies targeting CD44v isoforms.

The understanding of the molecular action of CD44v in preventing Fas-mediated apoptosis allows us to establish the relationship between CD44v upregulation and defective apoptosis observed in many diseases.
In a colitis model, CD44v6/v7 deficient mice showed increased number of apoptotic cells. Those mice, contrary to the wild type mice recovered from the disease (20).

Our *in vivo* data exposed in Chapter III demonstrate that CD44v isoforms are also involved in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS). In fact, we demonstrate that the specific v3, v7 and v10 isoforms are up-regulated in these disorders. In EAE and MS, CD44v isoforms probably participate in migration and extravasation as these functions have already been attributed to certain variant isoforms (21-24). But in addition, considering our findings about the molecular anti-apoptotic mechanism of CD44v described in chapter I, we also suggest that CD44v isoforms probably contribute in the progression of these diseases by preventing apoptosis of the autoreactive T cells. These cells infiltrate in the brain and due to their protection against Fas-mediated apoptosis maintain a persistent inflammation (Figure 5).

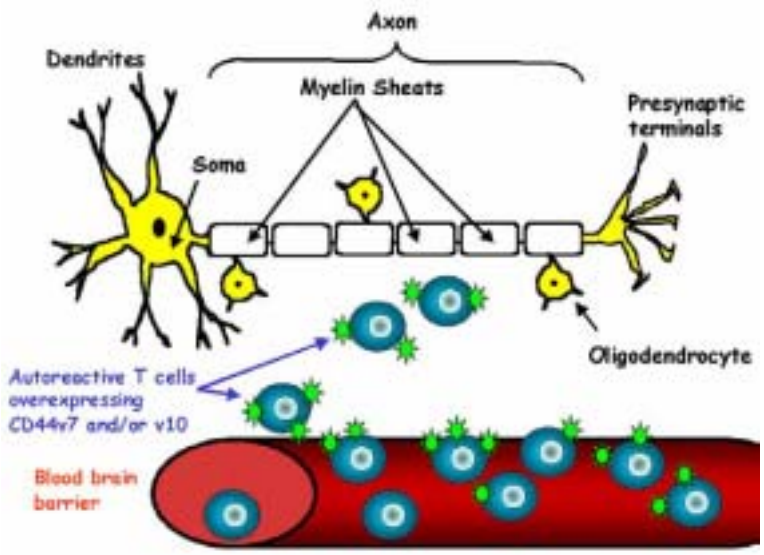

Figure 5. Model representing the implication of CD44v isoforms in MS and EAE. During an inflammatory response, the induction of adhesion molecules on endothelial cells and on the surface of leukocytes allows the extravasation process. Cells expressing CD44v isoforms are able to cross the blood brain barrier and are resistant to activation induced cell death, contributing to a persistent inflammation.

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Wanted: dead or alive?

In this thesis we could deepen our knowledge about the implication of CD44 in programmed cell death. However regulation of the homeostatic balance is very complex and Nature still keeps many secrets with jealousy. Understanding completely the process of apoptosis requires multidisciplinary approaches that combine: cellular biology to identify all the molecular players; functional genomics to select the most important targets; structural biology to predict protein structure and interactions; computational pharmacology to design the best possible inhibitors; and animal models to increase the chances of success of drug candidates in the clinic. Combining the contributions of all these disciplines, we might one day be able to control and decide if we want a cell to die or to live, but until then, try to enjoy and appreciate your healthy life!

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EDUCATION

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EXPERIENCE

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- **May 2004** **Obtention of the Paul Basset Prize for young scientists.**
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- **June 2000** **Obtention of the grant "Introducción a la investigación".**
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PUBLICATIONS

- A novel anti-apoptotic mechanism based on Fas sequestration by CD44 variant isoforms.
  Ainhoa Mielgo, Marjolein van Driel, Lukas Landmann, Ursula Günthert. (submitted).

- The complex CD44 standard/ezrin regulates Fas mediated apoptosis.
  Ainhoa Mielgo, Lukas Landmann, Ursula Günthert. (ready for submission).

- Fas-ligand gene silencing in basal cell carcinoma tissue with small interfering RNA. Ji J, Marion Wernly, Ainhoa Mielgo, Stanislaw A. Buechner, Peter Erb.
  Gene Therapy, 2004.

- Involvement of specific CD44 variant isoforms in multiple sclerosis and experimental autoimmune encephalomyelitis.
  Lizette Visser, Britt Johansson, Ainhoa Mielgo, Debby van Riel, Marie-José Melief, Marjan van Meurs, Jon D. Laman, and Ursula Günthert (in preparation).

Meetings

- 11th Euroconference on Apoptosis. Ghent, Belgium, 2003.
  Poster presentation: CD44 variant isoforms can block Fas-mediated apoptosis.

- SPO (Schwerpunkt Onkologie) meeting. Augst, Switzerland, 2003.
  Oral presentation: CD44 variant isoforms confer resistance to Fas-mediated apoptosis.

- Swiss meeting for PhD students in Immunology. Wolfsberg, Switzerland, 2004.
  Oral presentation: CD44 variant isoforms can block Fas-mediated apoptosis.

- USGEB meeting. Fribourg, Switzerland, 2004.
  Poster presentation: CD44 variant isoforms can block Fas-mediated apoptosis.

- EuCC (European Cancer Center) meeting, Alsace, France, 2004.
  Oral presentation: A novel anti-apoptotic mechanism based on Fas sequestration by CD44 variant isoforms. Awarded with a prize offered by the ATGC (Alsace Thérapie Génique et Cancers).