CD44 as a Hematopoietic stem cell marker

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“Never give up on what you want to do. A person with real big dreams is more powerful than all the facts”

To my loving parents and my family….

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Abbreviations

Ab: Antibody

Ag: Antigen

ALDH: Aldehyde dehydrogenase

BMC: Bone marrow cell

BMP: Bone morphogenetic protein

BMT: Bone marrow transplantation

BSA: Bovine serum albumin

CD: Cluster of differentiation

CD44s: CD44 standard

CD44v: CD44 variant

CFSE: Carboxyfluorescein succinimidyl ester

CK2: Casein kinase-2

CLP: Common lymphoid progenitor

cm: Centimeter

CMP: Common myeloid progenitor

CXCR4: Chemokine receptor-4

d: Day

ECM: Extra cellular matrix

EGF: Epidermal Growth Factor

ERM: Ezrin, Radixin, Moesin

ES: Embryonic stem cells

FACS: Fluorescent activated cell sorting

FAK: Focal adhesion kinase

FCS: Fetal calf serum

FGF: Fibroblast growth factor

Flt-3: fms-like tyrosine kinase receptor-3

GAGs: Glycosaminoglycans

G-CSF: Granulocyte-colony stimulating factor

GM-CSF: Granulocyte macrophages -colony stimulating factor

GVDH: Graft vs host disease

HA: Hyaluronic acid

HEK: Human embryonic kidney

HLA: Human leukocyte antogen

hr: Hour

HSC: Hematopoietic stem cell

i.v.: Intravenous

ICAM: intracellular adhesion moleclue

Ig: Immunoglobulin

IL: Interleukin

IP: Immunoprecipitation

JNK: Jun kinase

LFA: Leukocyte function antigen

Lin: Lineage

LSC: Leukemic stem cell

LTBMC: Long-term bone marrow culture

M: Molar

MAP Kinase: Mitogen activated protein kinase

MDR: Minimal drug resistance

MHC: Major histocompatibility

MIP: Macrophage inflammatory protein

ml: Millilitre

MMP: Matrix metalloproteinase

MPB: Mobilized peripheral blood

MPP: Multipotent progenitor

ng: Nanogram

NK: Natural killer

PAGE: Polyacrylamide gel electrophoresis

PAK2: p21-active kinase 2

PBS: Phosphate buffer saline

PI: Propidium iodide

PIP2: Phosphatidyl inositol 3,4 bisphosphate

PKC: Protein kinase C

PP: Protein phosphatase

PTK: Phoshp tyrosine kinase

Rb: Retinoblastoma

RPM: Revolution per minute

RTK: Receptor tyrosine kinase

RT-PCR: Reverse transcriptase polymerase chain reaction

s.c.: Sub cutaneous

SCA: Stem cell antigen

SCF: Stem cell factor

SD: Standard deviation

SDF-1: Stromal cell-derived factor-1

SLAM: Signalling lymphocyte activation molecule

Sm7: Soluble molecule CD44v7

Sms: Slouble molecule CD44 standard

TCR: T cell receptor

TGF: Tumor growth factor

TNF: Tumor necrosis factor

UCB: Umbilical cord blood

VEGF: Vascular endothelial growth factor

VLA: Very late antigen

w/v: weight/volume

WB: Western blot

Zap-70: Zeta associated protein

%: Percentages

˚C: Degree Celcius

µg: Micro gram

Zusammenfassung

Die CD44 Expression auf hämatopoetischen Stammzellen (HSC) ist essentiell für das Homing in die osteogene Nische und die Aufrechterhaltung der Quieszenz. Es ist nicht bekannt, welche CD44-Isoform, die CD44-Standardform oder varianten Isoformen (CD44s und CD44v), nötig ist. Ebenso wurde die mögliche Rolle des auf Stromazellen exprimierten CD44 bis jetzt nicht untersucht.

Eine Analyse der CD44 Expression auf HSC und Stromazellen, die aus langzeit-kultiviertem Knochenmark generiert wurden (Long Term Bone Marrow Culture-stroma cells; LTBMC-stroma cells) zeigte eine dominante CD44s Expression in $CD117^{+}SCA1^{+}$ HSC, während CD44v6, CD44v7 und CD44v10 niedrig exprimiert waren. Die LTBMC-Stromazellen zeigten überwiegend CD44v7 Expression.

Des weiteren wurden Adhäsionsmoleküle wie CD49d und CD54, einige wichtige Vorläufermarker wie CD16, CD24 und Cytokinrezeptoren wie CD184 (SDF-1 Rezeptor) ebenfalls auf HSC und LTBMC-Stroma gefunden. Die CD44-Moleküle auf HSC und LTBMC-Stroma zeigen distinkte Komplexbildungen. In beiden Zellpopulationen co-immunoprezipitiert CD44 mit CD49d und CD54, während CD44v7 im LTBMC-Stroma ausschließlich mit CD49d assoziiert. Funktionelle Studien deuten daraufhin, dass die Interaktion des CD44-CD49d-Komplexes auf HSC mit der Nische für deren Quieszenz verantwortlich ist. Des Weiteren spielt CD44 auf HSC durch eine erhöhte Expression anti-apoptotischer Proteine eine wichtige Rolle bei der Apoptoseresistenz. Diese Funktionen sind durch die koordinierte Interaktion beider Zellpopulationen möglich.

Da HSC und LTBMC-Stroma über CD44 interagieren, kann CD44 möglicherweise auch die Interaktion von HSC versus leukämische Zellen (LSC) mit der osteogen Nische gezielt beeinflussen. Tatsächlich wird durch die Blockade von CD44 nicht nur das Homing der hämatopoetischen Stammzellen

und der leukämischen Zellen verhindert, sondern es wurde beschrieben, dass in leukämischen Zellen Differenzierung und Apoptose ausgelöst wird.

Eine Analyse der CD44-Blockade auf das Wachstum der T-Lymphomazelllinie EL4 zeigte ein verlangsamtes Wachstum *in vivo* und erhöhte Apoptose der leukämischen Zellen. In mit Knochenmarkszellen rekonstituierten Mäusen induzieren anti-CD44-Antikörper Apoptose in T-Vorläuferzellen stärker als in Tumorzellen, so dass die Überlebenszeit der Mäuse verkürzt und die Metastasierung erhöht ist.

In vitro, führt die CD44-Blockade zu einer 2-4-fach erhöhten Apoptoserate in EL4-Zellen. Die Expression der „Todesrezeptoren“ CD95, TRAIL, und TNFRI und auch die durch CD95-Aktivierung ausgelöste Apoptose bleibt unverändert: Anstatt dessen wird die Apoptose durch eine Depolarisierung der Mitochondrien ausgelöst, welche mit der Spaltung der Caspase-9 einhergeht und durch einen Caspase-9-Inhibitor gehemmt wird. Die Apoptose wird durch die Aktivierung der CD44-assoziierten Phosphatase 2A (PP2A) initiiert und über ERK1/2-Dephosphorylierung weitergeleitet, ohne ERK1/ 2 abzubauen.

Das bedeutet, dass anti-CD44-Antikörper leukämische T-Zellen über den mitochondrialen Weg durch die Assoziation von CD44 mit PP2A, in Apoptose treiben.

1: Introduction

1. Stem cells and Hematopoietic stem cells:

It has long been known that stem cells are capable of renewing themselves and that they can generate multiple cell types. Efforts are now underway to harness stem cells and to take advantage of this new found capability, with the goal of devising new and more effective treatments for a host of diseases and disabilities. The stem cells that form blood and immune cells are known as hematopoietic stem cells (HSC). They are ultimately responsible for the constant renewal of blood—the production of billions of new blood cells each day.

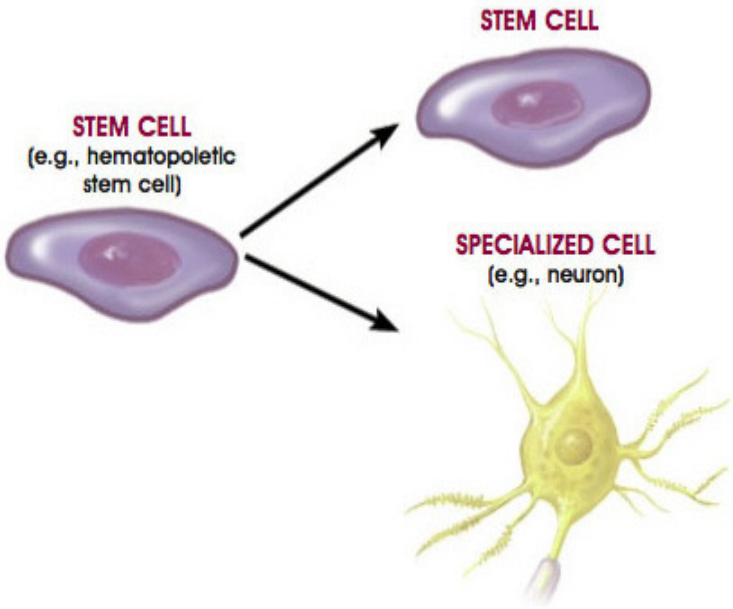
1.1 Definition of stem cells:

A stem cell is an unspecialized cell that is capable of replicating or self renewing itself and developing into specialized cells of a variety of cell types. The product of a stem cell undergoing division is at least one additional stem cell that has the same capabilities of the originating cell.

The critical balance between stem and differentiated cell populations is crucial for the long term maintenance of functional tissue types. Stem cells maintain this balance by choosing one of several alternate fates: self-renewal, commitment to differentiate, and senescence or cell death. These characteristics comprise the core criteria by which these cells are usually defined. The self-renewal property is important, as it allows for extended production of the corresponding differentiated cells throughout the life span [1, 2].

The best example of a stem cell is the bone marrow stem cell that is unspecialized and able to specialize into blood cells, such as white blood cells and red blood cells, and these new cell types have special functions, such as being able to produce antibodies, act as scavengers to combat infection and transport gases. Basically, a stem cell remains uncommitted until it receives a signal to develop into a specialized cell. Stem cells serve as a repair system by being able to divide without limit to replenish other cells.

Today, stem cells have been isolated from preimplantation embryos, fetuses, adults and the umbilical cord and under certain conditions, these undifferentiated stem cells can be pluripotent (ability to give rise to cells from all three germ layers, viz. ectoderm, mesoderm and endoderm) or multipotent (ability to give rise to a limited number of other specialized cell types).

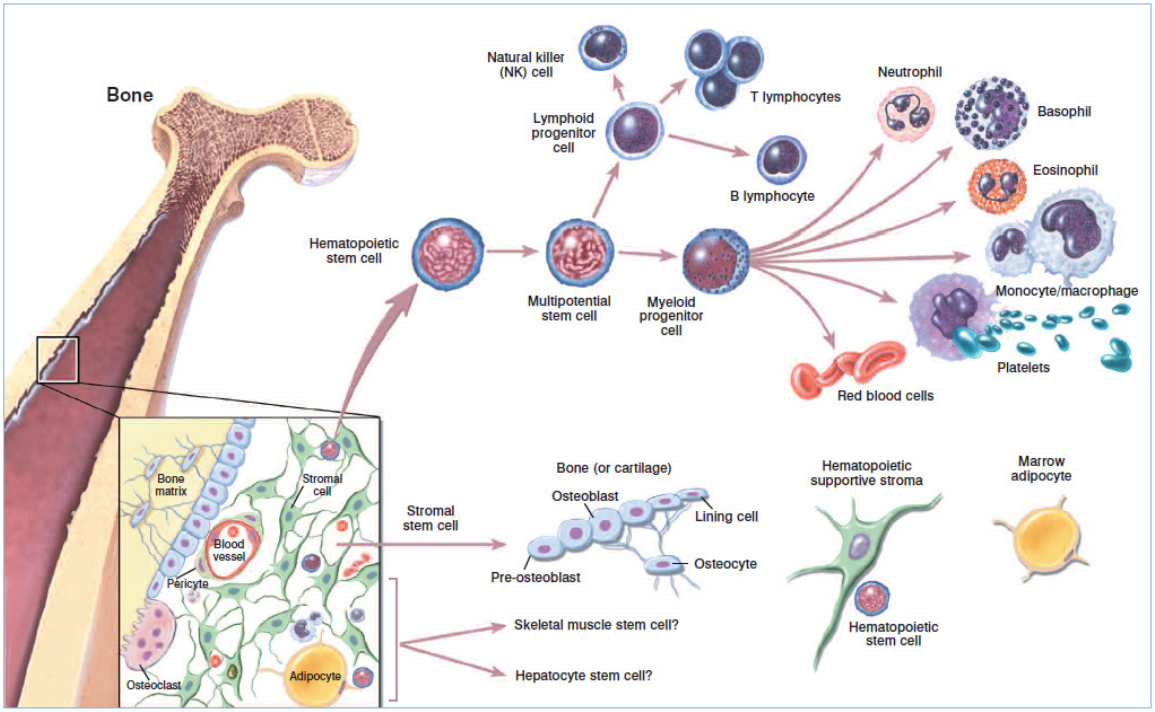


Scheme 1: Schematic representation of stem cells and differentiation [2]

1.2 Hematopoitic stem cells and Hematopoiesis

1.2.1 Hematopoitic stem cells:

A hematopoietic stem cell is a cell isolated from the blood or bone marrow that can renew itself, can mobilize out of the bone marrow into circulating blood, and can undergo programmed cell death, called apoptosis—a process by which cells that are detrimental or unneeded self-destruct.



Scheme 2: Hematopoietic and stromal cell differentiation. (stem cell information at stemcells.nih.gov)

All of the mature blood cells in the body are generated from a relatively small number of hematopoietic stem cells (HSC) and progenitors [3, 4]. Murine models, particularly short- and long-term transplant studies, have provided a number of insights into the biology of HSC and progenitors [5, 6]. The results of these studies have demonstrated that HSC are able to generate every lineage found in the hematopoietic system including red blood cells, platelets, and a variety of lymphoid and myeloid cells [3-6]. Some of the most important

lymphoid cells include natural killer (NK) cells,T cells, and B cells, while important myeloid cells include granulocytes, monocytes, macrophages, microglial cells, and dendritic cells [7]. Each of these cell types can be generated from a single HSC, and each HSC has an enormous capacity to generate large numbers of these cells over many years and perhaps even decades. In the mouse, a single HSC can reconstitute the entire hematopoietic system for the natural lifespan of the animal [8]. HSC are detected in the bone marrow at a frequency between 1: 10^7 - 10^8 [9]. While HSC are primarily found in the bone marrow, they are present in a variety of other tissues including peripheral blood and umbilical cord blood, and are found at low numbers in the liver, spleen, and perhaps many organs [10]. These HSC may have somewhat different properties, but they all have the ability to generate all the different blood lineages in large numbers for a prolonged period of time.

Downstream of the common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) are more mature progenitors that are further restricted in the number and type of lineages that they can generate [11, 12]. When a bone marrow or blood stem cell transplant is performed, it appears that progenitors contribute to engraftment for only a short period of time, while long-term blood production is derived primarily from HSC [13].

1.2.2 Hematopoiesis

The process of hematopoiesis involves a complex interplay between the intrinsic genetic processes of blood cells and their environment. This interplay determines whether HSC, progenitors, and mature blood cells remain quiescent, proliferate, differentiate, self-renew, or undergo apoptosis.[14-16] All of the genetic and environmental mechanisms that govern blood production operate by affecting the relative balance of these fundamental cellular processes. Under normal conditions, the majority of HSC and many progenitors are quiescent in the G0 phase of the cell cycle; however, many of the more mature progenitors are proliferating and producing mature offspring [17]. In the absence of any stresses, this is balanced by the rate of apoptosis in progenitors and mature cells [15]. In the event of a stress such as bleeding or infection, several processes occur. Stored pools of cells in the marrow or adherent to the endothelium are quickly released into the circulation in order to localize to the site of injury [18]. Fewer progenitors and mature cells undergo apoptosis [19, 20]. In addition, quiescent progenitors and HSC are stimulated by a variety of growth factors to proliferate and differentiate into mature white cells, red blood cells, and platelets. When the bleeding, infection, or other underlying stress ceases and the demand for blood cells returns to normal, the antiapoptotic and proliferative processes wind down, blood cells are redistributed back to their storage sites, and the kinetics of hematopoiesis return to baseline levels. This process repeats itself innumerable times during the lifespan of an individual and is seen in an exaggerated form following chemotherapy or bone marrow transplantation.

Probably the best characterized environmental regulators of hematopoiesis are cellular microenvironment, known as niche. It functions as an extrinsic regulatory system, which maintains and governs the location, adhesiveness, retention, homing, mobilization, quiescence/activation, symmetric/asymmetric division and differentiation. [21]

Cytokines are a broad family of proteins that mediate positive and negative affects on cellular quiescence, apoptosis, proliferation, and differentiation. In general, cytokines function by engaging a specific receptor and activating a

variety of signaling pathways. This includes activation of tyrosine kinases such as focal adhesion kinase, pp60src, and c-Abl, MAP kinases, jun Kinase (JNK), and protein kinase C (PKC) [22]. Mediators of cell growth and differentiation such as c-src, phosphoinositides, protein kinase C, and growth factor-mediated signaling pathways are also modulated by cytokines. Cytokines including interleukin-3 and GM-CSF induce cell proliferation, while other cytokines including flt-3 ligand and kit ligand protect cells from apoptosis and sensitize them to the effects of growth promoting cytokines [23-25]. Cytokines may also facilitate the interactions between stem cells and elements in the microenvironment including extracellular matrix (ECM) components [26]. Regulators of HSC including transforming growth factor-beta (TGF-β) and tumor necrosis factor-alpha (TNF-α) modulate cell cycle activity and engraftment [27]. Some cytokines including Wnt and the notch ligand family may also have important effects on stem cell biology [28, 29]. Some cytokines, including TNF-α, may be either inhibitory or activating depending on their concentration and other ongoing physiologic processes [30]. Several known cytokines, such as kit ligand, exist in either a soluble or membrane-bound form and have different activities depending on whether they are bound or soluble and on the environmental context in which they are acting [25]. Hematopoietic regulatory cytokines are produced through both autocrine and paracrine mechanisms and in many cases are produced by non-hematopoietic cells including bone marrow stroma and endothelium.

Chemokines are another class of compounds that are important regulators of hematopoiesis [31-33]. These molecules regulate blood cell trafficking and homing to sites of need and may also be negative and positive growth regulators [34]. Chemokines are composed of a large family of proteins that mediate a variety of processes including inflammation, leukocyte migration and development, angiogenesis, and tumor cell growth and metastasis. Chemokines bind to one or more of a large family of structurally related guanine protein-coupled transmembrane receptors. In hematopoiesis, chemokines inhibit progenitor growth, regulate migration of hematopoietic progenitors. For example, the chemokine SDF-1 (which binds the receptor CCXR4) is essential for trafficking of hematopoietic cells in the developing embryo, mediating

homing of HSC and progenitors to the bone marrow following transplantation and, in stem cell mobilization, for collecting peripheral blood stem cells for transplant purposes [31]. A number of other chemokines likely play important roles in hematopoiesis and are under active investigation.

HSC and progenitors bind tightly to a number of ECM components including heparin sulfates, chemokines, collagens, laminin, thrombospondin-1, fibronectin, and others. These molecules provide a scaffold for colocalizing progenitors and HSC with a wide array of positive and negative cytokines and other growth regulators. In addition, ECM and stromal components may directly mediate signaling to HSC to activate growth, protect cells from apoptosis, or modulate responses to positive and negative regulatory factors. The adhesion molecules on HSC and progenitors that mediate binding to these ECM components include CD44, integrins, selectins, and mucins. Adherence of cells to microenvironmental elements can trigger a variety of signaling pathways and can lead to changes in intracellular ions such as proton (pH), calcium, and the small GTPase Rho as well as lipid mediators such as phosphoinositides, diacylglycerol, and arachidonic acid metabolites [35]. Adhesion may also regulate expression of immediate-early genes such as c-fos and key cell cycle events such as kinase activity of cyclin-cdk complexes and phosphorylation of the retinoblastoma (Rb) protein [36]. Cell adhesion may potentiate the responses to growth factors and by modulating the downstream components of growth factor signaling cascades including PI-3 kinase, AKT, and p70rsk [37]. Hematopoietic and non-hematopoietic cells that may regulate hematopoiesis include NK cells, T cells, macrophages, fibroblasts, osteoblasts, adipocytes, and perhaps even neurons [38, 39]. These cells may produce important growth factors, facilitate engraftment, or induce apoptosis. A number of nutrients, trace elements, and vitamins (zinc, selenium, copper, vitamins A, D, and E) are also critical to hematopoiesis. Retinoids and particularly retinoid antagonists play important roles in differentiation at even low concentrations.

1.3 Characterization of HSC:

HSC have an identity problem. First, the ones with long-term replicating ability are rare. Second, there are multiple types of stem cells and third, the stem cells look like many other blood or bone marrow cells. The most common approach is through markers that appear on the surface of cells. These are useful, but not perfect tools for the clinic and research laboratory [40, 41].

Phenotypically, murine HSCs are small cells with minimal cytoplasm, and they express high levels of the multidrug resistant (MDR) proteins and high levels of aldehyde dehydrogenase (ALDH) [42, 43].

CD34 mainly expressed on a small fraction of human bone marrow cells. The $CD34^+$ enriched cell population from marrow or mobilized peripheral blood appears responsible for most of the hematopoietic activity. CD34 has therefore been considered to be the most critical marker for hematopoietic stem cells (HSC).

$SCA\text{-}1^+$ (Stem cell antigen 1, Ly-6A/E) and $C\text{-}kit^+$ (CD117) are hematopoietic marker for murine HSC. SCA-1 is the most recognized HSC marker in mice with both Ly-6 haplotypes as it is expressed on multipotent HSC. $Sca\text{-}1^+$ HSC can be found in the adult bone marrow, fetal liver and mobilized peripheral blood and spleen within the adult animal. CD117 is a cytokine receptor expressed on the surface of hematopoietic stem cells. CD117 is an important cell surface marker used to identify certain types of hematopoietic (blood) progenitors in the bone marrow. It is also a marker for mouse prostate stem cells.

The hematopoietic stem cells are negative for the markers that are used for detection of lineage commitment, and are, thus, called Lin^-; and, during their purification by FACS, a bunch of up to 14 different mature blood-lineage marker, e.g., CD13 & CD33 for myeloid, CD71 for erythroid, CD19 for B cells, CD61 for megakaryocytic, etc. for humans; and, B220 (murine CD45) for B cells, Mac-1 (CD11b/CD18) for monocytes, Gr-1 for granulocytes, Ter119 for erythroid cells, CD3, CD4, CD5, CD8 for T cells etc. (for mice) antibodies are used as a mixture to deplete the lin^+ cells or late multipotent progenitors (MPP).

Thy-1 can be considered as a surrogate marker for hematopoietic stem cells. It is one of the popular combinatorial surface markers for FACS for stem cells in combination with other markers like CD34. During sorting, mouse HSC fall under $Thy1^{+}$/low subpopulation, while human HSC fall under the $Thy1^{+}$ subpopulation. In humans, Thy-1 is only expressed on neurons [44]. Thy-1 is also a marker of other kind of stem cells, for example: mesenchymal stem cells, hepatic stem cells ("oval cells"), [45] keratinocyte stem cells [46].

There are many differences between the human and mice hematopoietic cell markers for the commonly-accepted type of hematopoietic stem cells.

Mouse HSC: $CD34^{lo/-}$, $SCA\text{-}1^{+}$, $Thy1.1^{+/lo}$, $CD38^{+}$, $C\text{-}kit^{+}$, lin^{-}

Human HSC: $CD34^{+}$, $CD59^{+}$, $Thy1/CD90^{+}$, $CD38^{lo/-}$, $C\text{-}kit/CD117^{+}$, lin^{-}

1.4 The importance of the HSC niche:

Key properties of stem cells such as their self-renewal and developmental capacity can be controlled in a non autonomous manner by their cellular microenvironment. Such a microenvironment is usually referred to as a stem cell niche. A niche is a group of cells that allows a stem cell to maintain its identity [21]. The cells of a niche will prevent a previously specified cell from losing its stemness through loss of quiescence and potency or precocious differentiation. In the best demonstrations of a niche, a specific signaling pathway or a cell adhesion molecule is identified that allows the niche cells to maintain contact with stem cells and typically in the absence of such a mechanism, the stem cells leave their niche and either divide, differentiate, or apoptose [21].

The niche and the stem cells may arise from the same progenitor population, as is the case of the origin of HSC and endothelial cells of the dorsal aorta, which share a common progenitor in their ancestry [47], and the placenta in mice [48].

A niche could be derived completely separately from the stem cell, as is the case for the bone marrow hematopoietic niche, which utilizes signals derived from osteoblasts and mesenchymal stromal cells, both of which, although mesodermal, have different developmental origins from the HSC [49].In the mouse adult bone marrow, N-cadherin is expressed in the HSC and the spindle-shaped osteoblastic cells of the niche [50]. Mammalian hematopoiesis gives rise to long-term reconstituting HSC that, in turn, generate short-term repopulating HSC [51]. From these stem cells a number of more restricted progenitors emerge that give rise to all differentiated blood cells in adult circulation, such as lymphoid, myeloid, and erythroid cells [52]. Each of these progenitors can be distinguished by a subset of cell surface markers. Development of the initial definitive HSC requires Runx-1 [53], and its expression later continues in differentiating myeloid and lymphoid cells [54]. Later inactivation of Runx1 within the bone marrow is not essential for adult hematopoiesis, but it does affect maturation of lymphocytes and platelets [55].

Osteoblast-specific loss of BMP-receptor 1 in mouse causes an increase in the number of osteoblasts and also a concomitant growth in the HSC pool [50]. The high calcium concentration present in the osteoblast niche is essential for homing and retention of HSC to this niche and for this purpose, HSC express the calcium-sensing receptor that enables them to respond to the calcium signal [56]. Osteopontin, produced by osteoblasts in response to various stimuli and secreted into the extracellular matrix is another important regulator of the number of HSC. Low levels of osteopontin lead to an increase in the number of HSC, suggesting that it is a negative regulator of stem cell number [57]. Conversely, targeting the osteoblasts with a suicide-gene causes metabolic death of the osteoblasts and also perturbs hematopoiesis [58]. Likewise, stimulation of the parathyroid receptor by overexpression of PTH–PTHRP results in an increase in both the trabecular bone and the number of HSC [59]. Taken together these results identified the osteoblast as an integral component of the hematopoietic niche. More recently, a second niche, in relation to endothelial cells, has been identified and called the vascular niche [60, 61]. HSC interact with endothelial cells via signaling lymphocyte activation molecule (SLAM) receptors on sinusoidal cells both in the bone marrow as well as the spleen [62]. The current view is that perhaps the osteoblastic niche provides a quiescent environment for HSC maintenance while the vascular niche provides the environment required for differentiation and mobilization.

The microenvironment in the stem cell niche is composed of cell-to-cell interactions via cell-surface molecule expression, chemokines and growth factors. This orchestra determines the fate of the cell(s) within the niche. The list of molecular mediators that regulate the HSC is increasing and includes angiopoietin-1/Tie-2, SDF-1 (CXCL12)/CXCR4, very late antigen 4 (VLA-4), leukocyte function antigen 1 (LFA-1), FGF-4, VEGF, SCF/c-kit, Wnt, N-cadherin and indirectly G-CSF, GM-CSF, FLT-ligand and possibly many others [63, 64, 65].

It has been proposed that adhesive contacts through N-cadherin and Tie2/angiopoietin interactions hold the HSCs anchored to the endosteal niche promoting HSC quiescence [63]. C-myc has been shown to be an important cell intrinsic regulator required for HSC homeostasis regulating the release of HSC

from the quiescence-promoting niche. Upon conditional ablation of c-myc in bone marrow HSC, these stem cells are unable to differentiate as they increase adhesion molecules on their surface and remain anchored to the niche that retains them in a quiescent, undifferentiated state [66]. Although the osteoblast endosteal niche of the mouse bone marrow is so far the best characterized hematopoietic niche, the majority of the HSC that engraft in transplantation assays actually localize in perivascular spaces, in contact with sinusoidal venous endothelium [67], leading to the speculation that these surfaces may provide additional niche-like interactions for the maintenance of adult HSC.

1.5 The clinical demand on HSC:

Bone Marrow Transplantation (BMT) is a life saving procedure for a number of malignant and non-malignant life threatening diseases [68]. Ever since the first successful BMT in the late 1960s, an increasing number of BMT and related procedures are being performed world wide. Allogeneic stem cell (unrelated human leucocyte antigen, HLA identical donor) transplantation is now a curative treatment modality for a number of disorders, including malignant diseases, dyserythropoiesis, bone marrow aplasia, immunodeficiency states, and a number of inherited disorders [69].

The indications for hematopoietic stem cell transplantation [69] can be conveniently divided into two groups: (a) Malignant disorders: like leukemias, lymphomas, multiple myeloma and solid tumors like breast cancer, testicular cancer. In all these indications, the cure or palliation is by the high doses of chemotherapy or radiation therapy, while the transplant serves to rescue the patient from the myelotoxic effects of the anti-cancer therapy. In allogeneic type of transplants, there is an additional immunological advantage of graft vs cancer effect, which contributes to the disease relief; (b) Non-malignant diseases: like aplastic anemia, thalassemia, Gaucher's disease, etc. In these conditions the abnormal marrow is deliberately destroyed and replaced by the healthy donor marrow. In this setting autologous (patient's own stem cells) transplantation cannot be effective for obvious reasons.

Bone marrow and stem cell transplantation is curative in many potentially fatal conditions. The graft vs tumor effect seen after allogeneic transplants for human malignancies represents the clearest example of the power of the human immune system to eradicate cancer [70, 71]. It is likely that in future the high dose preparative regimens used in allogeneic bone marrow and stem cell transplants will be replaced with less toxic therapy leading to a safer transplant procedure. Autologous transplants are mainly done for lymphomas, solid malignancies like neuroblastoma and germinal cancers, hematological malignancies like multiple myeloma [72], and in patients with acute leukemia who do not have an HLA identical donor. The advantage of autologous transplant over allogeneic transplant is that there is no graft vs host disease (GVHD), and once engraftment occurs then graft rejection is unlikely. Thus there is a significant decrease in the complication rate as compared to the allogenic transplantation; however risk of tumor relapse is higher as compared to the allogeneic transplantation.

1.6 Sources of HSC:

1.6.1 Bone Marrow and Mobilized Peripheral Blood

The best-known location for HSC is bone marrow, and bone marrow transplantation has become synonymous with hematopoietic cell transplantation.

In adults, under steady state conditions, the majority of HSC reside in bone marrow. However, cytokine mobilization can result in the release of large numbers of HSC into the blood. As a clinical source of HSC, mobilized peripheral blood (MPB) is now replacing bone marrow, as harvesting peripheral blood is easier for the donors than harvesting bone marrow.

1.6.2 Umbilical Cord Blood

In the late 1980s, umbilical cord blood (UCB) was recognized as an important clinical source of HSC [73, 74]. Blood from the placenta and umbilical cord is a rich source of hematopoietic stem cells, and these cells are typically discarded

with the afterbirth. Increasingly, UCB is harvested, frozen, and stored in cord blood banks, as an individual resource (donor-specific source) or as a general resource, directly available when needed. Cord blood has been used successfully to transplant children and (far less frequently) adults. Specific limitations of UCB include the limited number of cells that can be harvested and the delayed immune reconstitution observed following UCB transplant, which leaves patients vulnerable to infections for a longer period of time. Advantages of cord blood include its availability, ease of harvest, and the reduced risk of graft-versus-host-disease (GVHD). In addition, cord blood HSC have been noted to have a greater proliferative capacity than adult HSC.

1.6.3 Embryonic Stem Cells

Embryonic stem (ES) cells form a potential future source of HSC. Both mouse and human ES cells have yielded hematopoietic cells in tissue culture [75]. However, recognizing the actual HSC in these cultures has proven problematic, which may reflect the variability in HSC markers or the altered reconstitution behavior of these HSC, which are expected to mimic fetal HSC. This, combined with the potential risks of including undifferentiated cells in an ES-cell-derived graft means that, based on the current science, clinical use of ES cell-derived HSC remains only a theoretical possibility for now.

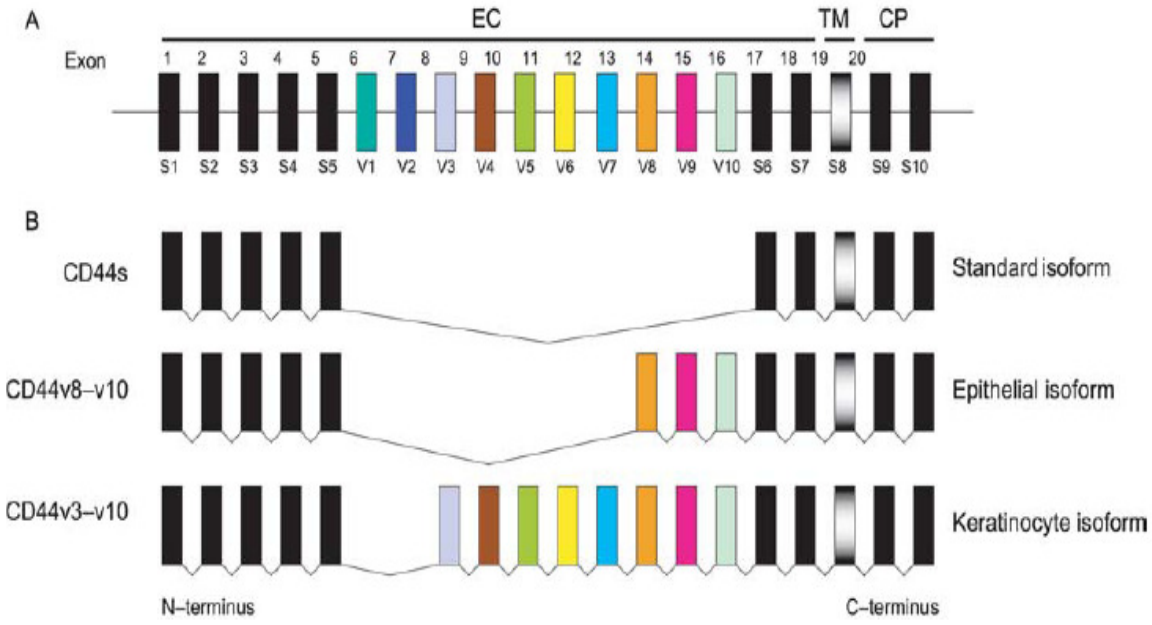
2. CD44 Molecule

CD44 is the most important molecule in the interaction between HSC and the osteogenic niche. The CD44 proteins form a ubiquitously expressed family of cell surface adhesion molecules that have a large extracellular domain, a transmembrane domain and an intracellular cytoplasmic domain. These molecules are termed adhesion since they bind strongly to specific ligands. This interaction is more complex in terms of sensing the extracellular environment and sending signals to adjacent cells. CD44, particularly its variants, may be useful as a diagnostic or prognostic marker of malignancy and, in at least some human cancers, it may be a potential target for cancer therapy [76].

CD44 glycoproteins are the major receptor to HA (Hyaluronic Acid) as the major ligand. HA is an extracellular polysaccharide present abundantly in extracelullar cell matrix (ECM) [77]. The CD44 epitope was first discovered as an antigen using a monoclonal antibody that was raised against human white blood cells [78]. It was then identified that a group of polymorphic proteins had this epitope in common (80-200kD in size).

2.1 Structure

CD44 is encoded by a single gene. It has 20 exons from which various protein products are further generated through alternative mRNA splicing. The standard form is most abundant and consists of an N-terminal signal sequence (exon 1), a hyaluron binding module (exon 2 and 3), a stem region (exon 4, 5, 16 and 17), a single transmembrane domain (exon 18) and a cytoplasmic domain (exon 20). Alternative exon splicing of CD44 involves variable insertions of combinations of exons from 6-15, also known as variants 1-10 into the stem region. These variant isoforms of CD44 are seen mostly in epithelial cells and upregulated during diseases [79]. Little attention has been paid to isoforms that included exon 19, because RT-PCR studies revealed such transcripts are of very low abundance.



Scheme 3: Structure of CD44 Molecule [80]

2.2 Hyaluron binding, amino terminal domain

In vitro studies have revealed that interaction of CD44 with hyaluronic acid (HA), collagen, laminin and fibronectin promote matrix dependent cell migration [78, 81]. However, there is no *in vivo* evidence. Apart from the hyaluron binding sites other sites have not been mapped precisely. A stretch of 90 amino acids in the amino terminal globular region form the link domain, that enables the binding of CD44 with HA and also other glycosaminoglycans (GAGs) [82, 83]. The affinity for GAGs depends on post translational modifications of CD44 such as glycosylations. More than one CD44 molecule binds to HA since only HA-derived oligosaccharides larger than 20 residues bind to CD44 [84].

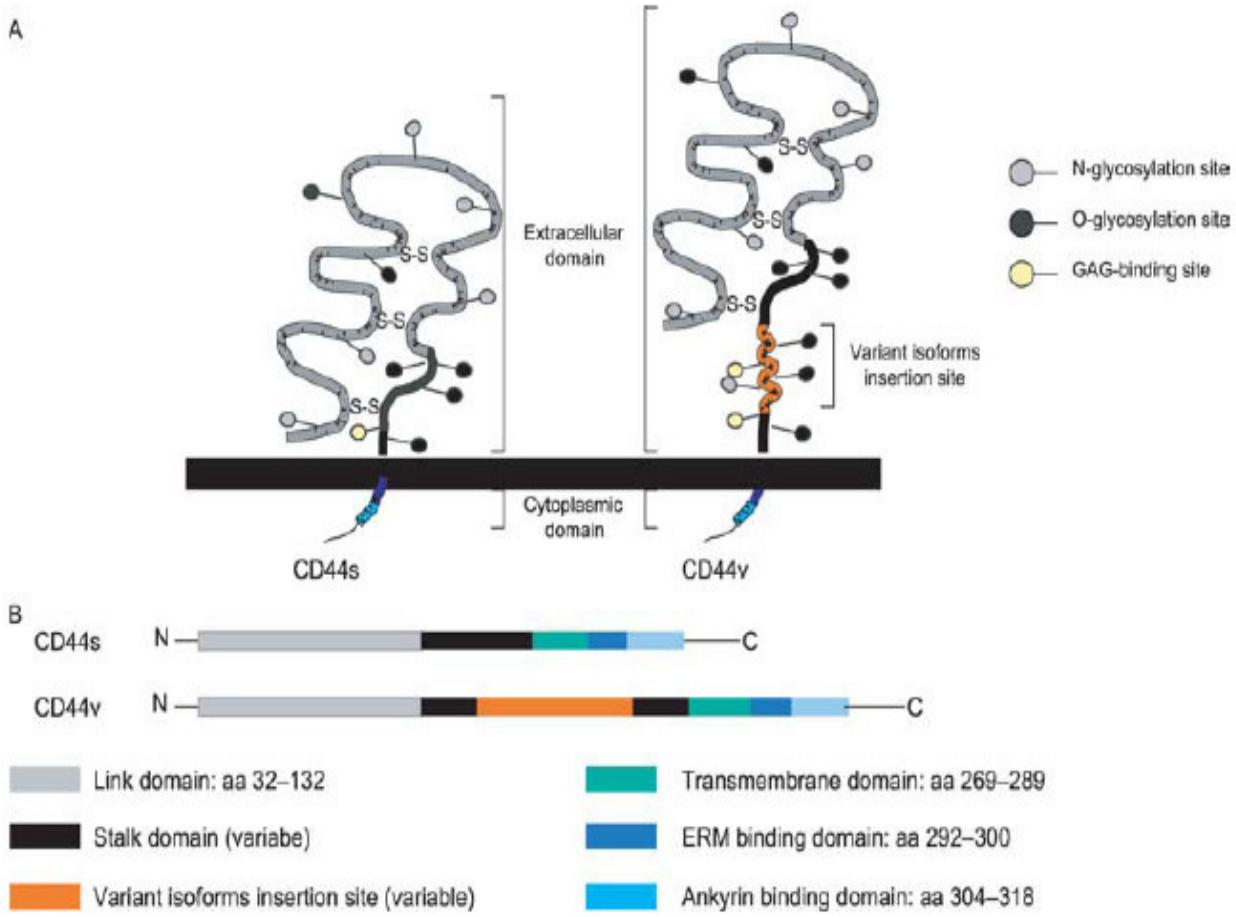
2.3 Stem structure

The amino terminal globular domain of the smallest CD44 isoform (CD44 standard) is separated from the plasma membrane by a 46 amino acids stem structure. It contains proteolytic cleavage sites. It also contains the variant exon region of CD44. The inclusion of several combinations of variant exons depends on mitogenic signals including stimulation of Ras-MEK-ERK pathway that regulate splicing events [85, 86] Some variant exon segments include motifs for post translational modifications but little is known about the exact structure of these variants-exon-encoded segments. [78]

2.4 Transmembrane and cytoplasmic domain

The transmembrane and cytoplasmic domain are required for efficient ligand binding [87]. The transmembrane domain consists of 23 hydrophobic amino acids and a cysteine residue (Cys_{286}) that seems to be involved in CD44 oligomer formation. The cysteine residue is also responsible for recruiting CD44 molecule into lipid rafts. Transmembrane region has also been proposed to be associated with distinct proteins in lipid rafts. However, the recruitment into lipid rafts is cell type specific. Some molecules like cadherins might be responsible for displacing CD44 from lipid rafts. The functional significance of this incorporation into lipid rafts is not well known [88].

The cytoplasmic domain is encoded by exon 19 or 20 and it has sites mediating interaction with ezrin, and also a putative ankyrin binding site. Intracellular association of CD44 with these cytoskeletal linker proteins could function in post-ligand binding events mediated by CD44. The cytoplasmic domain of CD44 may be phosphorylated at Ser_{325} and Ser_{291} residues [79], and this phosphorylation has been implicated in mediating cell migration on HA and in the interaction of CD44 with ezrin [89]. Intracellular partners of CD44 and motifs in the cytoplasmic domain are essential for subcellular localization for e.g. leading edge of lamellipodia of migrating cells as well as accessory functions of CD44 in signal transduction [78]. The cytoplasmic domain is known to promote Hyaluron internalization and thus migration of cells [90].



Scheme 4: CD44 domains [80]

2.5 CD44 Functions

CD44 is widely expressed in different tissue types and has structural diversity that enables it to interact with several signaling molecules and facilitate various functional outcomes such as adhesion, migration, homing, proliferation, cell survival and apoptosis. These functions are discussed in detail below.

2.5.1 Cell adhesion and migration

Cell adhesion and migration are crucial for leukocytes and tumor cells in case of inflammation and metastasis, respectively. CD44 accumulates during several processes such as angiogenesis, wound healing and migration of cells across an HA substrate. HA being one of the important components of the ECM and CD44 being its receptor, this suggests an obvious role for CD44 in cell migration and adhesion [78]. Studies in a melanoma line transfected with CD44 showed that these cells exhibit motility on HA coated surfaces. Accordingly, an isoform of CD44 that does not bind to hyaluronic acid is not able to promote migration on HA. In addition, antibodies blocking the HA binding site of CD44 were able to block the migration of these cells [87].

Leukocytes and metastasizing tumor cells need to migrate and localize in special tissues and microenvironments for their functions and nutrition in the case of tumor cells [91]. Rolling of cells is generally mediated by selectins and carbohydrate ligands where they promote leukocyte entry into sites of inflammation [92]. In addition it is also mediated by CD44 and HA interactions. These processes are mediated by CD44v4-v7 isoforms as well as by different glycosylation patterns of CD44. CD44 gets localized at the leading edge of the cell and in lamellipodia showing evidence for cytoskeletal anchoring of CD44 on an HA matrix. Metalloproteinase bound protein of type-1(MT1-MMP) is recruited into lamallipodia through CD44. MT1-MMP in turn cleaves CD44 and allows new CD44-HA interactions during which migration of cells takes place. This process is mediated through Ras, PI3K (phosphoinositide 3-OH kinase), cdc42/Rac and serine proteases might also support CD44-mediated cellular mobility by cleavage of the molecule [93-96].

Endothelial cells bind to HA through their CD44 molecules and proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1β (IL-1 β) stimulate CD44 thus strengthening the endothelial cell HA binding and mediating leukocyte extravasation. Leukocyte immigration into sites of inflammation and delayed type hypersensitivity reactions can be inhibited using antibodies specific for CD44. It has been proposed that IM7 (anti-CD44) binds to CD44 and reduces its expression on leukocytes and induces proteolytic cleavage of CD44 thus blocking migration. [97] Antigen presenting cells, Langerhans cells and dendritic cells up regulate CD44 variants v4, v5, v6 and v9 and these epitopes of CD44 are essential in binding to T cells in the lymph nodes. Antibodies against these isoforms of CD44 block the migration of these antigen presenting cells [98].

2.5.2 Interaction with the cytoskeleton

Reorganisation of the cytoskeleton is important for cell migration and other physiological processes. Since the cytoplasmic domain of CD44 does not have actin binding sites, CD44 binds to the actin cytoskeleton through cytoskeleton associated proteins ankyrin, ERM family of proteins (ezrin, radixin and moesin) and related protein merlin [99].

Ankyrin is a group of homologous proteins that is involved in segregation of integral membrane proteins that have their functional domain on the plasma membrane and is mainly involved in linking cytoplasmic domains of integral membrane proteins to spectrin-actin based membrane cytoskeleton. Ankyrin has several isoforms generated through alternative gene splicing and different gene products coding for the protein. This is how the protein can bind several membrane proteins and mediate diverse interactions with cytoskeleton [100]. CD44 binds to ankyrin cytoskeletal protein linking the plasma membrane to underlying cytoskeleton. CD44 gets phosphorylated by protein kinase C. These two post translation modifications enhance the binding of CD44 to ankyrin. The binding affinity is further enhanced through GTP binding proteins [101-103].

ERM are band 4.1 superfamily proteins acting as key linkers between transmembrane proteins and cytoskeleton. The ERM proteins have a 300 amino acid domain at the N-terminus, α-helical central region and a C-terminal domain which has the F-actin binding site. ERM proteins are activated by phosphorylation and by binding to membrane phospholipids. It is the phoshorylated (active) form of ERM that binds to CD44 [99]. ERM activation is regulated via Rho GTPase family. Binding of ROK, PKC and phosphatidyl inositol 4,5 bisphosphate (PIP2) to ERM proteins results in phosphorylation of these proteins [104-106]. Thr_{567} Ezrin, Thr_{564} Radixin and Thr_{558} Moesin phosphorylations result in rearrangement of the cytoskeleton [107]. CD44 binding to cytoskeleton is further regulated by its cytoplasmic domain. In resting cells CD44 is phosphorylated at Ser325. The switch from Ser325 phosphorylation to Ser291 is triggered via PKC and leads to a break in the association between ezrin and CD44. In addition, Ser291 phosphorylation is also involved in directional migration of cells. How exactly CD44 is phosphorylated and dephosphorylated is yet to be determined. But, CD44 binding to HA triggers various signaling pathways including activation of PKC. A good example is ezrin that associates with PKC and thus promotes phosphorylation of CD44 and regulates binding to the cytoskeleton [89].

Merlin is a member of 4.1 protein ERM family that has 65% homology to ERM proteins. Its activity is also regulated through phosphorylation and dephosphorylation [108]. Merlin does not have an actin binding site and merlin-CD44 complex cannot bind to cytoskeleton (Morrison, Sherman et al. 2001). The ability of ERM/merlin proteins to switch between phosphorylated and dephosphorylated forms along with the competition between ERM and merlin to bind to CD44 leads to making and breaking of CD44 interactions with the cytoskeleton [89]. When cells are in the growth phase phosphorylated ERM binds to CD44 mediating cytoskeletal interactions. During this time merlin gets phosphorylated by PAK2 (p21-active kinase -2) and is now unable to bind to CD44. During growth arrest the opposite is seen and MAPK activation is blocked [109, 110].

2.5.3 Interaction with cytokines, chemokines and enzymes

Apart from interacting with extracellular matrix (ECM), CD44 also interacts with extracellular proteins and harbors growth factors. CD44 molecules have two consensus sequences in exon 5 and variant 3 exon which allow increased binding of interferon-γ and osteopontin, thus suppressing proliferation and inducing MHC-II expression [111]. CD44v3 has been shown to immobilize chemokines such as osteopontin, scatter factor, endothelial growth factor, MIP-I β and RANTES, reviewed in [80].

Cytokines play an essential role in regulating hematopoiesis that is regulated by interaction between bone marrow microenvironment and progenitor cells. Antibodies against CD44 v4 and v6 stimulate the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) [112]. Extracellular matrix degrading enzymes are required during tissue remodeling, wound healing, angiogenesis and tumor progression. CD44 plays a role by harboring matrix metalloproteinases such as MMP2 and MMP9 at the cell surface. CD44 monoclonal antibody has been shown to upregulate the expression of MMP-2 which would have implications in tumor metastasis, growth and lymphocyte function. [113]

CD44 binds to the chemokine, osteopontin. CD44 binding to osteopontin results in downregulation of the anti-inflammatory cytokine IL-10 and survival of cells by IL-3 and GM-CSF cytokines. These two cytokines share a common beta subunit and the distal cytoplasmic tail participates in cell survival. Binding to osteopontin also activates PI3K and Akt and thus is involved in the survival pathway of IL-3 [114].

CD44v3 heparan sulphate proteoglycan and v6 bind to epidermal growth factor, heparin binding epidermal growth factor and fibroblast growth factor and present in an efficient way to their receptors for survival and proliferation of leukocytes and tumor cells. One example is CD44v3 heparan sulphate proteoglycan that recruits proteolytically active MMP-9 and heparin binding epidermal growth factor precursor (pro-HB-EGF) that in addition activates its

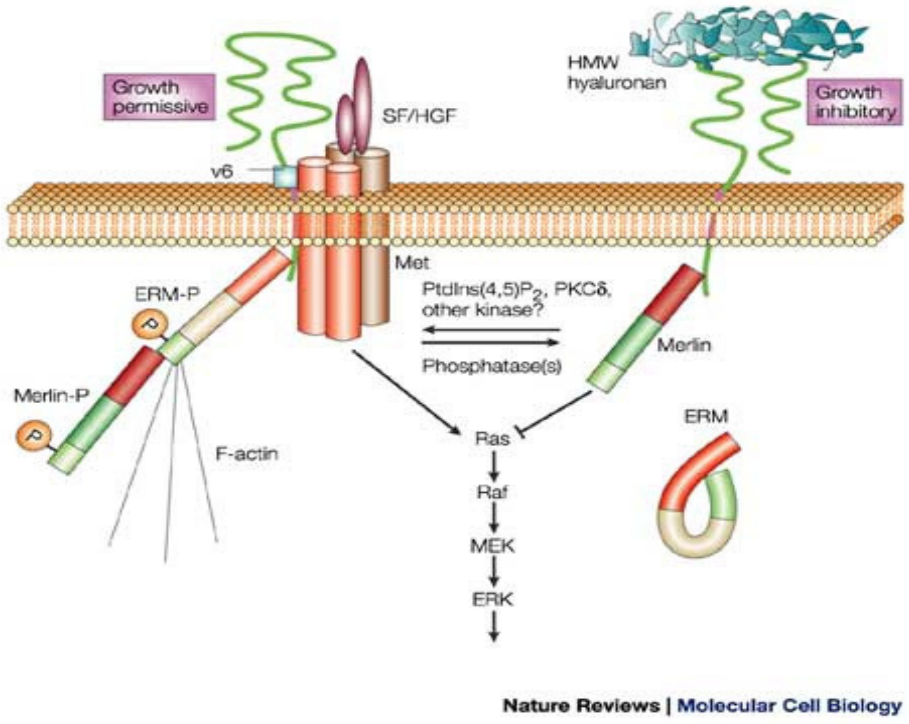
receptor ErbB4 which is involved in cell survival. [115] CD44v3 isoform efficiently binds to hepatocyte growth factor/scatter factor (ligand for receptor tyrosine kinase) through its heparin sulphate chain. The functional outcome is promotion of c-met phosphorylation, MAPK activation and increased tumor growth and metastasis [116].

Taken together the binding of cytokines, enzymes and chemokines to CD44 is essential for organogenesis, hematopoesis, inflammatory and autoimmune responses and tumor progression.

2.5.4 CD44 as a co-receptor and signaling

Many if not all signaling events are initiated through extracellular stimuli, followed by activation of receptor tyrosine kinases (RTKs). These receptors span the plasma membrane and their cytoplasmic domains have catalytic kinase activities that get phosphorylated upon ligand binding or other sources of stimulation and further serve as docking sites for several components of intracellular signaling. Receptor activation is mediated by several other proteins that are associated with them and which are devoid of catalytic activity (co-receptor activity). Cell adhesion molecules are now being known for their co-receptor activity [117] and CD44 is one of them. CD44 acts as a co-receptor for the ErbB family of receptor tyrosine kinases and for the c-Met receptor, and these associations are essential for activation of receptor kinase activity and the regulation of diverse cellular processes, including cell survival, proliferation and differentiation.

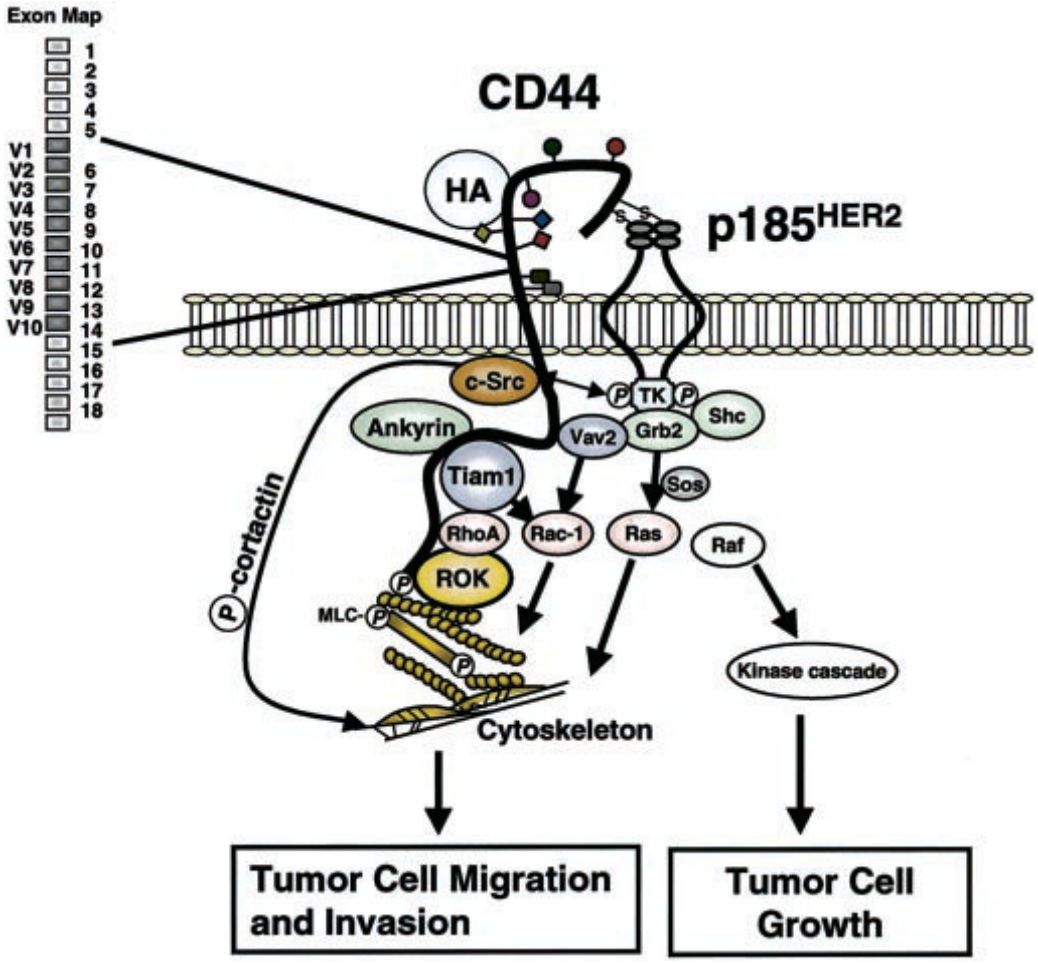
Since cytoskeletal organization and cell migration have been dealt with in the previous sections this section will mainly focus on cell growth proliferation, survival, apoptosis and anti-apoptosis.



Scheme 5: Signaling mediated by CD44 [77]

One main reason for the involvement of CD44 in multiple signaling events is its association with the Src family of phosphotyrosine kinases (PTKs). Src kinases act as molecular switches on the cell membranes linking extracellular events to intracellular signaling. They are activated through engagement with many receptors such as TCR/CD3 complex, CD4, CD8, B cell receptor, Fc receptors, integrins, GPI anchored receptors and growth factor receptors, reviewed in [80]. When activated (they are also phosphorylated on regulatory tyr residue c-terminal when inactive) they mediate several signaling events including activating of additional PTKs such as ZAP-70 or Syk, phospholipases, cytoskeletal proteins and adaptor proteins.

CD44 signaling can result in opposing effects depending on the cellular context, the expressed variant isoform and the associated signaling partner. For example, CD44 engagement can lead to proliferation or inhibition of proliferation, apoptosis or inhibition of apoptosis, resulting in up regulation and down regulation of several signaling pathways involved in cell activation.



Scheme 6: Accessory functions and signaling via CD44 [118]

CD44 associates with Lck and Lyn Src kinases in non stimulated T and B cells. It was shown that synthetic peptide of CD44, pCD44 (ILAVCIAVNSRRR) has high binding capacity towards these Src kinases. This peptide region corresponds to plasma membrane-cytoplasmic domain interface of CD44. A single mutation in the cysteine residue is enough to abolish the interaction [119]. Src kinase co-translocate with CD44 by binding to CD44 carboxyl-terminal ankyrin domain which in turn facilitates integrin β1 recruitment and activation in lipid rafts and mediates matrix derived cell survival [120]. Signaling of CD44 mediated through its association with Lck leads to phosphorylation of Pyk2, a focal adhesion kinase which leads to cytoskeleton reorganization and cell spreading [121].

T cell activation requires two signals one from the TCR and the other from a co-stimulatory molecule. CD44 has been described to deliver co-stimulatory signals for T cell activation [122]. CD44 and the associated Lck are recruited into membrane microdomains (low density sucrose gradient fractions) where they interact with the CD3/TCR complex. Association of CD44 with Lck and Fyn and in turn their co-localisation with the TCR allow for recruitment of several other kinases necessary for T cell activation [122, 123]. The co-stimulatory function of CD44 is demonstrated by its ability to induce cell proliferation on freshly isolated lymph node cells in the presence of sub-threshold levels of anti-CD3. This is mediated by activation of several tyrosine kinases and is accompanied by strong activation of erk and c-jun and involvement of MAPK. Consequently, IL-2 production and CD69 and CD25 expression are up regulated in T cells. The situation differs in the T helper line IP-12 where it was observed that CD44-cross-linking with CD3 leads to upregulation of CD95 and CD95L expression resulting in apoptosis induction (or activation induced cell death (AICD). This argues for the differential effects of CD44 on different cell types. It is important to note that, in both peripheral T cells and T helper line (IP12) CD44 cross linking alone did not exert any effect on signaling by itself [122].

The effects of anti-CD44 Ab treatment are specific for the epitope of CD44 where it binds i.e. co-stimulatory functions of CD44 inducing proliferation or apoptosis are exhibited only by cross-linking with IM-7 that does not bind to hyaluron binding site and not by KM81 binding to hyaluron binding domain of CD44 or K926 that binds to CD44v10 [122]. Some studies have shown that CD44 by itself is capable of activating human resting T cells and mouse cytotoxic T cells by itself like CD3 and can promote proliferation of T cells that is dependent on IL-2 production. In this study, anti-CD44 cross-linking does not phosphorylate the Zeta chain of the TCR complex indicating that the signals generated in this case are independent of TCR engagement. Indeed, it was demonstrated that this process is mediated by tyrosine kinases associated with CD44 [124]. In a TNBS-induced colitis mouse model, CD44v7 was shown to deliver anti-apoptotic signals and protect T cells from activation induced cell death. Cells were protected from apoptosis by upregulation of anti-apoptotic

proteins Bcl-2 and Bcl-xl and down regulation of CD95L [125]. It was also demonstrated in this work that the anti-apoptotic effect of CD44v7 is mediated via Akt and phosphorylation of the pro-apoptotic molecule Bad [125].

Using a mouse model for the autoimmune disease alopecia areata Marhaba *et al.*, have shown an association between CD44 and CD49d. This association resulted in the formation of a signaling complex between CD44, CD49d and the underlying signaling machinery allowing for each surface molecule (CD44 or CD49d) to avail from the associated signaling molecules of the other. In this case, cross-linking of CD44 resulted in the activation of the focal adhesion kinase (FAK) associated with CD49d, and CD49d cross-linking allowed the activation of Ezrin and Lck associated with CD44. Thus signaling pathways initiated through both the molecules CD44 and CD49d are triggered and this is important in lymphocyte activation and function [126].

CD44 signaling also mediates proliferation and apoptosis in several tumor lines depending on the cell type and engagement of CD44 isoform. The Src-kinase Lyn, was found to be activated via CD44v6 in a colorectal cancer cell line. The functional consequence was increased chemoresistance against the drug 1,3-bis (2-chloroethyl)-1-nitrosurea and this was mediated through the PI3K/Akt survival pathway [127]. In an ovarian tumor, CD44 association with c-Src was responsible for cytoskeletal reorganization mediated by phosphorylation of the cytoskeletal protein cortactin leading to increased migration and spreading [128]. A thymoma line (EL4) transfected with CD44v6 was subjected to much higher proliferation rates as compared to untransfected cells that express only CD44 standard isoform. Signaling molecules, erk, c-jun were activated and IkB was phosphorylated suggesting NF-kB activation. Indeed, NF-κB was found to be translocated to the nucleus. Consequently, IL-2 production was increased [129].

On the other hand, CD44 was also shown to strengthen dexamethasone induced apoptosis of a lymphoma line that was mediated through up regulation of the pro-apoptotic protein Bax and down regulation of the anti-apoptotic protein Bcl-Xl. The same effect by CD44 was also seen in thymic epithelial cells mediating apoptosis of a lymphoma line [130]. CD44 cross-linking in fibroblast

drives cells into apoptosis suggesting a role for CD44 in fibroblasts control [131]. CD44 ligation induced apoptosis of a promyelocytic leukemia line. Apoptosis was initiated via caspase 8 and 9 and in addition effector caspase 3 and 9. Serine protease dependent pathway was also proposed to be involved in mediating apoptosis of these cells [132].

With respect to tumors, it has been proposed that HA-CD44 interaction mediates proliferation and soluble CD44 inhibits the growth of cells. When a murine mammary cell line was transfected with soluble CD44, it was observed that the cells no longer attached to the peritoneal wall and also did not form tumors. This led to inhibition of anchorage dependent growth as well as tumor invasion. [133] CD44-HA interaction also promotes neo-angiogenesis and proliferation. Hyaluronic acid dependent clustering of CD44 promotes binding of MMP-9 to CD44 at the cell surface promoting its activity in addition to protecting it from tissue inhibitors for MMPs and thus promoting tumor invasion and angiogenesis [134].

One of the main events mediated by CD44 is cytoskeletal organization which is energy dependent and requires an intact actin and microtubuli system. Small GTPase Rac1 activation is required for this process. Rac1 is also known to co-localise with ezrin [135]. These events along with phosphotyrosine kinase activations are responsible for cytoskeletal re-organizations in the cell. In T lymphocytes cross-linking anti-CD44 Ab leads to CD44 dependent spreading through F-actin polymerization, accompanied by T cell adhesion, flattening and spreading [123]. Vav1 protein encoded by vav protooncogene is a 95KD protein that is an upstream regulator of Rac1. Vav catalyses the GDP to GTP exchange on Rac1. Vav1 activation involves phosphoinositides binding and tyrosine phosphorylation that is dependent on Src family kinases (Lck and Fyn) which associate with CD44. Thus, this could be a possible explanation for CD44 mediated effects on the cytoskeleton [123, 136, 137]. CD44 is co-localized in membrane microdomains (lipid rafts) where it interacts with Annexin II in a cholesterol dependent manner. The recruitment of CD44 in rafts further leads to stabilization of actin-cytoskeleton [138].

2.6 CD44 in Hematopoietic Stem Cells:

CD44 is important for Hematopoietic stem cell homing. Bone marrow stroma formation also requires CD44 that supports the process by induction of IL-6 secretion. Human and mouse HSC synthesize and express HA and HA expression correlates with selective migration of HSC to the endosteal niche. HSC homing can be blocked by anti-CD44 or soluble HA or hyaluronidase treatment. In addition, CXCL12 stimulates adhesion of progenitor cells via CD44, which demonstrate a cross-talk between CD44 and CXCR4 signaling and suggest a key role of HA and CD44 in CXCL12-dependent transendothelial migration of HSC and their anchorage within specific niches. Thus, CD44 contributes to homing and settlement of HSC in the bone marrow niche.

Taken together, the involvement of CD44 in HSC homing and niche embedding is known since over a decade and has been used as a means to mobilize HSC. Meanwhile, compelling evidence has been provided that LSC compete with HSC for embedding in the bone marrow niche and that LSC may be driven into differentiation or apoptosis by anti-CD44, which prevents their homing in the bone marrow niche.

3. AIM

CD44 has been identified as a leukemia initiating cell marker on several types of hematological malignancies. CD44 expression on hematopoietic stem cells (HSC) is required for homing into the osteogenic niche and maintenance of quiescence. CD44 plays an important role in leukemic stem cells (LSC) and hematopoietic stem cells (HSC) niche embedding, LSC and HSC apparently compete and a blockade of CD44 more efficiently prevents HSC than LSC embedding during HSC reconstitution.

Based on these findings and in view of the importance of CD44 for hematopoiesis we aimed to evaluate:

The CD44 isoform and associating molecules on HSC, leukemic cells and BM stroma cells, that contribute to the adhesion process.

The CD44 ligands on HSC, leukemic cells or BM stromal cells and their impact on functional consequences like maintenance of quiescence and apoptosis resistance.

2: Materials and methods

2.1 Materials

2.1.1 Animals

Mice		**Origin**
C57BL6 (H-2b)	Wild type	Charles River, Suzfeld, Germany
Balb/c	Wild type	Charles River, Suzfeld, Germany

2.1.2 Bacterial strain

E.coli DH5α	Genotype: F^-, Φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk^-,mk^+), *pho*A, *sup*E44, *thi*-1, *gyr*A96, *rel* A1, λ^- (Invitrogen, Darmstadt, Karlsruhe)

2.1.3 Cell Lines

Cell Line	**Origin**
EL-4	Mouse Thymoma American Type Tissue Culture (ATCC) number: TIB- 39
EL-4 transfected with CD44v6	[129]
HEK 293-EBNA 1	ATCC number: CRL-1573 derived; transfected with the EBNA1 viral gene.
Myc -9e10	mAB-9e10 producing hybridoma line ATCC CRL 1975
IM7.8.1	mAb IM7.8.1 producing hybridoma line ATCC TIB235
PS/2	mAb PS/2 producing hybridoma line ATCC
S17	Established from the adherent layer of Dexter-type long-term bone marrow cultures.

2.1.4 Primers

CD44s	**F** 5'-GATCCATGAGTCACAGTGCG-3'
	R 5'-GCCTACTGGAGATCAGGATG-3'
CD44v3	**F** 5'-AGTCAAATACCAACCCAACAGG-3'
	R 5'-TGGTACTGGAGATAAAATCTT-3'
CD44v6	**F** 5'-CTCCTAATAGTACAGCAGAA-3'
	R 5'-CAGTTGTCCCTTCTGTCA-3'
CD44v7	**F** 5'-CTTCGGCCCACAACAACCAT-3'
	R 5'-CCTTGCTTTCTGTTTGATGAC-3'
CD44v8	**F** 5'-ATACAGACTCCAGTCATAGTACAA-3'
	R 5'-GAGTTGTCACTGAAGTGGTC-3'
CD44v9	**F** 5'-CACAGAGTCATTCTCAGAACT-3'
	R 5'-TGCTAGATGGCAGAATAGAAG-3'
CD44v10	**F** 5'-TGCAAGAAGAGGTGGAAGTCTTCC-3'
	R 5'-CTGGTAAGGAGCCATCAACATTAA-3'
GAPDH	**F** 5'-GACCCCTTCATTGACCTCAAC-3'
	R 5'-CTTCTCCATGGTGGTGAAGAC-3'

2.1.5 Primary Antibodies

Antibody	**Company**
Anti-CD11a	Becton Dickinson, Heidelberg, Germany
Anti-CD11b/YBM6.6.10	European Association of Animal Cell Cultures
Anti-CD11c	European Association of Animal Cell Cultures
Anti-CD16	Immunotools, Friesoythe, Germany
Anti-CD18	Becton Dickinson, Heidelberg, Germany
Anti-CD24	Becton Dickinson, Heidelberg, Germany
Anti-CD30	Becton Dickinson, Heidelberg, Germany
Anti-CD31	Becton Dickinson, Heidelberg, Germany
Anti-CD34	Becton Dickinson, Heidelberg, Germany
Anti-CD38	Becton Dickinson, Heidelberg, Germany

Anti-CD43	Becton Dickinson, Heidelberg, Germany
Anti-CD44(IM7)	ATCC
Anti-CD44v6	Bender Medsystems
Anti-CD44v7	Calbiochem, Darmstadt, Germany
Anti-CD44v10 (K926)	[163]
Anti-CD45R	Becton Dickinson, Heidelberg, Germany
Anti-CD49a	Becton Dickinson, Heidelberg, Germany
Anti-CD49b	Becton Dickinson, Heidelberg, Germany
Anti-CD49d (PS/2)	Miyazaki *et al*
Anti-CD49e	Becton Dickinson, Heidelberg, Germany
Anti-CD49f	Becton Dickinson, Heidelberg, Germany
Anti-CD51	Becton Dickinson, Heidelberg, Germany
Anti-CD53	Becton Dickinson, Heidelberg, Germany
Anti-CD54 (YN1/1.7.4)	European Association of Animal Cell Cultures
Anti-CD61	Becton Dickinson, Heidelberg, Germany
Anti-CD62E	Becton Dickinson, Heidelberg, Germany
Anti-CD62L	Immunotools, , Friesoythe; Germany
Anti-CD62L-lig	Becton Dickinson, Heidelberg, Germany
Anti-CD62P	Becton Dickinson, Heidelberg, Germany
Anti-CD71	Becton Dickinson, Heidelberg, Germany
Anti-CD81	Becton Dickinson, Heidelberg, Germany
Anti-CD90	Immunotools, , Friesoythe; Germany
Anti-CD95	Becton Dickinson, Heidelberg, Germany
Anti-CD95l	Becton Dickinson, Heidelberg, Germany
Anti-CD102	Becton Dickinson, Heidelberg, Germany
Anti-CD103	Becton Dickinson, Heidelberg, Germany
Anti-CD104	Becton Dickinson, Heidelberg, Germany
Anti-CD105	Becton Dickinson, Heidelberg, Germany

Anti-CD106	Becton Dickinson, Heidelberg, Germany
Anti-CD117	Becton Dickinson, Heidelberg, Germany
Anti-CD123	Becton Dickinson, Heidelberg, Germany
Anti-CD126	Becton Dickinson, Heidelberg, Germany
Anti-CD127	Becton Dickinson, Heidelberg, Germany
Anti-CD184	Becton Dickinson, Heidelberg, Germany
Anti-CD195	Becton Dickinson, Heidelberg, Germany
Anti-CD209	Becton Dickinson, Heidelberg, Germany
Anti-CD284	Biolegend, Uithoorn, Netherlands
E13	ATCC
Anti-Flt3	Santa Cruz, Heidelberg, Germany
Anti-Gr1	Immunotools, , Friesoythe; Germany
Anti-GMCSF	Becton Dickinson, Heidelberg, Germany
Anti-SDF1	Becton Dickinson, Heidelberg, Germany
Anti-Trail	Becton Dickinson, Heidelberg, Germany
Anti-TNFRI	Becton Dickinson, Heidelberg, Germany
Anti-TNFRII	Becton Dickinson, Heidelberg, Germany
Anti-Ter119	Becton Dickinson, Heidelberg, Germany
Anti-Ly6	Becton Dickinson, Heidelberg, Germany
Anti –myc	ATCC
Anti-IL-3	Becton Dickinson, Heidelberg, Germany
Anti-IL-7	Bio Trend, Dortmund, Germany
Anti-pAkt	Becton Dickinson, Heidelberg, Germany
Anti-akt	Becton Dickinson, Heidelberg, Germany
Anti-Actin	Becton Dickinson, Heidelberg, Germany
Anti-Bax	Becton Dickinson, Heidelberg, Germany
Anti-Bcl2	Becton Dickinson, Heidelberg, Germany
Anti-BAD	Becton Dickinson, Heidelberg, Germany

Anti-pBAD	Cell Signalling, Frankfurt , Germany
Anti-Caspase 3	Becton Dickinson, Heidelberg, Germany
Anti-Caspase 9	Becton Dickinson, Heidelberg, Germany
Anti-Caspase 9 cleaved	Cell Signalling, Frankfurt , Germany
Anti-Casein Kinase 2	Becton Dickinson, Heidelberg, Germany
Anti-Cytochrome C	Becton Dickinson, Heidelberg, Germany
Anti-pERK1/2	Santa cruz, Heidelberg, Germany
Anti-ERK1/2	Becton Dickinson, Heidelberg, Germany
Anti-Histone H3	Cell Signalling, Frankfurt , Germany
Anti-NFkB	Santa Cruz, Heidelberg, Germany
Anti-pIkB	Cell Signalling, Frankfurt , Germany
Anti-PP1	Cell Signalling, Frankfurt , Germany
Anti-PP2a	Cell Signalling, Frankfurt , Germany
Anti-phosphothreonine (pThr)	Santa Cruz, Heidelberg, Germany
Anti-phosphotyrosine (py20)	Becton Dickinson, Heidelberg, Germany

2.1.6 Secondary antibodies

Name	**Company**
Anti-mouse IgG HRP	Amersham, Freiburg, Germany
Anti-rabbit IgG HRP	Amersham, Freiburg, Germany
Anti-rat IgG HRP	Amersham, Freiburg, Germany
Anti-mouse IgG PE	Jackson Laboratories, Bar Harbor,USA
Anti-hamster IgG FITC	Jackson Laboratories, Bar Harbor,USA
Anti-mouse IgG FITC	Jackson Laboratories, Bar Harbor,USA
Anti-mouse IgG APC	Jackson Laboratories, Bar Harbor,USA
Anti-rat IgG FITC	Jackson Laboratories, Bar Harbor,USA
Anti-rat IgG PE	Jackson Laboratories, Bar Harbor,USA

Anti-rat IgG APC	Becton Dickinson Heidelberg, Germany
Streptavidin FITC	Jackson Laboratories, Bar Harbor,USA
Streptavidin PE	Jackson Laboratories, Bar Harbor,USA
Streptavidin APC	Jackson Laboratories, Bar Harbor,USA
Streptavidin HRP	Rockland, PA USA

2.1.7 Inhibitors and Enzymes

MEK1/2 Inhibitor (SL327)	Calbiochem, Darmstadt, Germany
Caspase 9 Inhibitor	Calbiochem, Darmstadt, Germany
CK2 Inhibitor	Calbiochem, Darmstadt, Germany
Restriction enzymes	MBI Fermentas, St. Leon-Rot, Germany
Taq polymerase	MBI Fermentas, St. Leon-Rot, Germany

2.1.8 Instruments

Name	**Company**
Agitator for bacterial cultures	Edmund Buehler GmbH, Hechingen, Germany
Cell chamber Neubauer improved	Brand, Wertheim, Germany
Centrifuge Sorvall RC5B Plus	Kendro, USA
Centrifuge Biofuge fresco	Heraeus, Hanau, Germany
DNA-agarose gel electrophoresis chamber	Bio-Rad, Munich, Germany
Eagle eye (Mididoc)	Herolab, Wiesloch, Germany
ELISA plate reader	Anthos labtec, Wals, Austria
FACS Calibur	Becton-Dickinson, Heidelberg, Germany

Hyper processor (for processing films)	Amersham, Freiburg, Germany
Incubator for bacteria	Melag, Berlin, Germany
Incubator for cell culture	Labotec, Goettingen, Germany
Invert microscope DM-IL	Leica, Bensheim, Germany
Master cycler (PCR cycler)	Eppendorf, Hamburg, Germany
Magnetic stirrer 3000	Heidolph, Keilheim, Germany
Microscope DMBRE	Leica, Bensheim, Germany
Microwave	Phillips, Wiesbaden, Germany
Photocassette	Amersham, Freiburg, Germany
Ph-Meter-761 Calimatic	Knick, Berlin, Germany
Photometer Ultraspec III	Amersham, Freiburg, Germany
Pipettus-Akku	Hirschmann, Eberstadt, Germany
Pipettes	Eppendorf, Hamburg, Germany
Powersupply PS 9009	GIBCO, Darmstadt, Germany
Rotor GSA	Kendro, USA
Rotor SW34	Kendro, USA
Rotor SW41 Ti	Beckman Coulter, Krefeld, Germany
Sterile bench	Heraeus, Hanau, Germany
Sonicator Sonoplus	Bandelin, Berlin, Germany
Tabletop centrifuge	Heraeus, Hanau, Germany
Transferapparatus Mini Trans-Blot®	Bio-Rad, Munich, Germany
Thermo-mixer	Eppendorf, Hamburg, Germany
Ultrasound homogenizer	Bandelin Electronik, Germany
Water-bath	Julabo, Seelbach, Germany
Weighing scale RC210 D	Sartorius, Goettingen
Whirlmixer Vortex Genie	Si Inc., New York, USA

2.1.9 Miscellaneous Materials

Cell culture flasks 25cm^2, 75cm^2	Greiner, Frickenhausen, Germany
Cell culture 96-well, 24-well, 6-well plates	Greiner, Frickenhausen, Germany
Centrifugal concentrators Vivaspin 6ml, 20ml	Vivascience, Hannover, Germany
Cryovials	Greiner, Frickenhausen, Germany
Coverglass	R. Langenbrinck, Emmendingen, Germany
CFSE	Invitrogen, Frankfurt, Germany
Falcon tubes 15ml, 50ml	Greiner, Frickenhausen, Germany
Hyaluronan	SIGMA, Steinheim, Germany
Hyperfilm ECL	Amersham, Freiburg, Germany
Magnetic Beads	Miltenyl Biotec, Bergisch Gladbach, Germany
Needles	BD Biosciences, Heidelberg, Germany
Nitrocellulose membrane Hybond ECL	Amersham, Freiburg, Germany
Parafilm	American Nat. Can., Greenwich, Great Britain
Petriplates	Greiner, Frickenhausen, Germany
Pipette tips	Sarstedt, Numbrecht, Germany
Sterile filter 0,2µm	Renner, Darmstadt, Germany
Syringes	BD Biosciences, Heidelberg, Germany
Trans-well migration (Boyden) chambers 48 well	Neuroprobe, Gaithusberg, USA
WhatmanTM 3MM paper	Scleicher & Schull, Dassel

2.1.10 Chemicals:

Acetic acid	Riedel-de Haen, Seelze
Acetone	Fluka, Buchs, Switzerland
Agarose	Sigma, Steinheim
Ammonium persulphate (APS)	GIBCO, Darmstadt
Ampicillin sulphate	Calbiochem, Darmstadt
Annexin FITC V	Becton Dickinson,Heidelberg
Bactoagar	Fluka, Buchs, Switzerland
Bio-Rad, Munich Bradford reagent	Bio-Rad, Munich
Biotin-X-NHS	Calbiochem, Darmstadt
Bovine Serum Albumin (BSA)	PAA, Pasching, Austia
Brij 96	Fluka, Buchs, Switzerland
Bromo phenol blue	Merck, Darmstadt
Calcium chloride	Merck, Darmstadt
CFSE	Invitrogen, Darmstadt
Chloroform	Riedel-de Haen, Seelze
Coomassie R-250	Merck, Darmstadt
Crystal violet	Sigma, Steinheim
Dimethyl formamide	Merck, Darmstadt
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Ethanol	Riedel-de Haen, Seelze
Ethidium bromide	Merck, Darmstadt
Ethylenediamine tetraacitic acid (EDTA)	Sigma, Steinheim
Foetal Calf Serum (FCS)	PAA, Pasching, Austria
Formaldehyde (37%)	Merck, Darmstadt
G418 sulphate	PAA, Pasching, Austria
Gelatine (cold water fish skin)	Merck, Darmstadt
Glucose	Merck, Darmstadt

Ladder Gen ruler Protein	MBI Fermentas, St. Leon-Rot
Ladder Prestained Protein	MBI Fermentas, St. Leon-Rot
L-Glutamine	AppliChem, Darmstadt
Glycerine	Roth, Karlsruhe
Glycine	GERBU, Gaiberg
HEPES	GERBU, Gaiberg
HiPerfect-Reagent for transfection	Quiagen, Hilden
Hydrochloric acid (HCl)	Riedel-de Haen, Seelze
Hygromycin	PAA, Pasching, Austria
Isopropanol	Fluka, Buchs, Switzerland
Magnesium carbonate	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Magnesium sulphate	Merck, Darmstadt
Milk powder	Roth, Karlsruhe
Methanol	Riedel-de Haen, Seelze
N,N,N'N'-Tetramethylenediamine (TEMED)	Sigma, Steinheim
Paraformaldehyde	Sigma, Steinheim
Penicillin	Sigma, Steinheim
Phenylmethylsulphonylfluoride (PMSF)	Sigma, Steinheim
Phorbolmyristateacetate (PMA)	Sigma, Steinheim
Potassium acetate	Sigma, Steinheim
Potassium carbonate	Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Potassium dihydrogenphosphate	Merck, Darmstadt
Potassium tetrathionate	Merck, Darmstadt
Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim
Protein G Sepharose 4 Fast Flow	Amersham Biosciences, Freiburg
Rotipherose Gel 30 (Acrylamide-mix)	Roth, Karlsruhe

RPMI 1640	GIBCO, Darmstadt cell culture
Silver nitrate	Roth, Karlsruhe
Sodium acetate	Merck, Darmstadt
Sodium azide	AppliChem, Darmstadt
Sodium carbonate	AppliChem, Darmstadt
Sodium chloride	Fluka, Buchs, Switzerland
Sodium hydrogen phosphate	Merck, Darmstadt
Sodium dodecyl sulphate (SDS)	GERBU, Gaiberg
Sodium hydrogen carbonate	AppliChem, Darmstadt
Sodium hydroxide	Riedel-de Haen, Seelze
Sodium pyruvate	Merck, Darmstadt
Sodium thiosulphate	Merck, Darmstadt
Tris	Roth, Karlsruhe
Triton-X-100	Sigma, Steinheim
Trypan bue	Serva, Heidelberg
Trypsin	Sigma, Steinheim
Trypton	AppliChem, Darmstadt
Tween 20	Serva, Heidelberg
Yeast Extract	GIBCO, Darmstadt

2.1.11 Buffers and solutions

Name	Composition
Annexin FITC/PI Binding buffer	10mM HEPES pH7.4, 140nM NaCl, 25mM $CaCl_2$
Bicarbonate buffer pH 9.6	15mM Na_2CO_3, 35mM $NaHCO_3$. Fill to 900ml with distilled water. Adjust pH to 9.6 and make it upto 1l with water.
Blot buffer (5x)	10g SDS, 142g Glycine, 30.3g Tris base and make upto 1l with distilled water. 1X buffer was made fresh by taking 5x Blot buffer, methanol and distilled water in the ratio 1:1:3
DEPC water	200µl DEPC reagent in 1l distilled water and autoclave
Ethidium Bromide	0.2g Ethidium bromide, distilled water 20ml. Stored in dark.
Freezing medium	90%FCS, 10%DMSO
Glycine solution	0.2M glycine in PBS
HEPES buffer	25mM HEPES pH7.2, 150mM NaCl, 5mM $MgCl_2$, 1mM PMSF, 1x Protease inhibitor, 1mM $NaVO_4$, detergent as indicated in experiments
LB medium	10g Peptone, 5g Yeast extract, 10g NaCl, fill upto 1l with Distilled water. For LB plates 15g agar was added.
PBS (pH7.2)	137mM NaCl, 2.7KCl, 4.3mM Na_2HPO_4 in distilled water
Running buffer for protein gels (10x)	10g SDS, 144g Glycine, 30g Tris, filled upto 1l with distilled water
TAE Buffer	242g Tris base, 57.1ml Glacial acetic acid, 100ml 0.5m EDTA pH 8.0, Add distilled water 1l and adjust final pH to 8.5.
TNES buffer	50mM Tris, 0.4M NaCl, 100mM EDTA, 1% SDS.

2.2 Methods:

2.2.1 Molecular biology

2.2.1.1 Chemical Competent cells :

From a 50 ml DH5α overnight culture, 1 litre of LB medium (NO ANTIBIOTIC) was cultivated till the O.D.600nm reaches 0.5-0.6. Cells were then centrifuged in the Sorvall GSA rotor (250 ml centrifuge bottle) at 5,000 RPM for 10 minutes at 4^{o}C, bacteria pellet was gently resuspended in 1/4 volume of ice cold sterile Magnesium chloride ($MgCl_2$). Cells were again centrifuged 4000 RPM in the Sorvall GSA rotor for 10 minutes and bacterial pellet was now re-suspended in 1/20 volume of ice cold Calcium chloride ($CaCl_2$). Cells were centrifuged 4,000 RPM in the GSA rotor for 10 minutes and the cell pellet was re-suspended in 1/50 volume of ice cold, sterile 85 mM $CaCl_2$ in 15% glycerol w/v. cells were aliquoted in 50 µl each and stored at -80^{o}C.

2.2.1.2 Transformation:

Competent cells were thawed on ice and 1-10ng of plasmid was added and kept on ice for 30 mins. Competent cell plus plasmid was given a heat shock at 42°C for 2 mins and kept on ice for another 5 mins. To this 1 ml of LB medium was added and kept at shaker for 1 hr at 37°C. 10 to 100 µl was then plated on LB agar plate containing selection drug (ampicilline at 100µg/ml). Plates were incubated overnight at 37°C.

2.2.1.3 Mini-Prep:

Single colonies were recovered from LB agar plates in 2 ml LB medium containing selection agent (ampicilline at 100µg/ml) on a shaker set at 200 rpm at 37°C. overnight. 1ml bacterial suspension was used for miniprep following producer's recommendations (Miniprep kit, Qiagen). Positive clones were obtained by doing enzymatic digestion.

2.2.1.4 Enzymatic restriction digestion:

For double digestion 1 µg purified plasmid with insert was mixed with 0.5µl (5U) primary restriction enzyme and appropriate digestion buffer (1x).Total volume was completed with distilled water to 10 µl and digestion was performed 1-2 hr in a water bath at 37°C. After digestion, DNA was purified over Qiagen mini-column and a second enzymatic digestion was performed following the same procedure. Samples containing right sized fragments were then proceeded via midiprep for transfection.

2.2.1.5 Midi-Prep

To obtain sufficient amount of vector DNA necessary for transfection, overnight cultures of positive clones were incubated overnight on shaker set at 200 rpm at 37°C in 100ml of LB medium containing appropriate selection antibiotic (ampicilline at 100µg/ml) whole bacterial culture was used for midi-prepration following producer's recommadations (Midi-prep Kit,Qiagen).

2.2.1.6 RNA preparation, cDNA synthesis and amplification

Total RNA isolation was done from 10^7 cells with Tri reagent following manufacturer's instructions (Applichem, Darmstadt, Germany). Quality of RNA preparation was checked by running RNA sample diluted in RNA sample buffer on 1% agarose/formaldehyde gel.

cDNA synthesis and amplification was performed by RT–PCR (reverse transcriptase polymerase reaction). 1-2 µg template RNA was mixed with 0.5µg oligo dT primer and heated at 70°C, 5 min; chilled on ice, then mixed with 1µl Im Prom II reverse transcriptase (10U), 1-2mM dNTP, 1x Im Prom II buffer 5x, 6mM $MgCl_2$ and made upto a total of 20µl with nuclease free water.The program continues as 25°C-5min, 42°C-60min and 70°C-15min.

PCR was performed in 25 µl volume containing template, 1-2 mM dNTP, 1.5µl Red Taq polymerase, 2.5 µl 10x Taq buffer and 0.2 µM primer forward and reverse. Total volume was completed to 25 µl with autoclaved distilled water.

PCR Program:

CD44s : 94°C-5min, 94°C-30secs, 55°C-30secs, 72°C-1min -32cycles, 72°C-10min

CD44v3 : 94°C-5min, 94°C-30secs, 54°C-30secs, 72°C-1min, 32cycles, 72°C-10min

CD44v6 : 94°C-5min, 94°C-30secs, 53°C-30secs, 72°C-1min -32 cycles, 72°C-10min

CD44v7 : 94°C-5min, 94°C-30secs, 55°C-30secs, 72°C-1min -32 cycles, 72°C-10min

CD44v8 : 94°C-5min, 94°C-30secs, 54°C-30secs, 72°C-1min, 32cycles, 72°C-10min

CD44v9 : 94°C-5min, 94°C-30secs, 53°C-30secs, 72°C-1min -32 cycles, 72°C-10min

CD44v10 : 94°C-5min, 94°C-30secs, 53.5°C-30secs, 72°C-1min -32 cycles, 72°C-10min

GAPDH : 94°C-5min, 94°C-30secs, 55°C-30secs, 72°C-1min -32 cycles, 72°C-10min

2.2.1.7 DNA gel electrophoresis

PCR products or samples containing DNA of interest were checked by running an agarose gel of 1 to 2%, depending on the size of the product. When Red Taq polymerase was not used then DNA sample was mixed with DNA loading dye (6x) to locate the running front. Markers (1kb or 100bp) were run in parallel. The gel was run at 100 volts in a migration tank containing 1x TAE buffer. Bands were visualized on a U.V. transilluminator.

2.2.2 Protein Chemistry

2.2.2.1 Antibody purification

Antibody purification was done by affinity chromatography using a sepharose protein G-4B column. To purify IgG fractions, sterile filtered hybridomas supernatants were passed over a sepharose protein G-4B column. The column was washed with 0.1M phosphate buffer, pH 7.5. Bound IgG was eluted from the column with 0.1M Glycine buffer pH 2.7. Protein containing fractions were dialyzed against PBS, concentrated and filter-sterilized. The protein amounts obtained were photometrically analysed for protein concentration by Biorad assay.

2.2.2.2 Column preparation (CNBr coupling)

10mg purified anti-myc antibody (9e10 hybridoma) were covalently coupled to CNBr activated sepharose. For this purpose, CNBr coupling method was used as follows: After purification over a protein-G-sepharose 4B column, concentration and dialysis against PBS, the anti-myc antibody was further dialysed overnight against carbonate coupling buffer pH8.3 at 4°C and adjusted thereafter to 6 mg/ml in coupling buffer; 0.5g CNBr activated sepharose were swollen in 1mM HCl solution. Thereafter the gel was washed with 5ml coupling buffer and sucked off until cracks appeared in the cake. Gel was then immediately transferred to 15 ml tube containing the antibody solution and incubated under agitation overnight at 4°C. Beads were then spun down (2,000 rpm, 1min) and efficiency of the coupling procedure was measured, by quantifying the amount of protein remaining in the supernanant; Biorad reagent was used for this purpose. OD was read at 565nm. If OD was 10 fold lower comparing to starting ligand solution, coupled beads were further washed 3 times with 10 column volumes coupling buffer, incubated 2 hours at room temperature in 10 ml filtered blocking solution 0.2M glycine pH8.0 and then washed 4 times alternatively with wash buffer pH4.7 and coupling buffer pH8.3. OD 595nm was read at first and fourth wash. The gel was poured into chromatography column and washed with 5 volumes columns PBS-azide 0.01%pH7.3 and stored at 4°C until use.

2.2.2.3 Purification of the CD44 recombinant soluble protein

To purify the soluble molecules, the HEK293- EBNA1 transfectant supernanat were cleared by centrifugation15min at 3,000 rpm and sterile filtered. Filterate was supplemented with 0.05% Natrium azide and passed over anti-myc affinity column at 0.3ml/min over 24 hours. Elution was done using a 0.2M glycine pH2.7 buffer. Eluted fractions were collected in 1:20 volume 1M Tris buffer pH8.0 then concentrated and dialyzed against cold PBS using Vivaspin tubes (50 Kda cut off). Protein concentration was measured by Biorad assay.

2.2.2.4 Biorad assay

This test is based on the affinity of the coomassie dye G-250 for proteins. In microassay it allows detections of BSA amounts under 25µg/ml. To proceed with detection of the soluble proteins, 1µl of each sample to test were diluted in a flat micotiter well with 99µl Biorad reagent prediluted (1 :10 in distilled water). In parallel, a 10mg/ml BSA solution in water was serially diluted to allow drawing of a standard curve. After 5min incubations, optical densities were read at 595nm on an ELISA reader. Optical densities were reported to the standard curve for concentration calculation.

2.2.2.5 Pulldown assay

Cell lysates were passed over the soluble CD44s and soluble CD44v7 sepharose column. Column was washed with PBS and finally Elution was done using a 0.2M glycine pH2.7 buffer. Eluted fractions were collected in 1:20 volume 1M Tris buffer pH8.0 then concentrated and dialyzed against cold PBS using Vivaspin tubes (50 Kda cut off). Protein concentration was measured by Biorad assay. Proteins were separated using SDS-PAGE.

2.2.2.6 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

Protein components were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS, an anionic detergent denatures and imparts negative charge to all proteins in the sample. Proteins can be separated according to their size in an electrical field. Two gels were used for this purpose. At the bottom resolving gel was poured to separate the proteins and at the top a stacking gel. The stacking gel concentrates all proteins in one band and allows them to enter the resolving gel at the same time; it can be mounted with a comb to load samples in the wells. Before loading the gel, samples were boiled for 5 minutes at 95° C for proteins to allow complete protein denaturation. Around 25-30 µl of sample was loaded into wells. The inner and outer chambers were filled with running buffer (1x).

2.2.2.7 Immunoprecipitaion

Freshly harvested cells from bone marrow or cultured cells (10^7) washed 3 times in cold PBS were lysed in 1ml HEPES buffer containing protease inhibitor and phosphate inhibitors as well as detergent (mainly 1% Lubrol) for 1 hour at 4°C on a rocking platform. Lysates were then centrifuged 10min at 13,000 rpm, 4°C. Lysed supernatants were collected. Immunoprecipitation was done using 1 to 5 µg specific monoclonal or polyclonal antibodies at 4°C on a rocking platform for over night. Following over night incubation at 4°C, 5% of protein G-sepharose was added to the precleared lysates for 1 hour. Protein G coupled antibody was washed 3 times with lysis buffer completed with detergent. Sepharose pellet was resuspended in 80µl Laemmli buffer and boiled 5min at 95°C. After 1min centrifugation at maximum speed, 20µl of the supernatant were taken and load on acrylamide gel to be resolved by SDS-PAGE. If indicated, cells were biotinylated before lysis and immunoprecipitation. Washed cells were resuspended in HEPES buffer containing 0.1mg/ml NHS-water soluble biotin. Biotinylation was performed 30min under agitation at 4°C. Reaction was terminated by 3 washes with 0.2M glycine PBS solution. Cells were lysed 1 hour at 4°C in 1ml HEPES buffer containing protease and phosphatases inhibitors as well as detergent of choice. Following steps were similar as described above.

2.2.2.8 Western Blotting

Following gel electrophoresis proteins were electroblotted onto a nitrocellulose membrane (Amersham Biosciences) overnight at 30 volts and analysed by immunoblotting using specific primary and secondary antibodies. The gel, foam pads and 3MM Whattmann papers were equilibrated in blotting buffer; the gel was placed on the membrane which in turn was placed on Whattmann paper followed by foam pads on either side. The whole set was placed in a cassette holder, followed by a tank blotting apparatus such that the membrane was placed towards the anode side. After overnight transfer the blots were blocked with PBS/5% Milk powder or BSA (for phosphospecific antibodies) for 1 hour, followed by primary antibody for 1 hour. Blots were washed with PBS/0.1%Tween thrice and 5 minutes each wash. Then the blots were incubated in secondary antibody for an hour, followed by washing again. The blots were developed with Enhanced Chemiluminescence system (ECL, Amersham Biosciences) and exposed to X-ray film (Amersham Biosciences) for desired time points and developed.

2.2.2.9 Silver staining of protein gels

After separation of proteins by SDS-PAGE, gels were fixed overnight in 30% ethanol/10% acetic acid and sensitized for 45min in 0.3% potassium tetrathionate, 0.5M potassium acetate, 30% ethanol. This was followed by 6 washes, totally for 1h with bidest water. Gels were stained with 0.2% silver nitrate for 1-2hr, rinsed with bidest water and developed for upto 40min in developer (3% potassium carbonate, 31µl $Na_2S_2O_3$-$5H_2O$ (10%), 75µl formalin (37%) per 250ml). The reaction was stopped by adding 330mM Tris/ 2% acetic acid and gels were kept in bidest water.

2.2.3 Cell biology

2.2.3.1 Cell culture

Cells were grown in a humidified incubator at 37°C, 5% CO_2. Cells were maintained in RPMI or ISCOVE's medium in 10% FCS as per the requirements and split when they reached confluency. Cells were usually passaged at a ratio

of 1:4. Trypsin (0.25%) (w/v) in PBS was used to disperse adherent cells and reseeded to fresh culture flasks.

For long term cell storage, cells were washed once with medium and frozen in cryovials in FCS, 10% DMSO. The vials were placed for 1 hour at -80°C before transferring into liquid nitrogen. Cells were thawed from the cryovials by placing the vials from liquid nitrogen first on ice, followed by 37°C water bath and immediately placed in falcon containing medium and centrifuged at 1600 rpm for 5 minutes. The medium was sucked off and new medium was added and transferred to flasks at high density to maximise recovery.

Cell viability was determined using hemacytometer and trypan blue staining.

Tryptan blue (2x): 0.4% Trypan blue (4vol)

4.5% NaCl (1vol)

2.2.3.2 Magnetic Beads separation

Bone marrow cells (BMC) were stepwise depleted of $CD4^+$, $CD8^+$ and NK cells, $CD19^+$, $CD45^+$, $CD11c^+$ and $CD11b^+$, $Ter119^+$ and $Ly6C/G^+$ cells by magnetic beads coated with the respective antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). The depleted cells population was incubated with anti-CD117-coated beads collecting the adherent fraction. The $CD117^+$ cells mostly were further enriched for HSC by incubation with biotinylated anti-SCA1 and anti-biotin coated beads, again collecting the adherent population. The regain of $CD117^+$ cells varied between 2.6% to 5.3%. In BALB/C mice, roughly 30% of $CD117^+$ cells were $SCA1^+$, in SVEV the recovery of $SCA1^+$ cells from $CD117^+$ cells varied from 87% to 100%. Viability of the separated populations was >95%.

2.2.3.3 Flow cytometry

Fluorescent activated cell sorting (FACS) allows cell segregation based on size and volume and also allows detecting expression levels of proteins in cells. This method is based on diffraction of light and measure of fluorescence which reflects cell size and amount of fluorescent antibody labelled cells.

Cells were washed in PBS/0.5% BSA. About $5x10^5$ cells were added to round bottomed 96 well plates. After centrifugation cells were suspended in 50 µl of primary antibody diluted in PBS/0.5% BSA and incubated for half an hour on ice. This was followed by washing the cells thrice with PBS/0.5%BSA, 5min each wash. Secondary antibody (50µl) coupled to fluorochrome was added to each well and incubated for half an hour on ice in the dark. The cells were again washed. After the last wash cells were suspended in 200µl PBS/0.5%BSA for immediate measuring. Staining was evaluated using FACS-Calibur (Becton Dickinson, Heidelberg, Germany). In case of double or triple fluorescence the same procedure was repeated with adequate antibodies and blocking steps wherever necessary (e.g. different antibodies form the same species).

For cytokines, intracellular FACS was performed. The cells were first incubated with formalin 1% to fix them for 20 minutes on ice. The cells were washed with PBS 1%BSA 3x as mentioned above. Thereafter the cells were incubated with 0.1 % Tween for 15 minutes on ice to create pores on the cell membrane and facilitate the entry of antibodies against the cytokines into the cells. The cells were again washed and antibodies were added following the usual protocol.

Mitochondrial integrity was determined by DilC1 staining, measuring fluorescence in the FL4 channel. The cell cycle was controlled by PI staining (1µg/ml, 0.1% TritonX-100, RNAase A) after fixation in ethanol. Samples were processed in a FACS-Calibur using the Cell Quest program for analysis (BD, Heidelberg, Germany).

2.2.3.4 Apoptosis Assay

Apoptosis assay was performed using Annexin V-FITC and propidium iodide (PI) (R & D systems, Wiesbaden-Nordenstadt, Germany) double staining. Early apoptotic cells bind to Annexin V because of the exposed phosphatidylserine on the outer cell membrane. Late apoptotic cells are positive for Annexin V and PI. Necrotic cells bind only PI. BMC and HSC were seeded on plastic, HA or LTBMC-stroma for 24 hour and 48 hour in the presence or absence of 5µg/ml cisplatin. After the desired time points cells were transferred to 96 well plates and plates were centrifuged at 1600 rpm for 5 minutes and washed with PBS/1% BSA. Cell labelling was performed according to manufacturer's instructions. Cell were incubated in the dark at RT for 15 min and detected by FACS using the FL1 channel for Annexin FITC and FL-3 channel for PI.

2.2.3.5 Proliferation

BMC and HSC were labelled with CFSE and cells were seeded on plastic or HA or on LTBMC-stroma. To check for the proliferative activity, cells were evaluated for 72 hour in the presence or absence of anti-CD44 or anti-CD49d. Cell division was evaluated by CFSE dilution and detected by FACS using FL1 channel. To label the cells with CFSE, cells were washed with PBS 2 times and 2-3 µg/ml CFSE (pre diluted in PBS) was added to the cells. Cells were incubated at 37°C for 30min. To quench the reaction, 1 volume cold FCS was added to the cells. Cells were washed with RPMI medium 2 times and cells were resuspended in RPMI medium and kept at 37°C for 20min. After incubation, cells could be used for the seeding for different time points.

2.2.3.6 Phosphatase assay

Cell lysates, centrifuged at 12,000g for 10min, were incubated with anti-PP2A and ProteinG-agarose (Roche, Mannheim, Germany) (2h, 4°C). The immunoprecipitate was washed and incubated with 900µg/ml p-NPP (30min, 37°C). The amount of para-nitrophenol produced by dephosphorylation was determined by measuring the absorbance at 405nm.

2.2.3.7 Sucrose gradient centrifugation

Cells were lysed in 1% Lubrol, lysates were centrifuged (10min, 20,000g), adjusted to 40% sucrose (4.5ml) and were layered on 1.3ml of 50% sucrose and overlaid with 2.3ml of 30%, 2.3ml of 20%, and 1.3ml of 5% sucrose. After centrifugation (200,000g, 16h), 12 fractions (1ml) were collected from the top of the tubes. Fractions 1-4, 5-8 and 9-12 were pooled and precipitated with anti-CD44.

2.2.3.8 Cytosol, nuclei and mitochondria preparation

Cells were incubated in hypotonic buffer, homogenized and centrifuged at 800rpm for pelleting the nuclei. For separating the cytosolic from the mitochondrial fraction, $2.5x10^6$ cells were lysed in 0.5ml lysis buffer. After adding Nonidet-P40 (0.5%), vortexing, and centrifugation (1600rpm, 5min), cytosolic proteins are recovered from the supernatant. The pellet (mitochondria) and the pelleted nuclei were washed, resuspended in lysis buffer (1% TritonX-100, 1% SDS) and sonicated (7sec, 9cycles).

2.2.3.9 Transfection of adherent cells

HEK-293 EBNA cells were transfected using Polyfect reagent from Qiagen following manufacturer's instructions. Briefly, 2µg pCEP-puromycin modified vector were diluted in 0.1ml ISCOVE's (without serum and antibodies) with 20µl Polyfect and incubated 10 min at room temperature. Meanwhile, cells at 40-80% confluency in a 6-well-plate were given 1.5ml fresh complete ISCOVE's 10% FCS, 2mM L-glutamine, 250µg/ml neomycin per well. After 10min, 0.6ml complete medium was added to the DNA solution and distributed homogeneously on cells; the plate was rocked gently to assure equal distribution of DNA on the well, and then transferred to 37°C, 5% CO2 culture conditions. After 24hr culture, cells were passed in a $75cm^2$ flask and 24hr later puromycin selection was done with 0.5µg/ml. Cells were tested 2 weeks later for soluble molecule presence in the supernatant by western-blot.

2.2.4 Animal experiments

2.2.4.1 Long term bone marrow culture (LTBMC)

Bone marrow cells (BMC) were collected from femur and tibiae of 6-10 weeks old mice by flushing the bones with PBS through a 27 gauge needle. Cells were washed and seeded at a density of 2 X 10^6 cells / ml in ISCOVE's Minimal essential medium supplemented with 20% horse serum (HS), 25µm 2-ME (2-Mercapto Ethanol), 2mM L-glutamine and 10 µm Hydrocortisone. Medium was exchanged very carefully weekly. After 6 week of culture, the monolayer of adherent stroma cells (LTBMC-stroma) was used for further analysis.

2.2.4.2 Reconstitution and tumor cell injection

C57BL6 mice received an i.v. or s.c. injection of 10^4 EL4 cells or were lethally irradiated (9.5Gy) and reconstituted with $1x10^7$ T cell-depleted BMC and received EL4 cells 2days after reconstitution. Both groups of mice received twice per week an i.v. injection of 150µg control IgG or anti-panCD44 (IM7). Survival time and reconstitution were controlled. In a second setting, C57BL6 mice were treated as described above, but received $2x10^6$ CFSE-labeled EL4 cells. The % of CFSE-labeled EL4 cells and of apoptotic (AnnexinV-APC stained) CFSE-labeled EL4 cells in bone marrow, thymus and spleen was evaluated during 96hrs. Animal experimentations were approved by the governmental authorities of Baden-Wuerttemberg, Germany.

2.2.5 Statistical analysis

Significance of differences was calculated according to the Student's T test (*in vitro* studies). Functional assays were repeated at least 3 times. Mean±SD of *in vitro* studies are based on 3-4 replicates.

3: Results

Part I: The importance of CD44 in the crosstalk between HSC and bone marrow stroma

1. CD44 expression, associating molecules and ligands on HSC and niche cells

CD44 has been demonstrated to be required for HSC maintenance as well as their retention in the osteoblastic niche. It is largely unknown whether expression of CD44s or CD44v is required on HSC or the cells of the osteogenic niche. To answer these questions, I characterized bone marrow cells and as a surrogate for the osteogenic niche long term bone marrow culture stroma and S17 cells, a murine stromal cell line.

1.1 CD44 expression on BMC and LTBMC-stroma cells

HSC are a small subpopulation of BMC. To characterize this small subpopulation, it was essential to separate it from committed progenitors and mature leukocytes in the bone marrow. This has been achieved by selection with antibody coated magnetic beads. The efficacy of separation was controlled by flow cytometry, which evaluated several stem cells, progenitors and mature leukocyte marker expression in the selected sub populations.

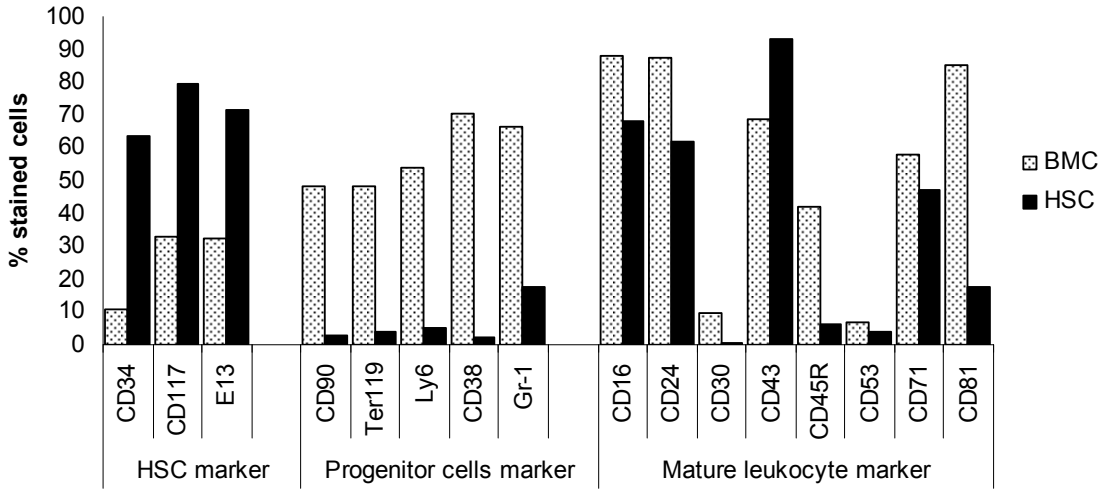


Figure 1A: Expression of stem cells, progenitors and mature leukocyte marker in BMC/HSC. Unseparated and separated populations of Bone marrow cells (BMC) including Hematopoietic stem cells (HSC) were analyzed for HSC, Progenitor and Mature leukocyte markers by flow cytometry.

These stem cells, progenitor and mature leukocyte markers were known to be expressed on mature progenitor cells in the hematopoietic lineage. CD117 and SCA-1 (E13 antibody) are known markers for HSC population and in Fig.1A expression of these markers was high in HSC. Progenitor cell markers i.e. CD90 (Thy-1), Ter119, Ly6 (hematopoietic marker), CD38 and Gr-1 (granulocyte marker) were highly expressed in unseparated BMC. Mature leukocyte markers CD16 (found on NK cells), CD24 (expressed on granulocytes and B cells), CD71 (expressed on activated T and B lymphocytes, macrophages) and CD43 (leukocyte marker) were expressed in high levels in BMC as well as in HSC while CD81 (leukocyte marker) was found to be high only on BMC.

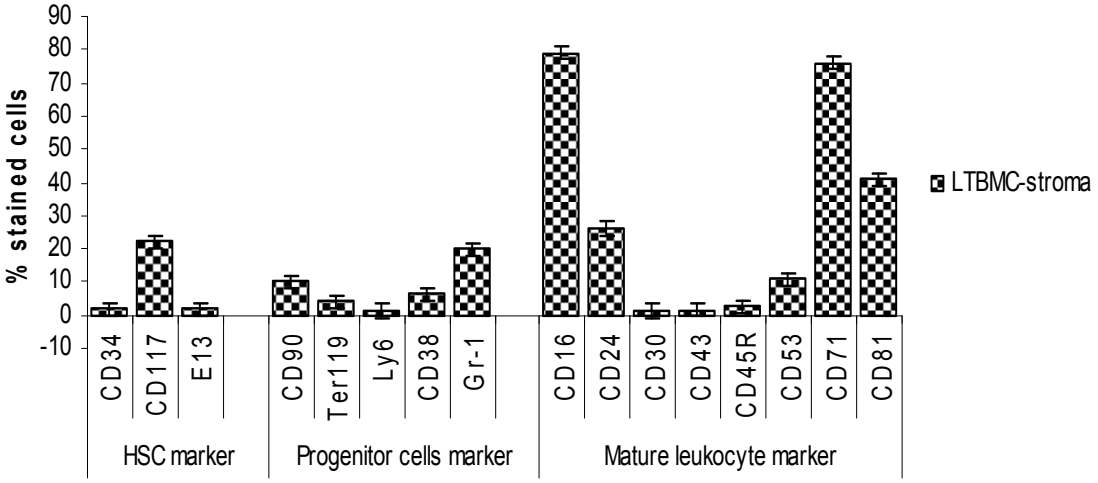


Figure 1B: Expression of stem cells, progenitors and mature leukocyte marker in LTBMC- stroma. Long term bone marrow culture –stroma (LTBMC-stroma) were analyzed for HSC, Progenitor and Mature leukocyte markers by flow cytometry.

Same markers were checked on LTBMC-stroma and expression of CD16 and CD71 was found to be high (Fig. 1B).

Next, I characterized CD44 and CD44 variant isoforms on BMC sub populations by flow cytometry and RT-PCR.

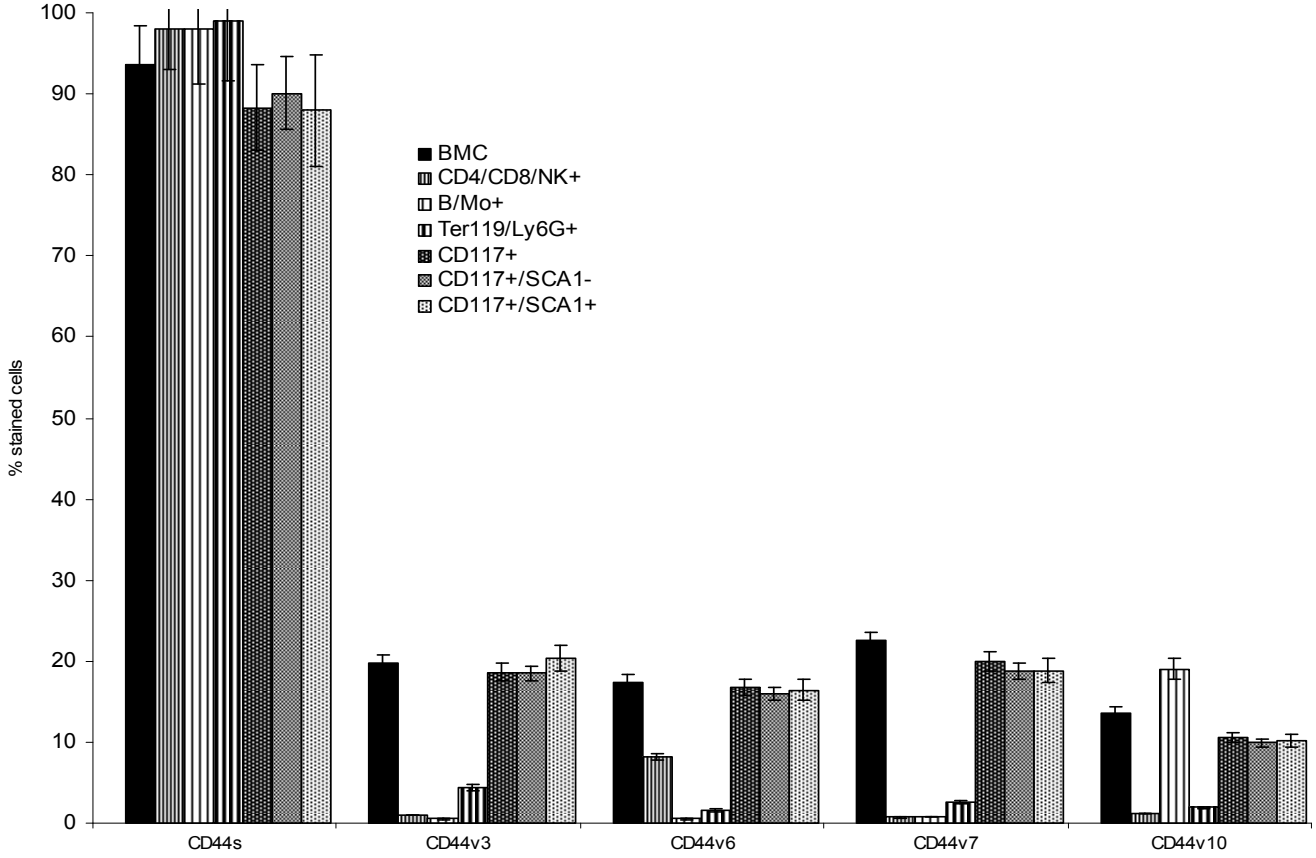


Figure 1C: Expression of CD44 and CD44v in BMC sub populations. Unseparated and separated populations of Bone marrow cells (BMC) including Hematopoietic stem cells (HSC) were analyzed for CD44 and CD44v expression by flow cytometry.

A flow cytometry analysis for CD44s, CD44v3, CD44v6, CD44v7 and CD44v10 on bone marrow cells (BMC), mature leukocytes within the bone marrow, committed progenitors of the erythroid and myeloid lineages, CD117+/SCA1- and CD117+/SCA1+ HSC showed high expression for CD44 while CD44v7, CD44v10 and CD44v6 were mainly expressed by HSC and committed progenitors (Fig. 1C).

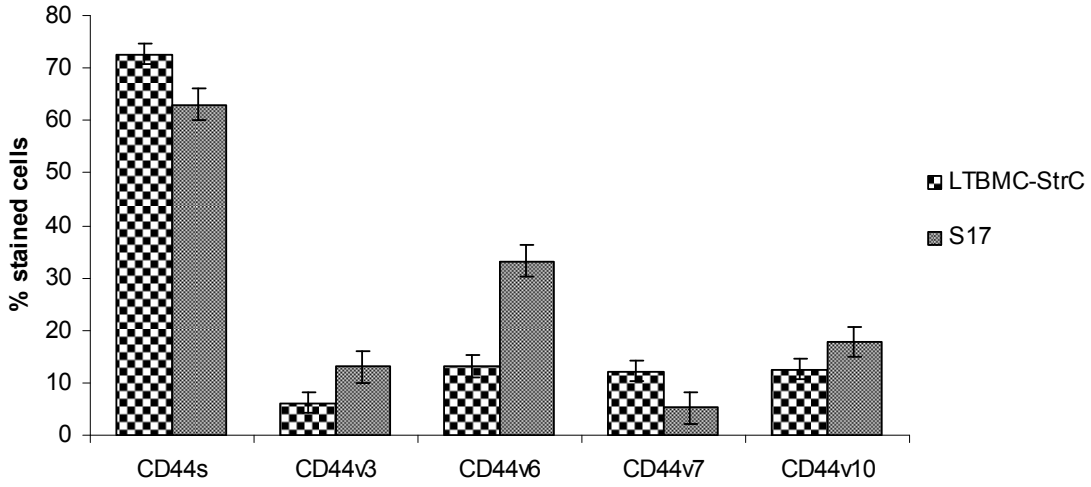


Figure 1D: Expression of CD44 and CD44v in LTBMC-stroma and S17 cells. Long term bone marrow culture-stroma cells (LTBMC-stroma) and S17 cells (murine stromal cell line) were analyzed for CD44 and CD44v expression by flow cytometry.

Expression of CD44s, CD44v3, CD44v6, CD44v7 and CD44v10 was checked on LTBMC-stroma and S17 cells, which were taken as surrogate niche cells. CD44s was highly expressed in both while CD44v6 was expressed by S17 cells and CD44v7 was predominantly expressed by LTBMC-stroma (Fig. 1D).

A semi-quantitative RT-PCR analysis of unseparated bone marrow and LTBMC-stroma revealed that in bone marrow CD44s, CD44v7 and CD44v10 were highly expressed and also revealed that CD44v8 and CD44v9 were always come together with CD44v10 isoforms. In LTBMC-stroma CD44v7 was highly expressed but CD44v3 and CD44v6 showed low expression, which was found to be very weak in the bone marrow. CD44v8 and CD44v9 showed expression in combination with CD44v10 (Fig. 1E).

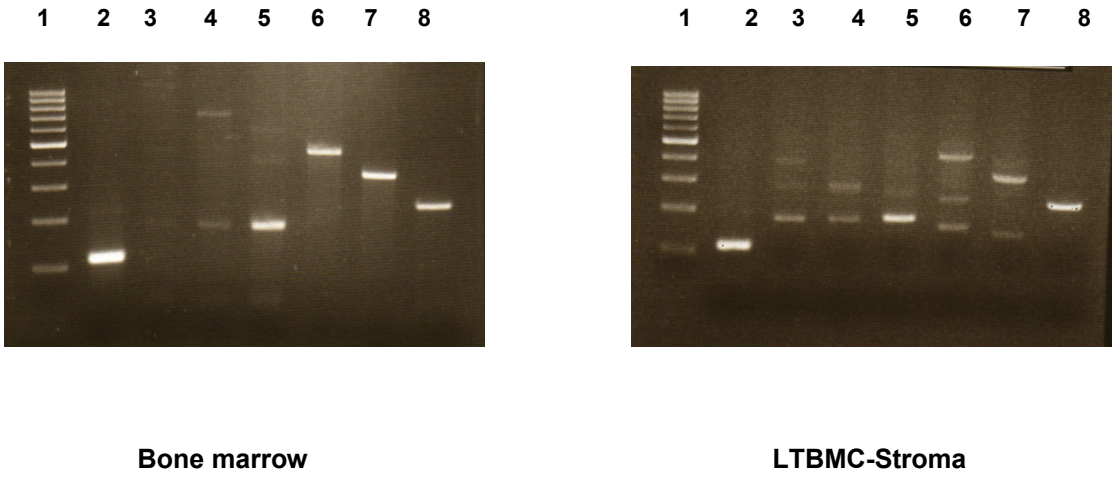


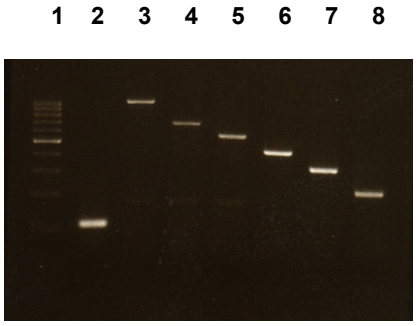
Figure 1E: RT-PCR for BM and LTBMC-Stroma: cDNA was prepared from bone marrow cells (BMC) and long term bone marrow culture-stroma (LTBMC-stroma) and semiquantitative RT-PCR was made under specific conditions.

Lane 1: Marker 100bp.

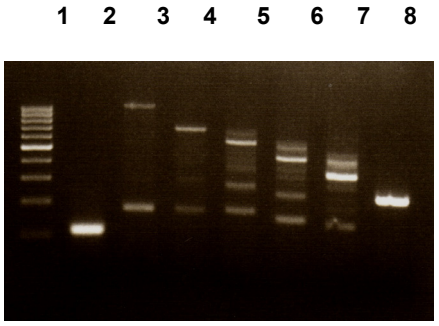
Lane 2,3,4,5,6,7,8: CD44s, CD44v3, CD44v6, CD44v7, CD44v8, CD44v9 and CD44v10 respectively.

1.1.1 RT-PCR profiling on BMC subpopulations

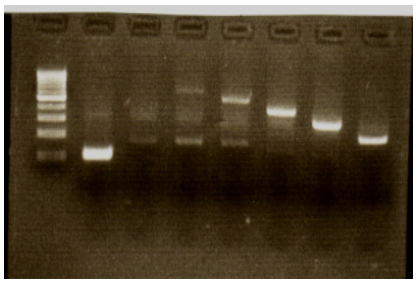
A semiquantitative RT-PCR analysis of mature leukocytes within the bone marrow, committed progenitors of the erythroid and myeloid lineage and CD117+/SCA1+ cells (HSC) revealed that HSC mostly express CD44v7 and CD44v10 while mature committed progenitors showed CD44v10 and CD44v10 combined with all other isoforms (Fig. 1F).



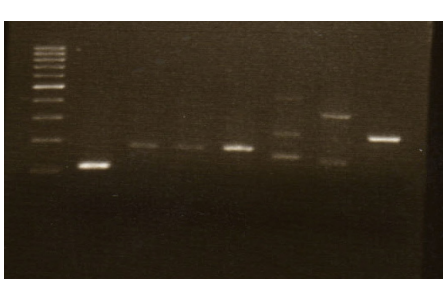
NK/CD4/CD8/ter/ly6/CD19/CD11c+



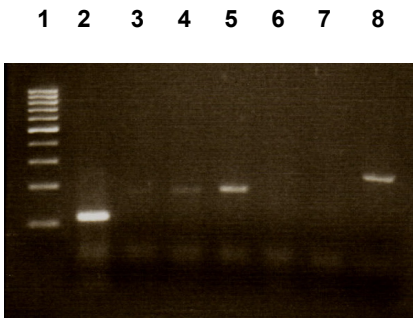
CD117-/SCA1-



CD117- /SCA1+



CD117+/SCA1-



CD117+/SCA1+

Figure 1F: RT-PCR of separated BMC subpopulations: cDNA was prepared for indicated subpopulations and semiquantitative RT-PCR was made under specific conditions.

Lane 1: Marker 100bp

Lane 2,3,4,5,6,7,8: CD44s, CD44v3, CD44v6, CD44v7, CD44v8, CD44v9 and CD44v10 respectively.

1.1.2 Adhesion molecule profile in BMC and LTBMC-stroma cells

CD44 has been described to be essential for the interaction between HSC and their niche. Yet other adhesion molecules have also been described to be involved. Furthermore, besides binding to HA, CD44 also binds to specific ligands and can associate with integrins or cell adhesion molecules. Therefore it became important to define the adhesion molecule profile on both HSC and LTBMC-stromal cells.

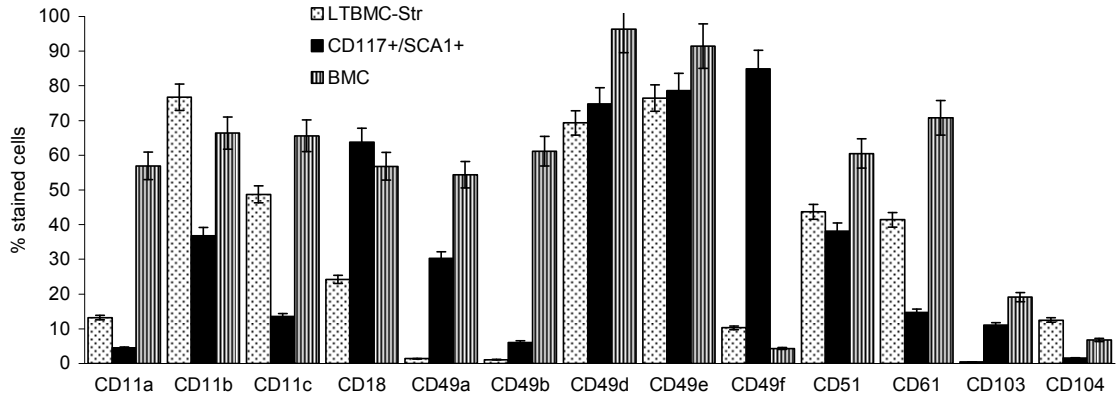


Figure 1G: Integrin expression profile on BMC, HSC and LTBMC-stroma cell. Unseparated bone marrow cells (BMC), hematopoietic stem cells (HSC) and long term bone marrow culture (LTBMC-stroma) were analyzed for Integrins by flow cytometry.

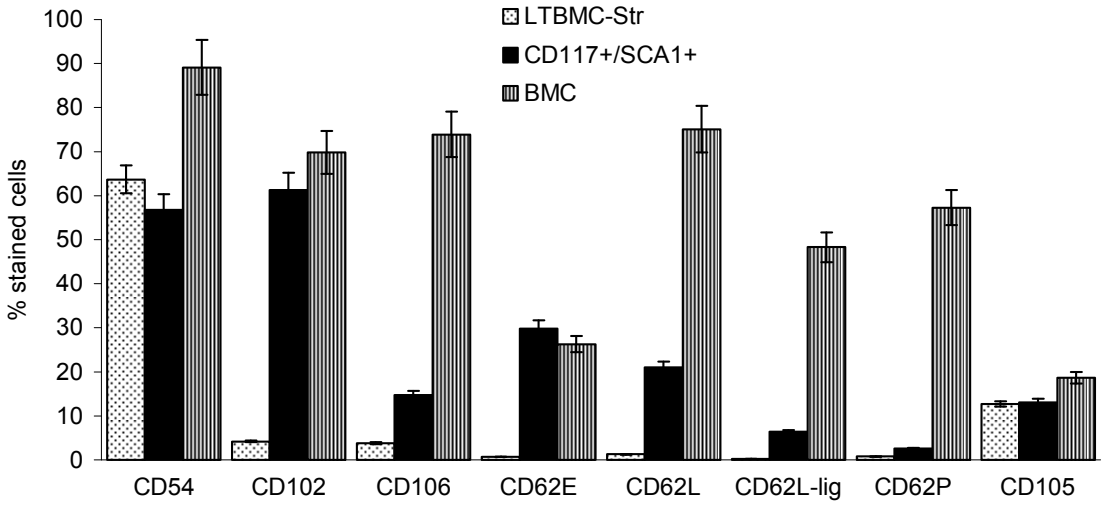


Figure 1H: Expression of cell adhesion molecule and selectins on BMC, HSC and LTBMC-stroma cell. Unseparated bone marrow cells (BMC), hematopoietic stem cells (HSC) and long term bone marrow culture (LTBMC-stroma) were analyzed for cell adhesion molecules and selectins by flow cytometry.

CD18 (Leukocyte marker), CD49d (alpha-4 integrin, essential to the differentiation and traffic of hematopoietic stem cells) and CD49e were highly expressed on BMC and HSC. CD11b (monocytic marker), CD49d and CD49e were found on LTBMC-Stroma cells. ICAM molecule CD54 was predominantly expressed in BMC, HSC and LTBMC-stroma (Fig. 1G & 1H).

1.1.3 Cytokines and cytokine/chemokine receptor expression on BMC and bone marrow stroma cells

Cytokines are a broad family of proteins that mediate HSC mobilization and homing. In BMC and HSC transendothelial trafficking is dependent on the expression of adhesion molecules, which is induced by different cytokines and chemokines and their receptors. Therefore, cytokines and cytokine receptor expression was evaluated by flow cytometry. IL-3 and IL-7 stimulates the differentiation of HSC into mature progenitors and SDF-1 is a known ligand for chemokine receptor, CD184 (stromal cell-derived factor-1 receptor) which was found to be very high in BMC, HSC and LTBMC-stroma. GMCSF, IL3, IL7 and SDF-1 were frequently expressed by HSC (Fig. 1I).

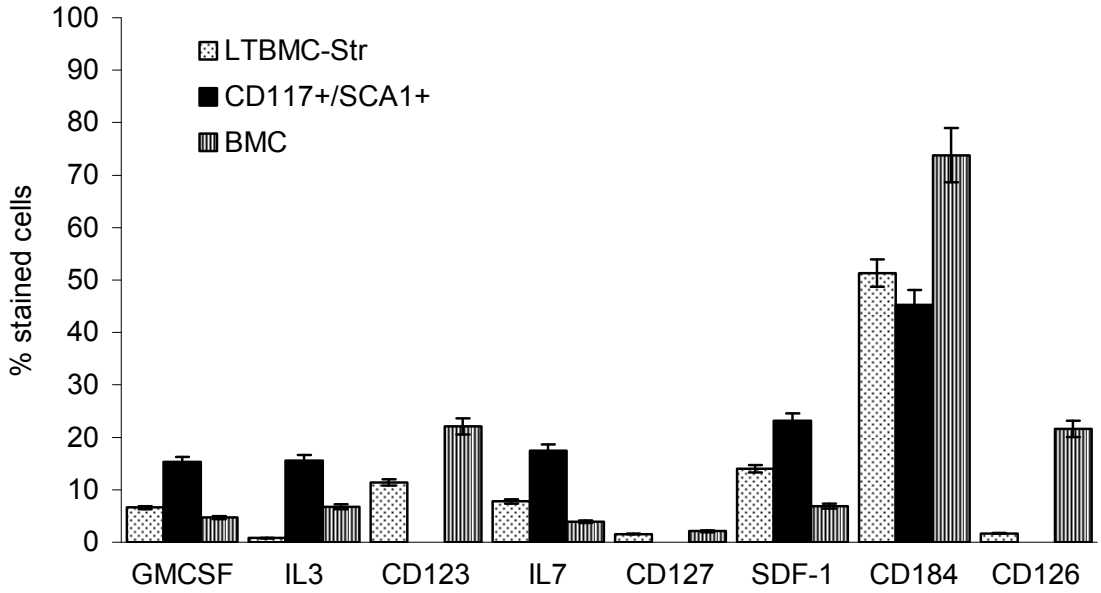


Figure 1I: Cytokines and cytokines receptors expression on BMC, HSC and LTBMC-stroma cells. Unseparated bone marrow cells (BMC), hematopoietic stem cells (HSC) and long term bone marrow culture (LTBMC-stroma) were analyzed for cytokines and cytokine receptors by flow cytometry.

These data showed that HSC and LTBMC-stroma share high expression of CD44. HSC differed from LTBMC-stroma in CD44 variant expression. HSC mostly expressed CD44v7 and CD44v10. LTBMC-stroma predominantly expressed CD44v7, mature leukocyte marker CD16 and CD71. Additional adhesion molecule like CD102 (ICAM-2, intercellular cell adhesion molecule-2), CD49e integrin were found in HSC and LTBMC-stroma as well.

Most prominent markers CD49d and CD54 were found on HSC and LTBMC-stroma cells. Similarly the chemokine receptor marker CD184 was predominantly expressed in BMC/HSC and LTBMC-stroma.

1.2 CD44 associating molecule in HSC and bone marrow stroma cells

1.2.1 CD44 co-expression with stem cell, progenitor cell and mature leukocyte markers on BMC/HSC and LTBMC-stroma cells

CD44 has been demonstrated to be essential for the interaction between HSC and their niche. Therefore, it became important to analyze the co-expression of CD44 with stem cell, progenitor cell and mature leukocyte markers on BMC/HSC and LTBMC-stroma.

Double fluorescence staining provided evidence that CD44 is co-expressed with different stem cells, progenitor and mature leukocyte markers on BMC. In BMC, CD44v6 and CD44v7 showed co-expression with leukocyte markers CD43 and CD81 (expressed on hematopoietic cells). They were also co-expressed with progenitor and stem cell markers, Gr-1 (granulocyte marker) and CD117. CD44v10 was co-expressed with majority of stem cell, progenitor and leukocyte markers, but showed low co-expression with CD30 (Fig.2A)

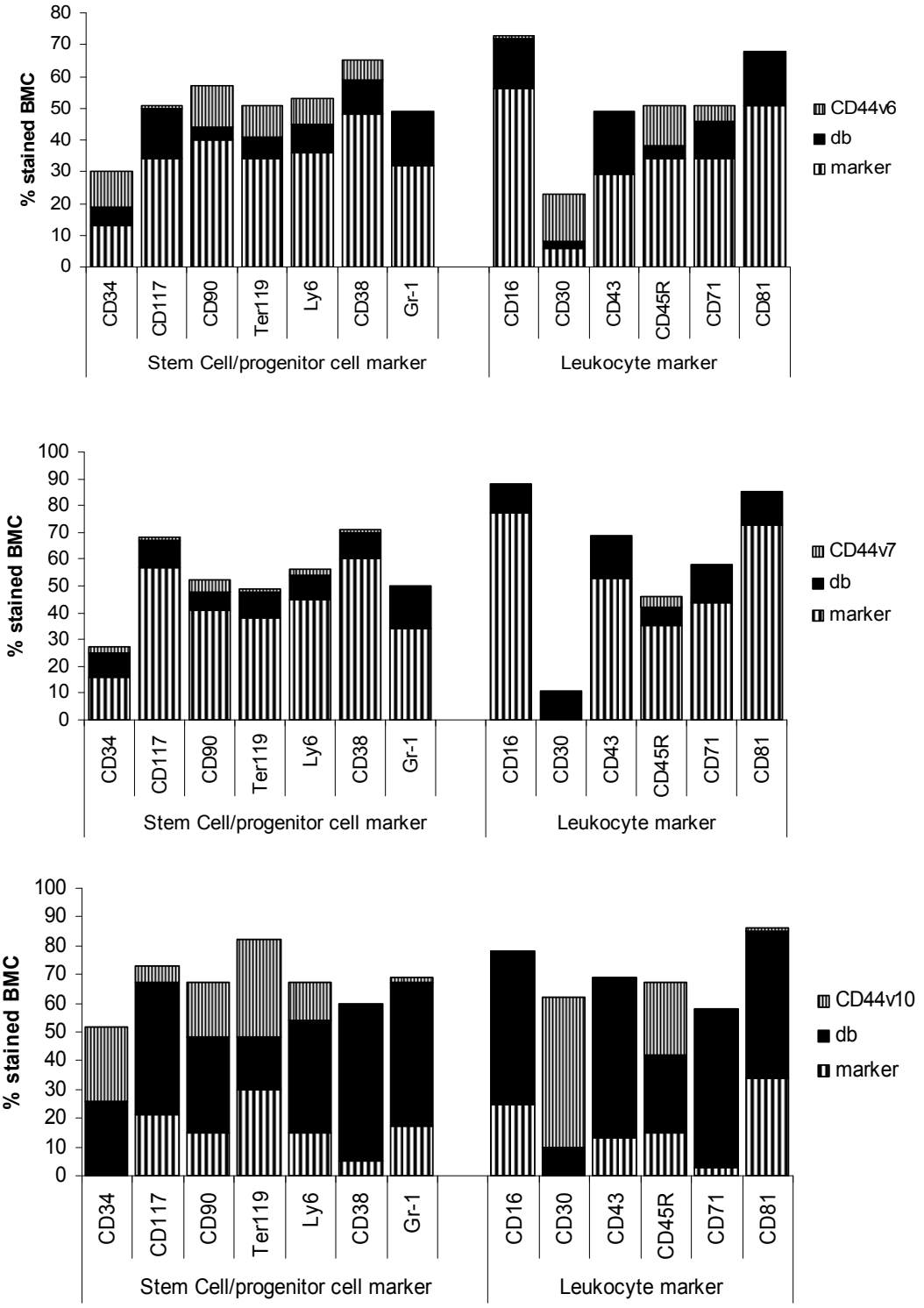


Figure 2A: CD44 co-expression with stem cells, progenitors and mature leukocyte markers on bone marrow cells (BMC). Bone marrow cells were double stained with anti-CD44v6, anti-CD44v7 and anti-CD44v10 and the indicated markers. The percentage of CD44+marker-, CD44+marker+ and CD44-marker+ cells was shown.

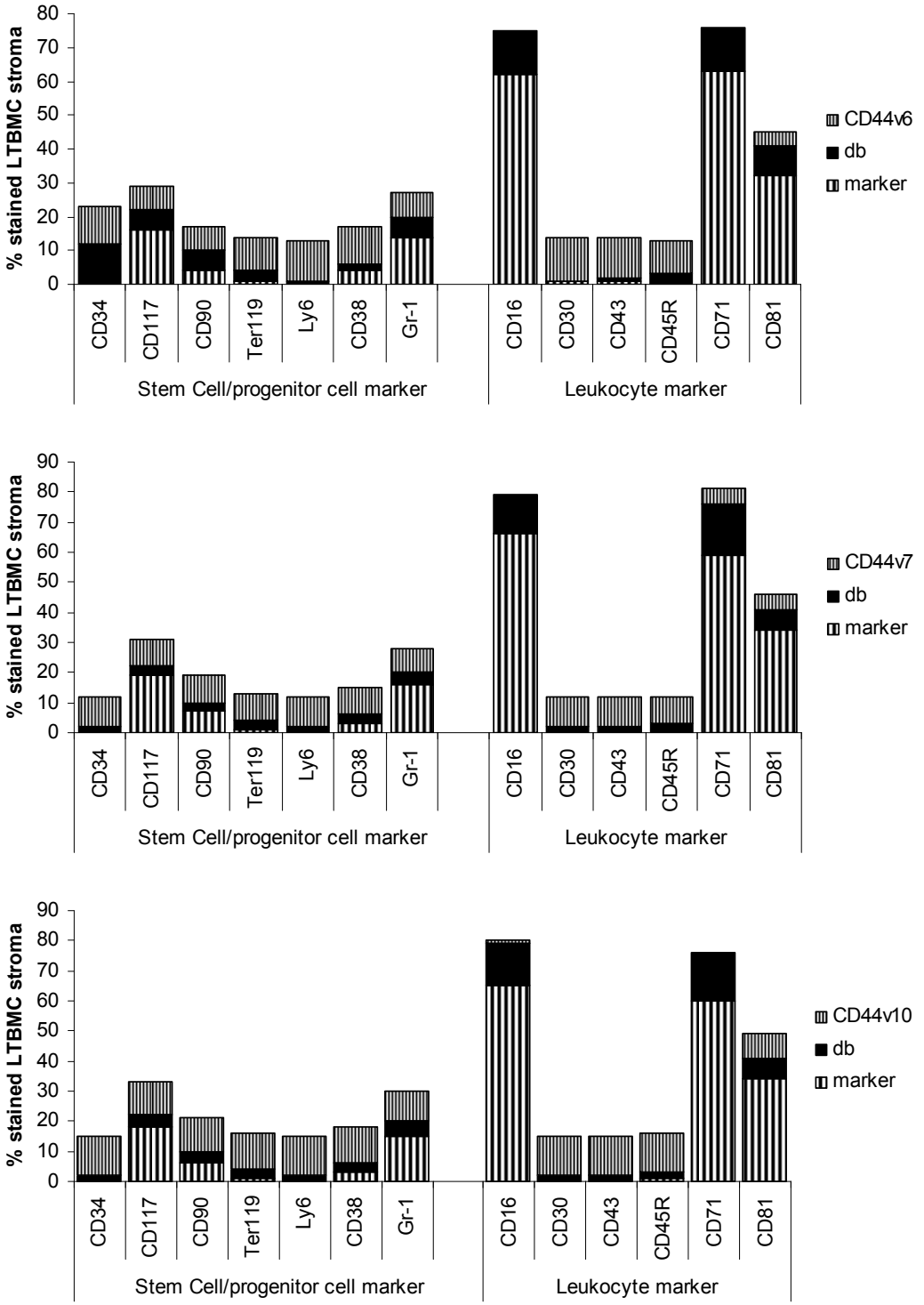


Figure 2B: CD44 co-expression with stem cells, progenitors and mature leukocyte markers on long term bone marrow culture stroma (LTBMC-stroma). Long term bone marrow culture-stroma cells (LTBMC-stroma) were double stained with anti-CD44v6, anti-CD44v7 and anti-CD44v10 and the indicated markers. The percentage of CD44+marker-, CD44+marker+ and CD44-marker+ cells was shown.

CD44 also co-expressed with different markers in LTBMC-stroma. CD44v6, CD44v7 and CD44v10 were co-expressed with CD16 and CD71, but partly with CD81, CD117 and Gr-1 (Fig. 2B).

1.2.2 CD44 co-expression with cytokine/chemokine receptors on BMC/HSC and long term bone marrow-stroma cells

As outlined above the chemokine receptors CD184 and cytokines IL-3 and IL-7 are supposed to mediate differentiation and proliferation of HSC. CD184 might promote homing of HSC into bone marrow niche and also mediates chemotaxis in mature and progenitor blood cells and, together with its ligand SDF-1, is essential for B lympho- and myelopoiesis. Therefore, I checked co-expression of CD44 with cytokine and chemokine receptors. CD44v6, CD44v7 and CD44v10 showed co-expression with CD184 (SDF-1 receptor) chemokine receptor on bone marrow cells. CD44v10 is also co-expressed with CD123 (IL3 receptor) and CD126 (IL6 receptor) on BMC (Fig.2C).

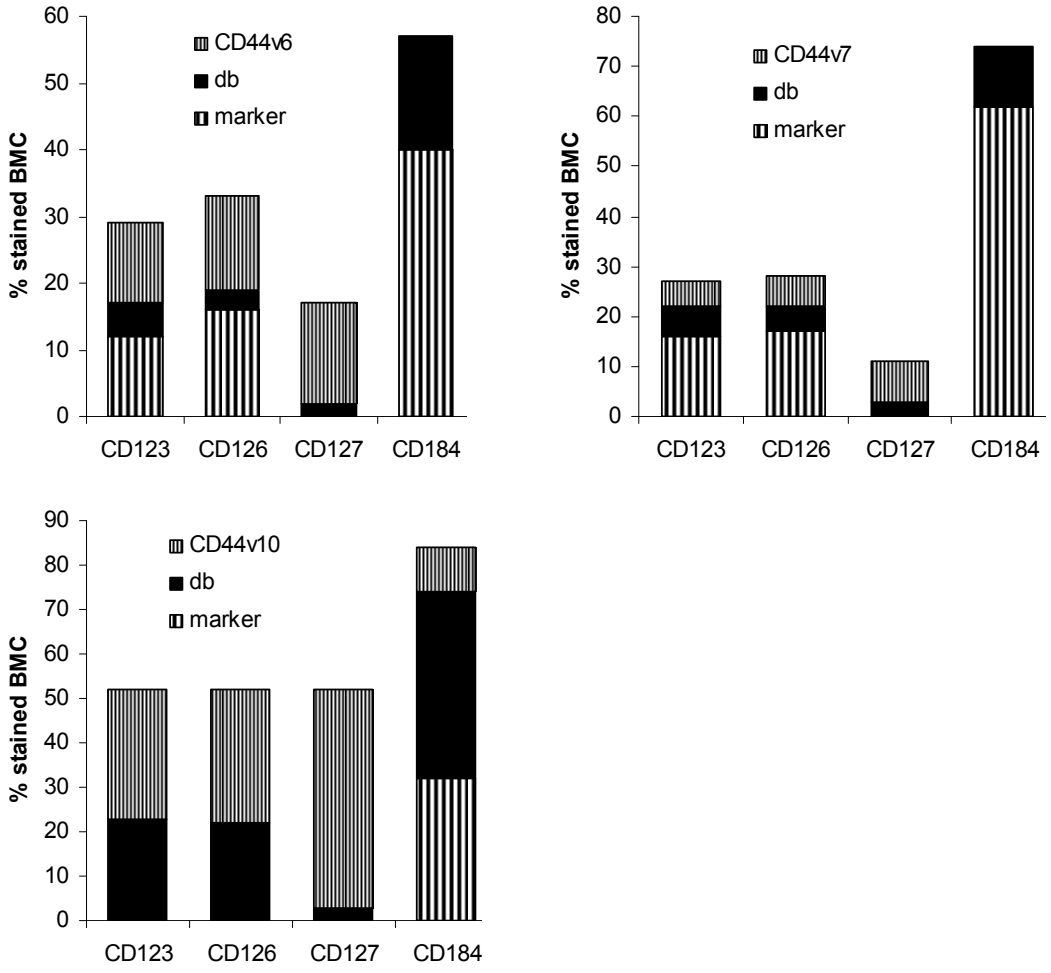


Figure 2C: CD44 co-expression with cytokine/chemokine receptors on bone marrow cells (BMC). Bone marrow cells were double stained with anti-CD44v6, anti-CD44v7 and anti-CD44v10 and cytokine and chemokine receptor markers. The percentage of CD44+marker-, CD44+marker+ and CD44-marker+ cells was shown.

In LTBMC-stroma cells, CD44 is pre-dominantly co-expressed with CD184 (Fig. 2D).

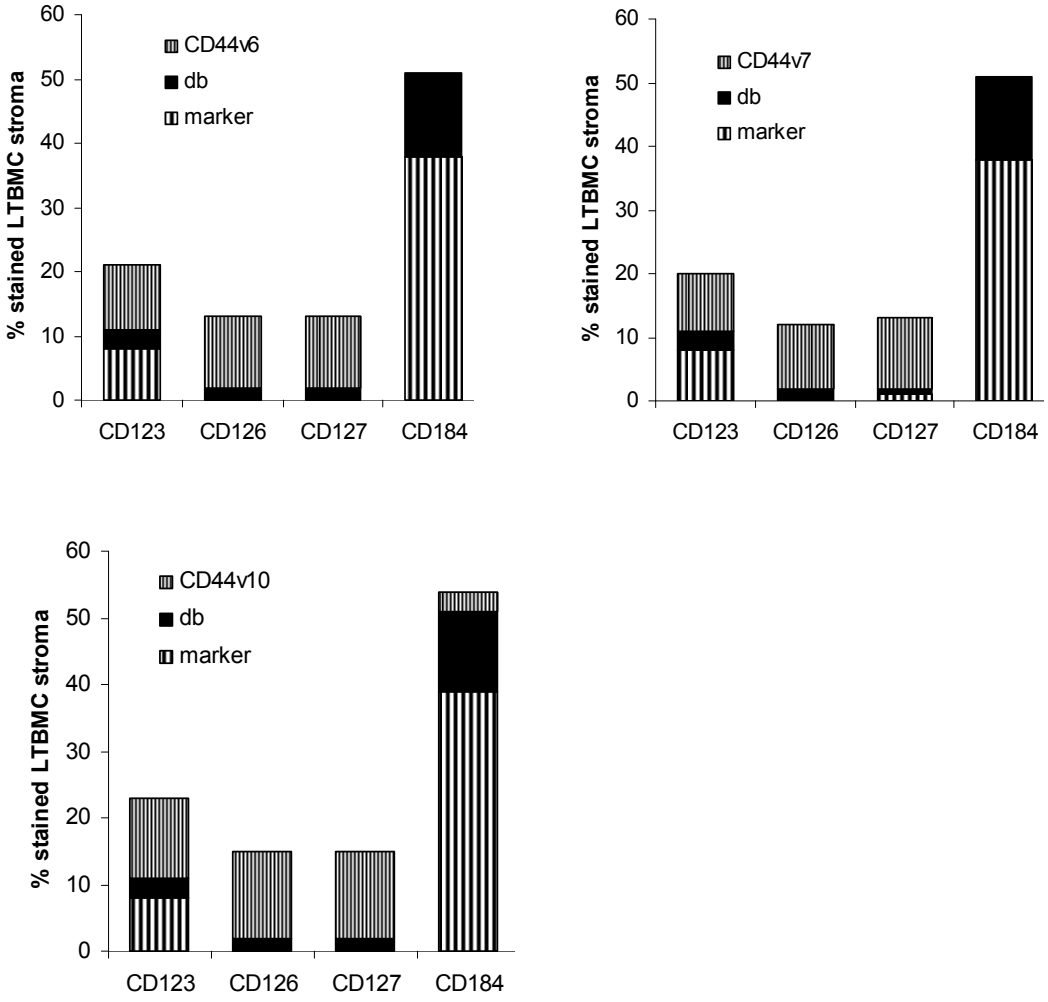


Figure 2D: CD44 co-expression with cytokine/chemokine receptors on long term bone marrow culture (LTBMC-stroma). Long term bone marrow cells were double stained with anti-CD44v6, anti-CD44v7 and anti-CD44v10 and cytokine and chemokine receptor markers. The percentage of CD44+marker-, CD44+marker+ and CD44-marker+ cells was shown.

1.2.3 CD44 co-expression with adhesion molecules on BMC/HSC and bone marrow stroma cells

The adhesion molecules are involved in the cellular interactions between HSC, LTBMC-stroma cells and BMC to regulate hematopoiesis and mediate mobilization and homing of HSC. Therefore, it became important to evaluate the co-expression of CD44 with adhesion molecules. In BMC, CD44v6 was co-expressed with CD49d, CD49e, CD51 (expressed on megakaryocyte and platelets), CD61, CD11b, CD11c (dendritic cell marker), CD62L (L-selectin) and CD62P (P-selectin). CD44v7 was co-expressed with CD49d, CD49e, CD54 and CD62L. CD44v10 was co-expressed mainly with CD51, CD61, CD49d, CD49e, CD54, CD102 (ICAM-2), CD11b and CD62L (Fig.2E).

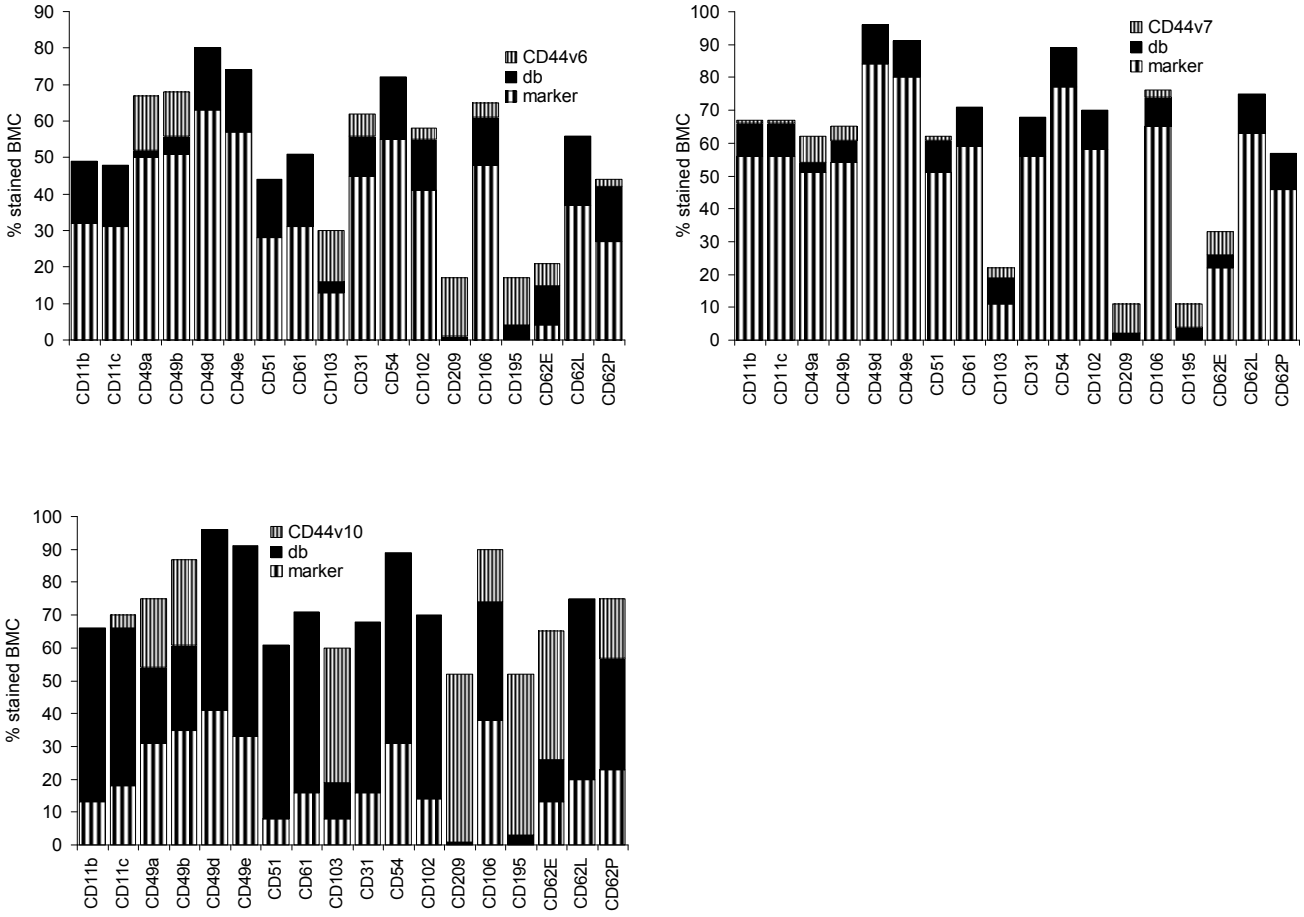


Figure 2E: CD44 co-expression with adhesion molecules on bone marrow cells (BMC). Bone marrow cells were double stained with anti-CD44v6, anti-CD44v7 and anti-CD44v10 and the indicated markers. The percentage of CD44+marker-, CD44+marker+ and CD44-marker+ cells was shown.

In LTBMC-stroma CD44v6, CD44v7 and CD44v10 were co-expressed with the integrins CD11b, CD49d, CD49e and CD54 but only partly with CD11c, CD51 and CD61 and hardly with CD49a and CD49b (Fig. 2F).

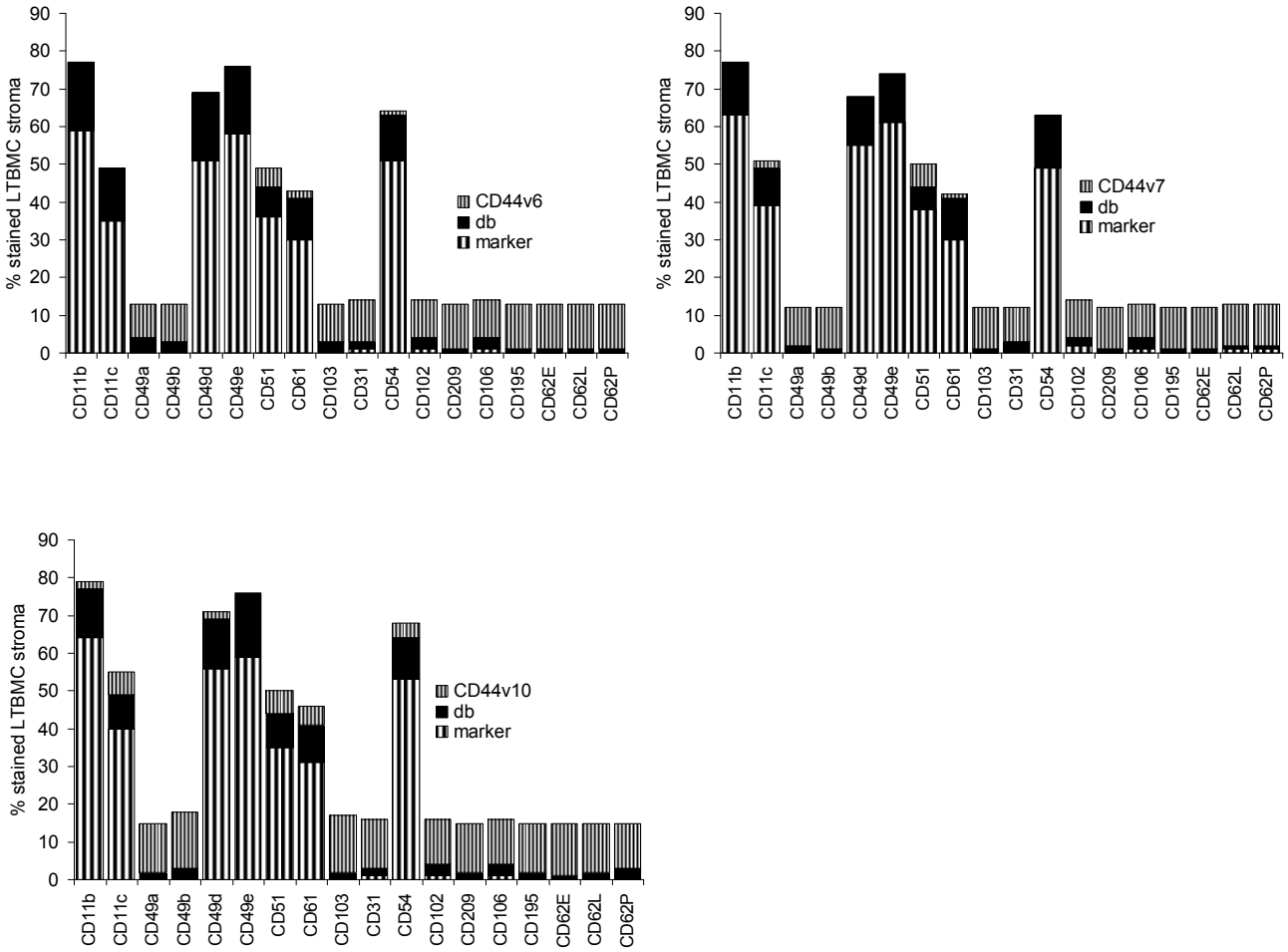


Figure 2F: CD44 co-expression with adhesion molecules on LTBMC-stroma cells. Long term bone marrow culture-stroma cells (LTBMC-stroma) were double stained with anti-CD44v6, anti-CD44v7 and anti-CD44v10 and the indicated markers. The percentage of CD44+marker-, CD44+marker+ and CD44-marker+ cells was shown.

1.2.4 CD44 associating molecules on BM, HSC and LTBMC-stroma cells

Co-localization does not imply an association of the “neighbouring” molecules. Thus, it became important to see, which of the co-localizing molecules actually interact with CD44. To answer the question, I performed co-immunoprecipitation analysis.

Co-immunoprecipitation studies revealed a direct association of CD44 with CD49d and CD54 in BMC, HSC and LTBMC-stroma cells (Fig. 2G & 2H).

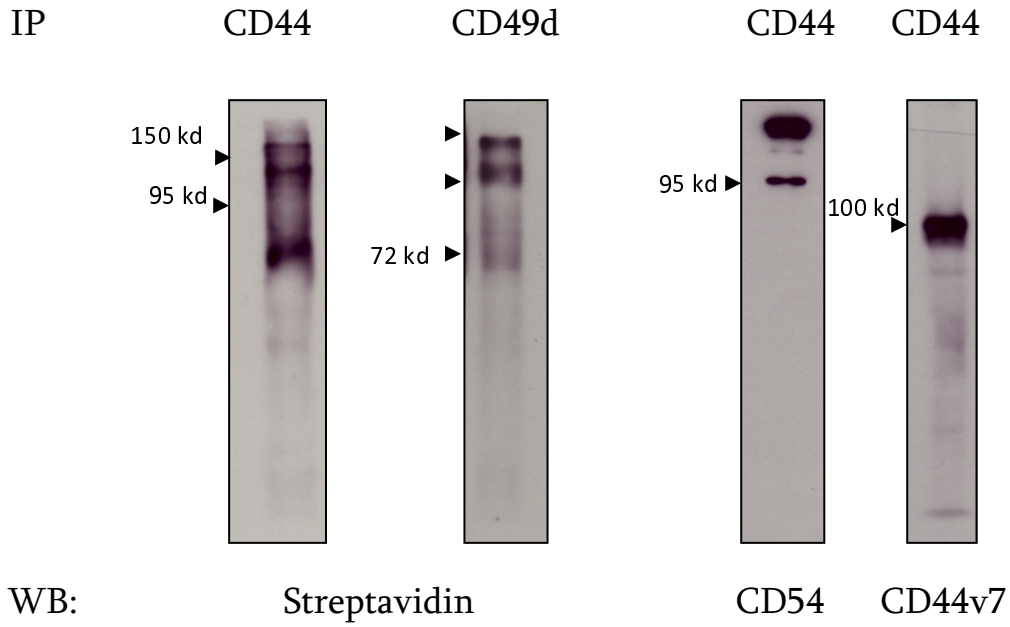


Figure 2G: CD44 associating molecules on BMC: Biotinylated bone marrow cells (BMC) lysate was immunoprecipitated with IM7 and precipitates were dissolved by SDS-PAGE. After transfer proteins were detected by streptavidin-HRP or the indicated antibodies. CD44, CD49d, CD54, CD44v7 indicated molecular weight 80kD, 150kD, 95kD, 100kD respectively.

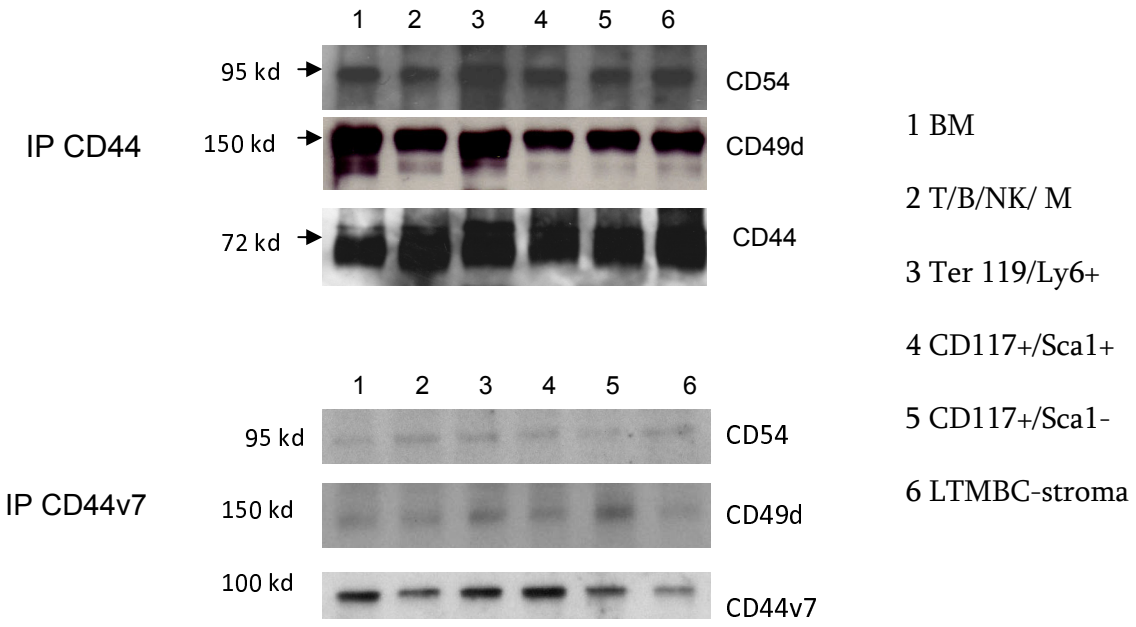


Figure 2H: CD44 associating molecules on BMC, subpopulations and LTBMC-stroma : Bone marrow cells **(**BMC), hematopoietic stem cells (HSC) and long term bone marrow culture-stroma (LTBMC-stroma) lysates were immunoprecipitated with IM7 and anti-CD44v7, precipitates were dissolved by SDS-PAGE. After transfer associated proteins were detected with indicated antibodies.

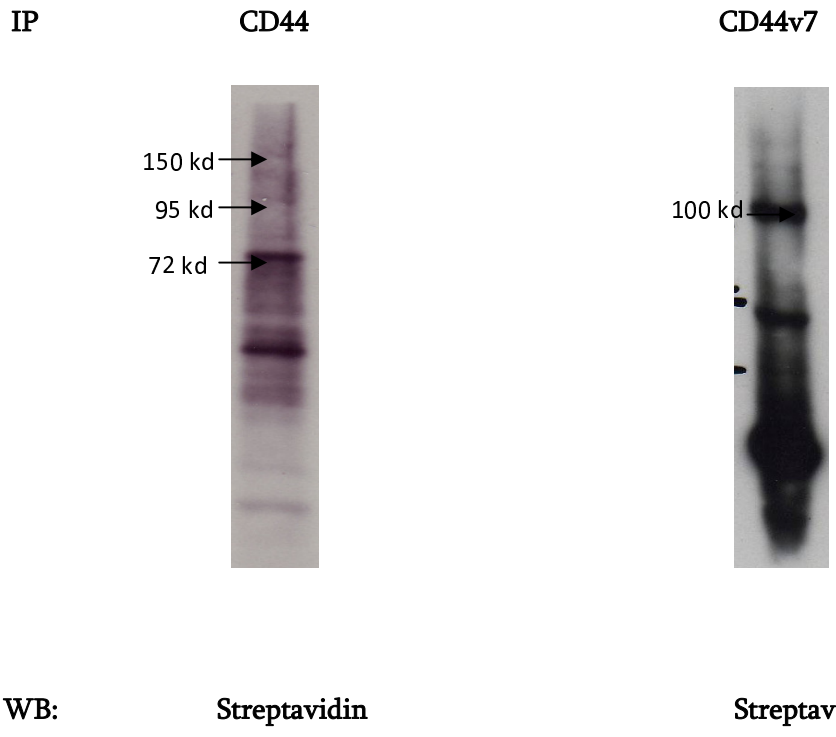


Figure 2I: CD44 associating molecules on LTBMC-stroma: Biotinylated long term bone marrow culture-stroma (LTBMC-stroma) lysate was immunoprecipitated with IM7 and anti-CD44v7 and

precipitates were dissolved by SDS-PAGE. After transfer proteins were detected by streptavidin-HRP. CD44, CD49d, CD54, CD44v7 indicated molecular weight 80kD, 150kD, 95kD, 100kD respectively.

In HSC and LTBMC-stroma CD44, pre-dominantly CD44v7 were associated with CD49d and CD54. Additional associating molecules are expected to be identified by MALDI-TOF.

1.3 Potential ligands of HSC and stroma cells for CD44 and CD44v

As mentioned, a blockade of CD44 does not only prevent HSC homing, but also can drive HSC into apoptosis and differentiation. Accordingly, it is expected and there is strong evidence that signal transduction is initiated by CD44 ligand binding. Thus, besides associating molecules, the CD44 ligand may play an important role in the fate of HSC. Therefore, I proceeded to search for CD44 ligands on HSC and bone marrow stromal cells and also, whether CD44s and CD44v isoforms display any distinct ligands.

1.3.1 Generation of soluble recombinant CD44s and CD44v7

To define potential ligands for CD44, we generated soluble rCD44 proteins. As CD44 is heavily glycosylated, it was important to choose an eukaryotic expression vector system. Expression of different CD44 variant isoforms as soluble molecules showed a precious tool for studying CD44 variant exon product binding properties. For purification and detection of the soluble recombinant CD44 molecules, the pCEP-4 vector was used. This vector was modified by insertion of a myc and his tag at the cloning region's 3' end. Restriction digestion was done to confirm the insert (Fig. 3A). The vector was stably transfected in the human embryonic kidney HEK-293-EBNA1 cell line using the lipid based reagent Polyfect (Qiagen). Positive selection of the transfectants was done using puromycin at 0.5µg/ml.

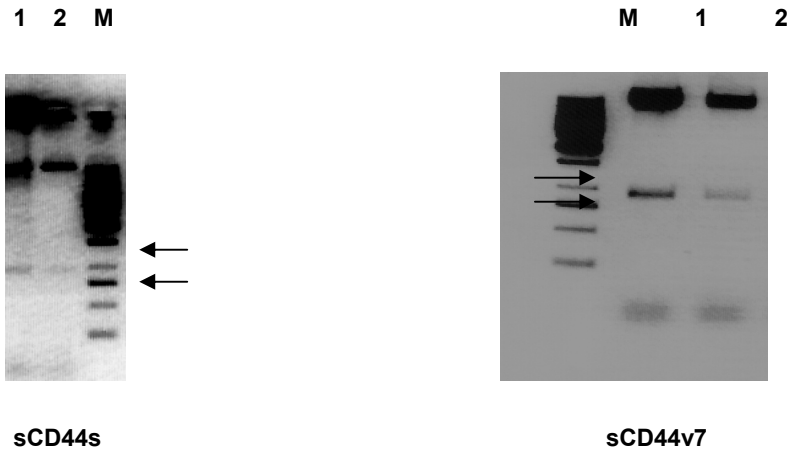


Figure 3A: Confirmation of cDNA of soluble CD44s and soluble CD44v7. Restriction digestion of plasmid was done with Kpn I and Xho I for confirmation. Band of interest indicated by arrow.

Lane 1 and 2 indicate plasmid and M indicates 1 Kb marker. DNA samples were run on 1% Agarose gel after restriction digestion at 37°C for 2h.

Release of the recombinant proteins in transfected cell supernatants were analyzed by immunoblot using an anti-myc mAb (9e10) (Fig. 3B) shows that transfected cells expressed and secreted the recombinant soluble CD44 molecule called sms for CD44s and sm7 for CD44v7.

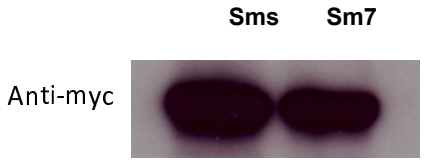


Figure 3B: Expression of sCD44s and sCD44v7 in transfected cells: Recovery of soluble molecule CD44s (Sms) and soluble molecule CD44v7 (Sm7) was evaluated in cultured supernatant of HEK-Ebna cells. Supernatant was 50-fold concentrated. After SDS-PAGE and protein transfer membranes were blotted with anti-myc (9e10).

1.3.2 Pull down assay

HSC have been shown to bind to the osteogenic niche via CD44. Whether this is due to matrix or cell binding is not known. It is also not known, whether LTBMC-stroma binds to CD44 and whether binding of HSC or LTBMC-stroma involves CD44v. To answer these questions we performed pulldown assays with BMC, HSC and LTBMC-stroma lysates passed over sepharose columns after coupling of sCD44s and sCD44v7.

Silver staining of eluted BMC and HSC proteins revealed protein bands to be analyzed by MALDI-TOF. The eluate of HSC did not contain additional bands not detected in BMC eluates, but several prominent bands in the BMC eluates were not or hardly detected in the HSC eluate. LTBMC-stroma eluate contains some additional bands which were not detected in BMC/HSC (Fig.3C).

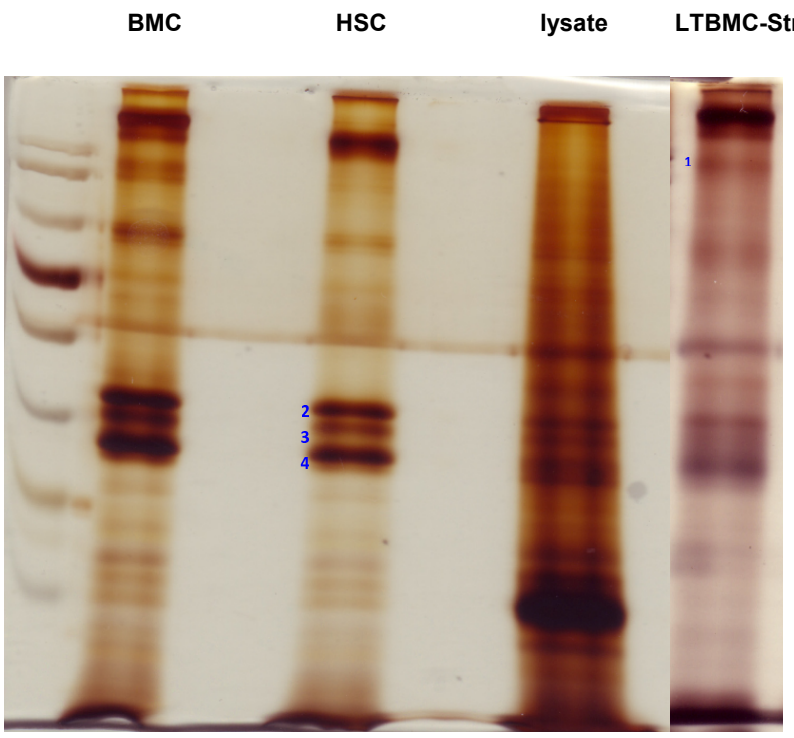


Figure 3C: sCD44v7 binding proteins. Pull down assay with BMC, HSC and LTBMC-stroma lysates were passed over sCD44v7 sepharose column. Eluates were concentrated and separated by SDS-PAGE. Fixed gels were silver stained to visualize the proteins. 1,2,3,4 indicated bands were sent for sequencing.

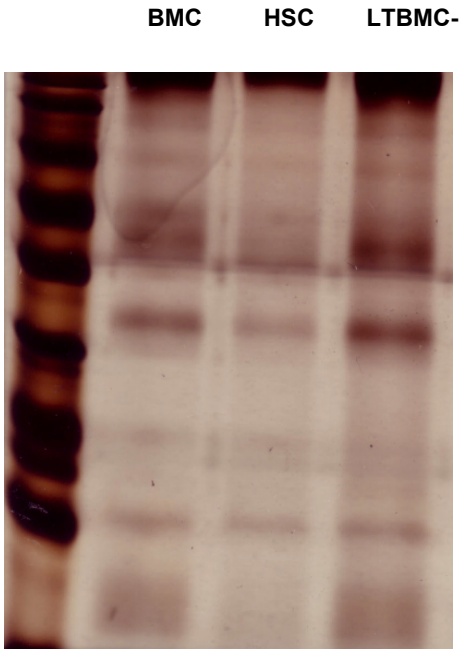


Figure 3D: sCD44s binding proteins. Pull down assay with BMC, HSC and LTBMC-stroma lysates were passed over sCD44s sepharose column. Eluates were concentrated and separated by SDS-PAGE. Fixed gels were silver stained to visualize the proteins.

Results of MALDI-TOF analysis for sCD44v7 molecule are summarized in Table-1.

Sample ID	Protein description	Protein score	Protein Mass in Da	No. of hits	pI
Band No.1	Protease, Serine	174	26802	10	4,75
	Collagen pro-alpha-1 type I	134	138885	5	5,84
	pro-alpha-2(I) collagen	83	130038	3	9,23
Band No.2	Put. Beta-actin (aa27-375)	1408	39446	217	5,78
	Actin, cytoplasmic 1	1355	42084	198	5,29
	Beta-actin like protein 2	569	42319	81	5,3
Band No.3	Put. Beta-actin (aa27-375)	1434	39446	167	5,78
	Bone marrow proteoglycan	56	24981	1	5,86
Band No.4	Actin, cytoplasmic 1	1779	42084	327	5,29

Taken together, HSC and Stroma cells CD44 bind distinct ligands. These ligands may play an important tole in the fate of HSC.

1.4 Functional consequences of HSC CD44 interaction with bone marrow stroma

It has been suggested that particularly the HSC CD44-LTBMC stroma interaction is not only required for homing but also influences quiescence and possibly provides a means of protection from apoptosis. I was particularly concerned about the latter two questions.

1.4.1 Role of HSC - CD44 interaction in the maintenance of quiescence

To check for the contribution of CD44 in maintaining quiescence BMC and HSC, proliferative activity was evaluated for 72h in the presence or absence of anti-CD44 or anti-CD49d. Cells were seeded on plastic or HA or on LTBMC-stroma. Cell division was evaluated by CFSE dilution and the percentage of cells that have not divided is shown.

First to note, HSC divide more slowly than bulk BMC, such that after 24h less than 10% of HSC have divided, while only about 80% of BMC remained resting. Furthermore, when cultured on plastic or HA, BMC division is not strongly altered by a CD44 or CD49d antibody blockade. Instead, when cultured on LTBMC-stroma, blocking CD44 or CD49d drives BMC into cell cycle. The slower cycling of HSC than of bulk BMC is more strikingly affected by the crosstalk with LTBMC-stroma, where even after 48h less than 20% had divided and less than 40% after 72h. On the other hand, blocking the HSC-LTBMC-stroma interaction by anti-CD44 promoted cycling more efficiently than that of bulk BMC. Notably, anti-CD49d was effective as anti-CD44, indicating that binding of the CD44/CD49d complex may account for maintaining quiescence (Fig. 4A).

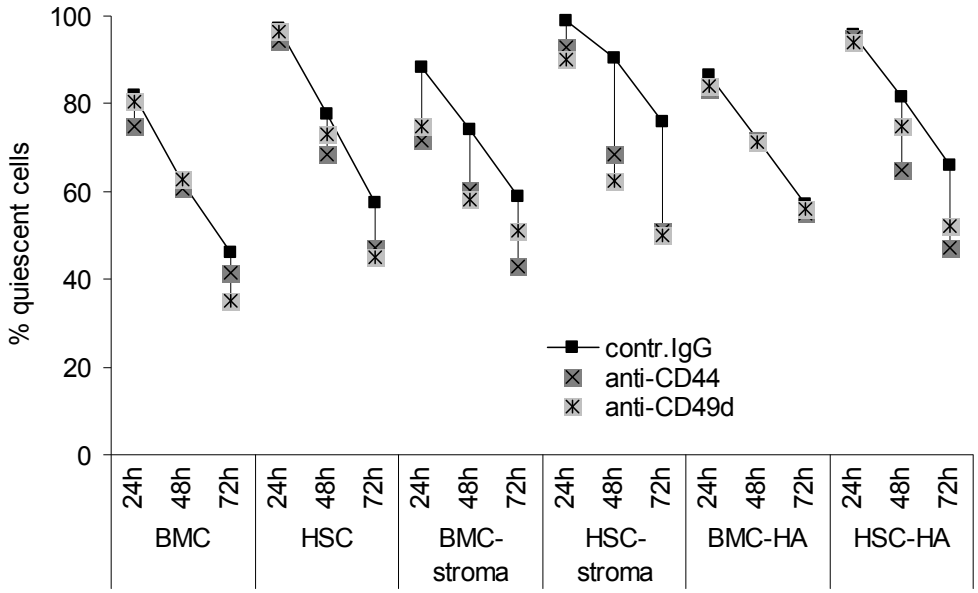


Figure 4A: Comparison of BMC vs HSC quiescence: CFSE-labeled BMC and HSC were seeded on BSA, HA or LTBMC-stroma cells coated plates. Where indicated cells were pre-incubated with anti-panCD44 or anti-CD49d. Cell cycle progression was evaluated after 24h, 48h and 72h. Mean value±SD (triplicates) of the percentage of non-cycling cells are shown.

1.4.2 Role of HSC-CD44 interaction in the apoptosis resistance

HSC are characterized by relative apoptosis resistance. This has been confirmed, when BMC and HSC, seeded on plastic, HA or LTBMC-stroma, were cultured for 48h in the absence or presence of cisplatin. HSC displayed a lower apoptosis rate than bulk BMC, the difference becoming more pronounced when cultured on LTBMC-stroma concomitantly with 5µg/ml cisplatin, which sufficed to significantly increase apoptosis in bulk BMC, but induced only a slight increase in apoptosis in HSC cultured on plastic or HA and none, when cultured on LTBMC-stroma. The importance of the HSC CD44 interaction with LTBMC- stroma became apparent when cultures contain anti-CD44. While the apoptosis rate of bulk BMC became significantly increased in the presence of anti-CD44 only in cisplatin–treated cultures and in co-cultures with LTBMC stroma, apoptosis of HSC was significantly increased in the presence of anti-CD44, irrespective of culture condition (Fig. 4B). Double staining for CD44 and Annexin V as well as triple staining for CD44, CD117 and Annexin V confirmed both, the higher apoptosis resistance of HSC as well as additional apoptosis

protection by LTBMC-stroma. Double and triple staining also confirmed the higher impact of anti-CD44 on HSC than bulk BMC, although due to the high degree of apoptosis resistance in LTBMC-stroma co-cultures, no significant increase in apoptosis of $CD44^+CD117^+$ was seen in the presence of anti-CD44 (Fig. 4C & 4D).

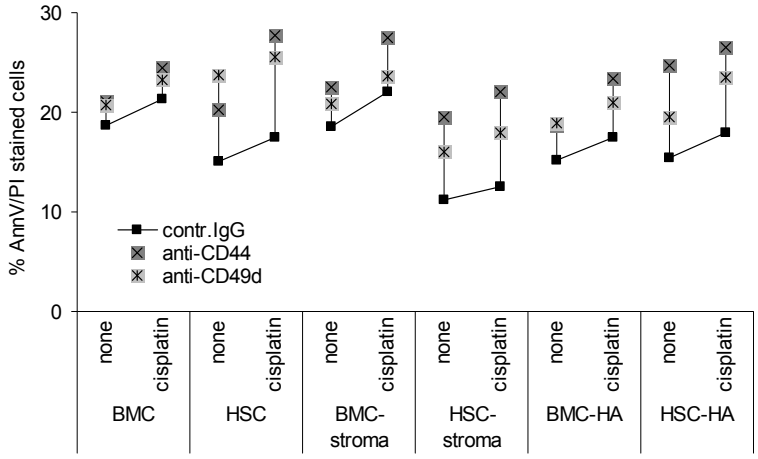


Figure 4B: Comparative analysis of the apoptosis resistance of HSC vs BMC. BMC and HSC were cultured for 48h in the absence or presence of 5µg/ml cisplatin on BSA, HA or LTBMC-stroma cells. Where indicated cultures contained anti-CD44 or anti-CD49d. Mean values±SD (triplicates) of the percentage of Ann V/PI stained cells are shown.

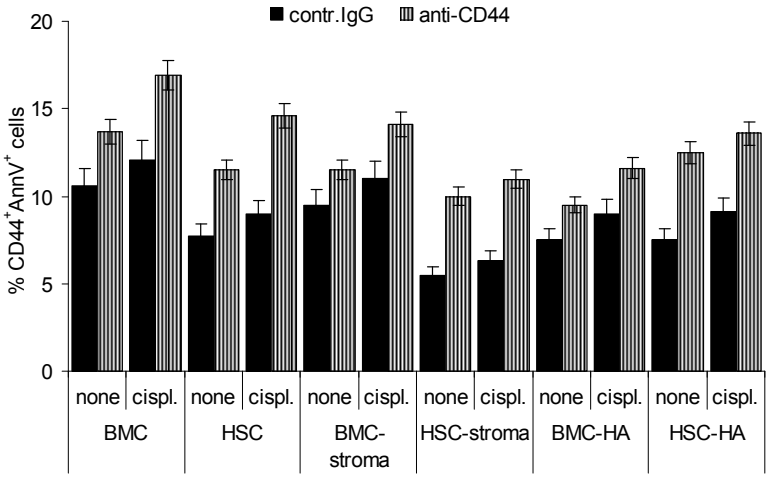


Figure 4C: Comparative analysis of apoptosis resistance of $CD44^+$ BMC and HSC. BMC and HSC were cultured for 48h in the absence or presence of 5µg/ml cisplatin on BSA, HA or LTBMC-stroma cells. Where indicated cultures contained anti-CD44. Mean values±SD (triplicates) of the percentage of $CD44^+$ Ann V^+ cells are shown.

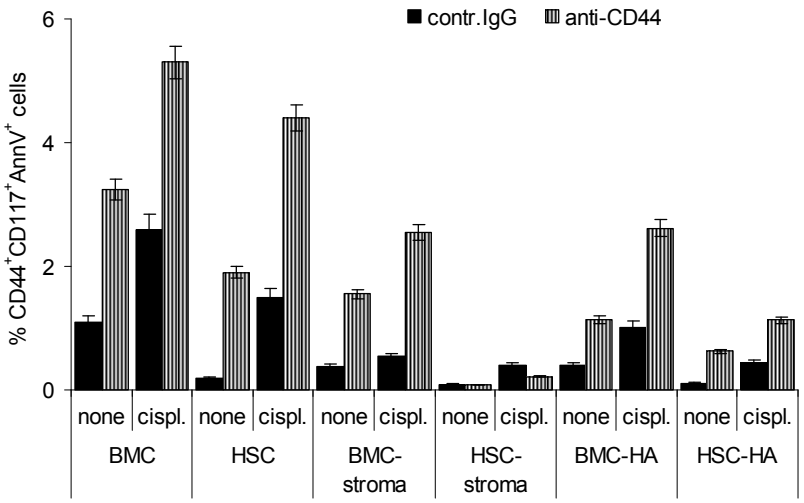


Figure 4D: The impact of a CD44 blockade on apoptosis resistance of $CD44^+CD117^+$ BMC and HSC. BMC and HSC were cultured for 48h in the absence or presence of 5µg/ml cisplatin on BSA, HA or LTBMC-stroma cells. Where indicated cultures contained anti-CD44. Mean values±SD (triplicates) of the percentage of $CD44^+$ $CD117^+$ Ann V^+ cells are shown.

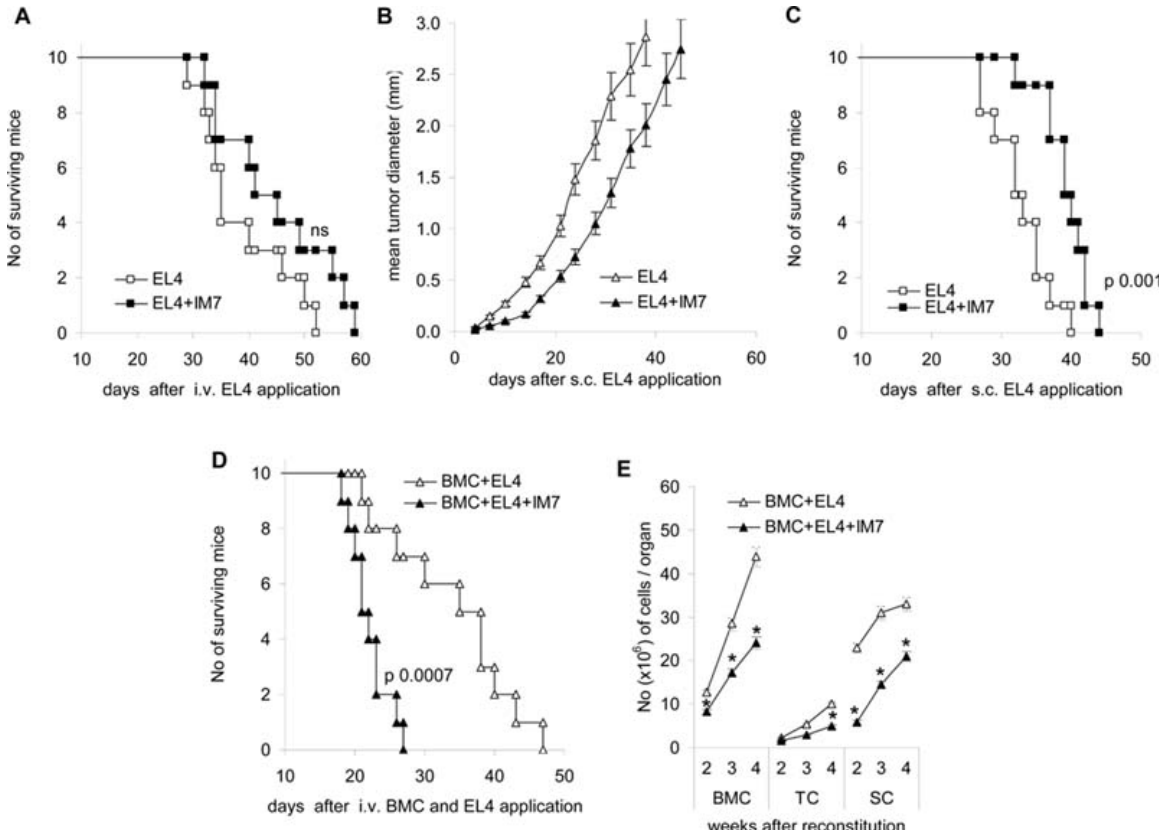
Part II The importance of CD44 in the crosstalk between leukemia and the bone marrow stroma

A blockade of CD44 can interfere with hematopoietic stem cell homing and also with leukemic cell homing. In addition, it has been described that anti-CD44 can drive leukemic cells into differentiation and apoptosis. The underlying mechanisms have not been explored. Based on the studies on the interaction of HSC CD44, I proceeded to evaluate the impact of a blockade of CD44 on T cell lymphoma growth and survival.

2.1 Anti-panCD44 interferes with hematopoiesis and thymoma growth:

C57BL6 mice received 10^4 cells i.v. or s.c. with/without anti-pan CD44 (IM7, 150µg/ml, i.v.) twice per week. After s.c. application of the lymphoma cells the mean tumor diameter was measured twice per week and mice were sacrificed, when the mean diameter of the s.c. growing tumor reached 2.5cm. After i.v. lymphoma cell application, mice were sacrificed upon weight loss and fatigue.

Subcutaneous lymphoma growth was retarded by IM7 and the survival time significantly exceeded the survival time of mice receiving control IgG. After i.v. application of lymphoma cells, the survival time of IM7 treated mice was prolonged, however, not at a statistically significant level. (Fig.1A). After s.c. EL4 application, the number of mice that developed metastases was also increased in IM7-treated as compared to control mice (Fig.1A-1C). Distinct to immunocompetent mice, application of IM7 accelerated tumor growth in irradiated and reconstituted mice (Fig.1D). In addition, bone marrow and thymus reconstitution was severely impaired by IM7 treatment in reconstituted, leukemia-bearing mice (Fig.1E).



Evaluating short term recovery of CFSE-labeled EL4 cells in spleen, bone marrow and thymus in dependence on concomitant IM7 application revealed that IM7 interfered with the settlement of EL4 cells in spleen, bone marrow and thymus in immunocompetent mice. Instead, in line with the survival study, this effect was not observed in irradiated and reconstituted mice (Fig.1F).

When analyzing the percentage of AnnexinV stained EL4 cells, it became obvious that hampering EL4 settlement by anti-CD44 was accompanied by a significant increase in the percentage of apoptotic cells between 24hrs-48hrs after intravenous application. Anti-CD44-induced apoptosis of lymphoma cells was not seen in concomitantly reconstituted mice (Fig.1G and 1H).

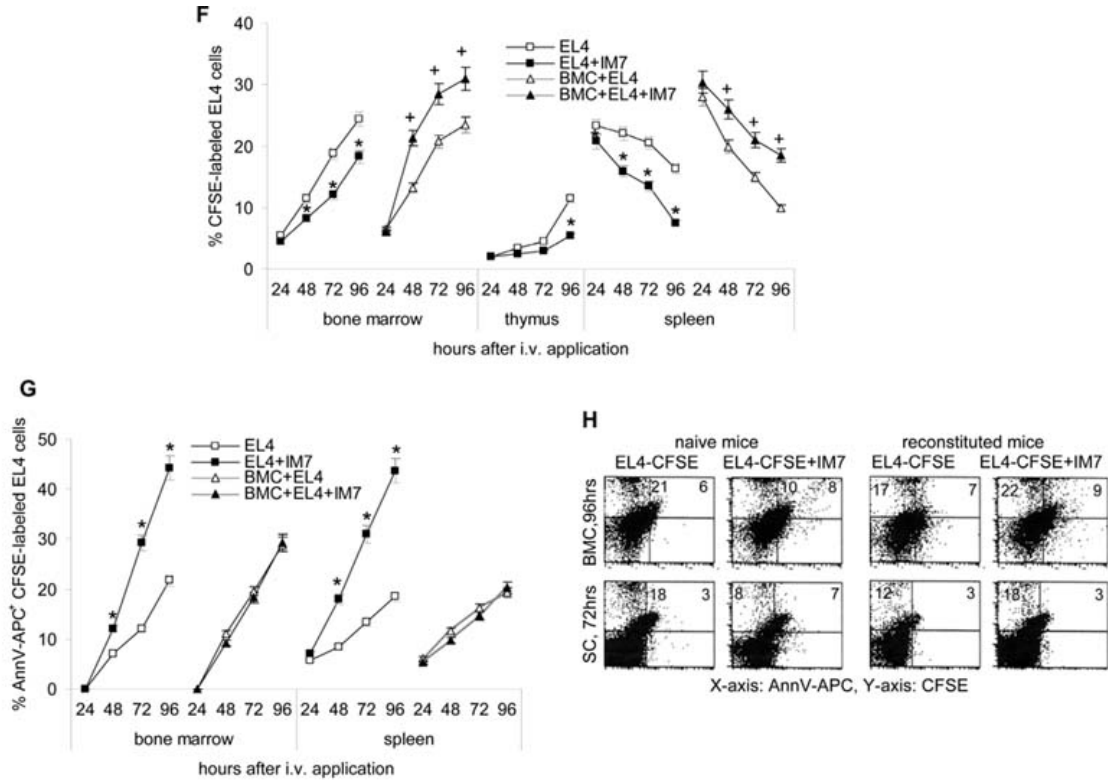


Figure 1: The impact of anti-CD44 on thymoma growth in vivo: (A-E) C57BL6 mice received an i.v. (A) or s.c. (B,C) injection of 10^4 EL4 cells or (C,D) were lethally irradiated and reconstituted with $1x10^7$ T cell-depleted BMC and received EL4 cells, i.v. 2 days after reconstitution. Mice received twice per week an i.v. injection of 150µg control IgG or IM7. (A) The survival time of mice receiving EL4 cells i.v. is shown. Survival was slightly, but not to a significant level prolonged by IM7 application. (B,C) After s.c. application of EL4 cells, IM7 retarded the start of the tumor growth and the survival time became significantly prolonged. (D) The survival time of IM7-treated, EL4 tumor-bearing reconstituted mice was significantly shortened as compared to mice receiving control IgG and (E) the recovery of BMC, TC and SC (mean number±SD of 3 mice / group) was significantly delayed (*). (F) C57BL6 mice were treated as described above, but received $2x10^6$ CFSE-labeled EL4 cells. The % of CFSE-labeled EL4 cells in bone marrow, thymus and spleen was evaluated during 96hrs. Mean values±SD of 3 mice are shown. The recovery of tumor cells is significantly reduced in IM7- treated, non-reconstituted mice (*), but unaltered or increased in IM7-treated reconstituted mice (+). (G) The percentage (mean±SD) of apoptotic CFSE-labeled EL4 cells during the starting 96hrs after i.v. application was evaluated by AnnexinV-APC staining. The percentage of apoptotic EL4 cells is significantly increased in non-reconstituted IM7-treated mice (*), but largely unaltered in reconstituted IM7-treated mice. (H) Examples of apoptotic EL4 cells in the bone marrow 96hrs and in the spleen 72hrs after application of CFSE-labeled EL4 cells in naive and reconstituted C57BL6 mice.

These data provided evidence that anti-CD44-mediated retardation of thymoma growth under steady state conditions was accompanied by an increase in apoptotic tumor cells. Thus, it became important to know, whether death was a consequence solely of a failure to embed or whether IM7 may have actively triggered apoptosis.

2.2 Anti-CD44 promotes thymoma cell apoptosis:

To evaluate the underlying mechanism(s), EL4 and EL4v6 cells were cultured in the presence of 10µg/ml anti-CD44. After 16h of culture in the presence of IM7 the percentage of apoptotic EL4 / EL4v6 cells was measured by AnnexinV-FITC/PI staining. In the presence of soluble antipanCD44 the apoptosis rate was increased after 6hrs from ~2% to ~25% and after o/n culture from ~10% to ~30% (Fig.2A). In order to check for the specificity of apoptosis induction through CD44, we used HA and for EL4v6 cells anti-CD44v6. HA and anti-CD44v6 induced apoptosis in EL4 and EL4v6 cells comparably to anti-panCD44. Instead, the percentage of apoptotic cells was not increased in cultures containing an anti-MHC antibody or anti-CD49d, CD49d being expressed at a high level on EL4 cells (Fig.2B). The increase in apoptotic cells obviously is not a consequence of activation induced cell death, as the distribution of cells in G1, S and G2 or M phase did not vary significantly depending on the culture condition (Fig.2C).

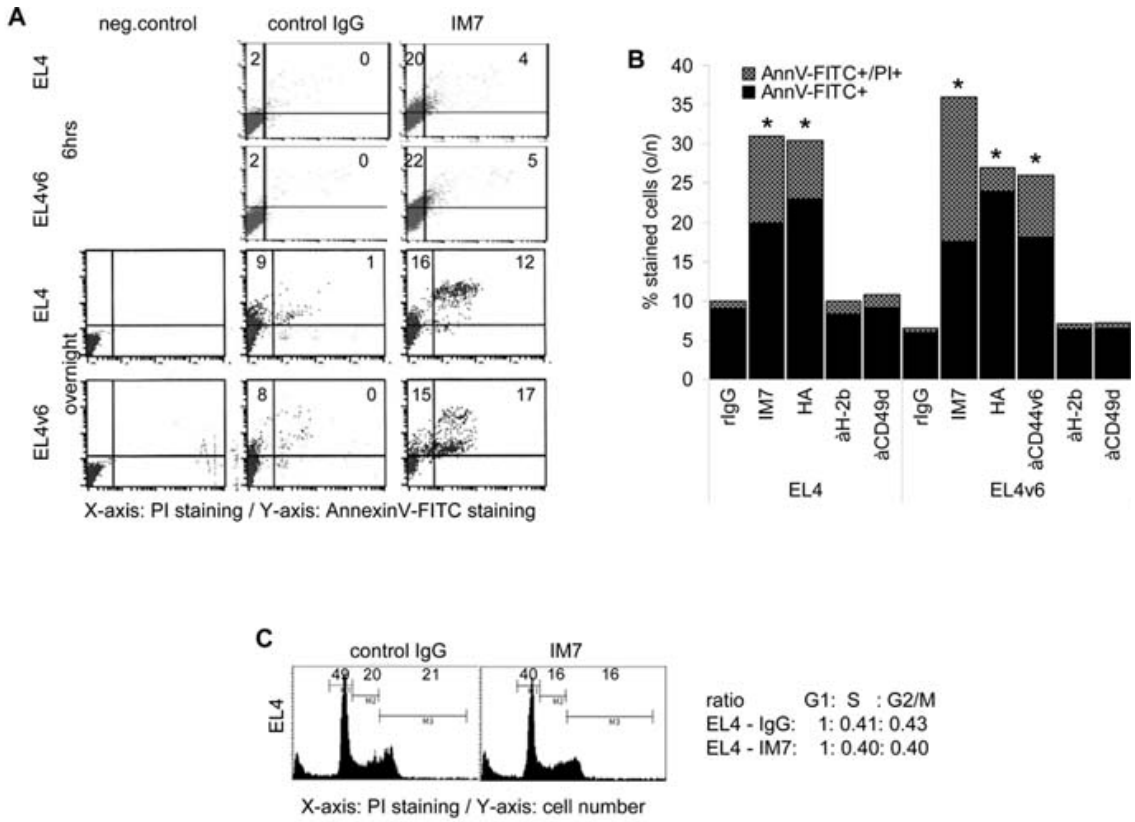


Figure 2: CD44 ligation induces apoptosis in EL4 cells: (A and B) EL4 and EL4v6 cells were cultured for 6hrs or o/n in the presence of 10µg/ml IM7, anti-CD44v6 or rIgG or 15µg/ml HA and, as controls, 10µg/ml anti-H-2b or anti-CD49d. Cells were stained with AnnexinV-FITC/PI. A representative example (A) and mean values±SD of apoptotic cells are shown (B). Significant differences in comparison to cells cultured in the presence of rIgG are indicated by *. (C) Cells were stained with PI and cultured o/n in the presence of rIgG or IM7. The percentage of cells in G1, S and G2 or M phase was evaluated by flow cytometry. Cell cycle progression did not vary significantly in dependence on the presence of IM7.

Thus, occupancy of CD44, independently of the particular epitope, can induce apoptosis in T lymphoma cells.

2.3 Apoptosis induction, loss of mitochondrial membrane polarization and caspase-9 activation:

To explore the mechanism for CD44-induced apoptosis, expression levels of Fas, FasL, Trail, TNFRI and TNFRII were evaluated. EL4 and EL4v6 cells express Fas at about 55-60%, FasL at less than 10%, TRAIL at >70%, TNFRI and TNFRII at ~10% to ~20%. Expression and intensity of expression of these death receptors was not changed after overnight culture in the presence of anti-CD44 (Fig 3A and 3B). Death receptor cross-linking also did not strengthen anti-CD44-induced apoptosis (Fig 3C). In view of these results the possibility of death receptor-induced apoptosis by a cross-talk between CD44 and the Fas machinery becomes unlikely.

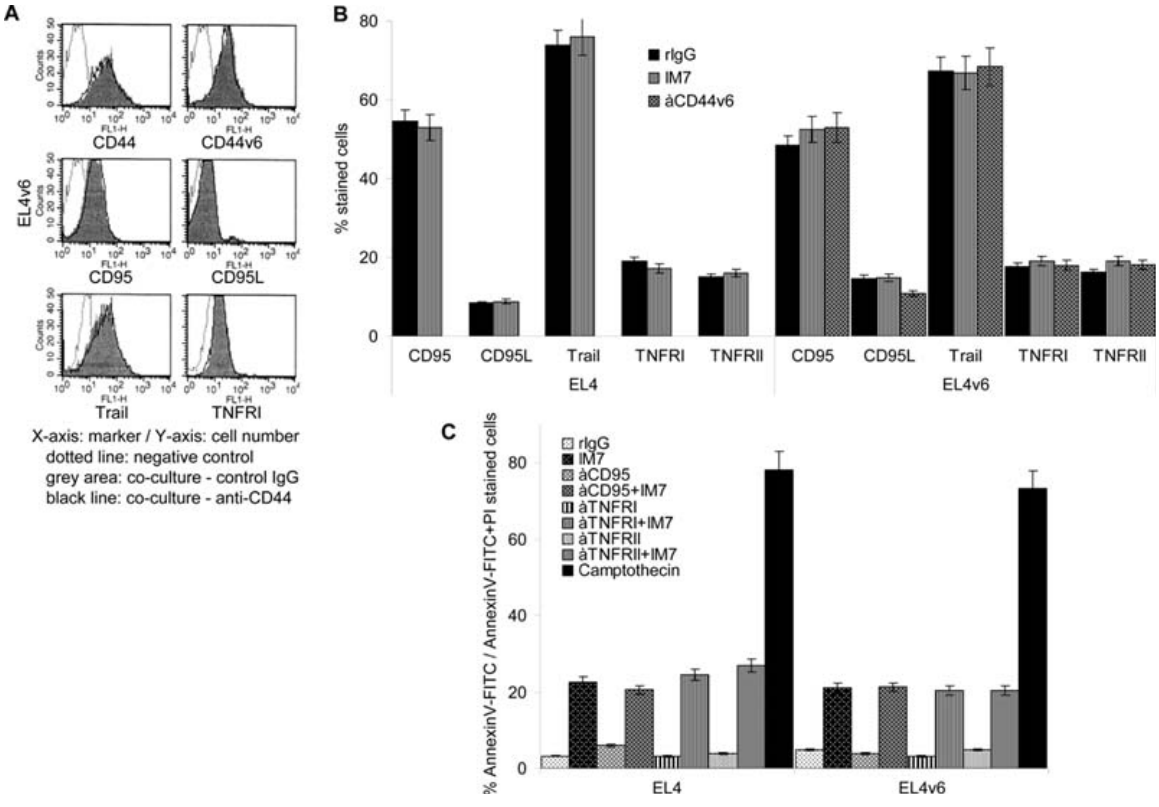


Figure 3: EL4 and EL4v6 cells are resistant towards Fas-induced apoptosis: (A and B) CD44, CD44v6, CD95, CD95L, Trail, TNFRI and TNFRII expression on EL4 cells, EL4v6 cells and thymocytes was evaluated after o/n incubation in the presence of rIgG, IM7 or anti-CD44v6 (10µg/ml). (A) A representative example and (B) mean values±SD are shown. IM7 had no impact on CD44 and apoptosis receptor expression. (C) EL4 and EL4v6 cells were cultured o/n on uncoated or anti-CD95-, anti-TNFRI-

or anti-TNFRII-coated plates. Cultures contained 10µg/ml rIgG or IM7 or 1µM/ml camptothecin (positive control). The percentage (mean±SD of triplicates) of AnnexinV-FITC/PI stained cells is shown. EL4 and EL4v6 cells were resistant towards receptor-induced apoptosis and IM7-induced apoptosis was independent of death receptor cross-linking.

To strengthen this interpretation, the involvement of caspase-3 and caspase-9 in anti-CD44-initiated apoptosis was evaluated. EL4 and EL4v6 cells were cultured overnight in the presence of IM7 and caspase-3 or caspase-9 inhibitors. Apoptosis was measured by annexinV-FITC / PI staining. Caspase-3 and caspase-9 inhibitors reduced IM7-induced apoptosis in EL4 and EL4v6 cells (Fig.4A and 4B). As revealed by WB, cleavage of caspase-9 and caspase-3 in EL4 and EL4v6 cells was more pronounced when cultured in the presence of IM7 and caspase-9 cleavage was partly inhibited in the presence of a caspase-9 inhibitor (Fig.4C). Caspase-9 becoming activated within the apoptosome after cytochrome c release from the mitochondria, this result points towards anti-CD44-initiated activation of the intrinsic apoptosis pathway, which is characterized by loss of mitochondrial membrane polarization. To confirm the involvement of the mitochondrial pathway, we measured mitochondrial membrane depolarization using DilC1, which accumulates in intact mitochondria. Untreated cells show high level of dye intensity. However in IM7 treated EL4 cells the intensity of fluorescence was significantly decreased after a culture period of 12hrs (Fig.4D). Also, cytochrome c was recovered at low level in the cytosol only in IM7-treated EL4 and EL4v6 cells (Fig.4E).

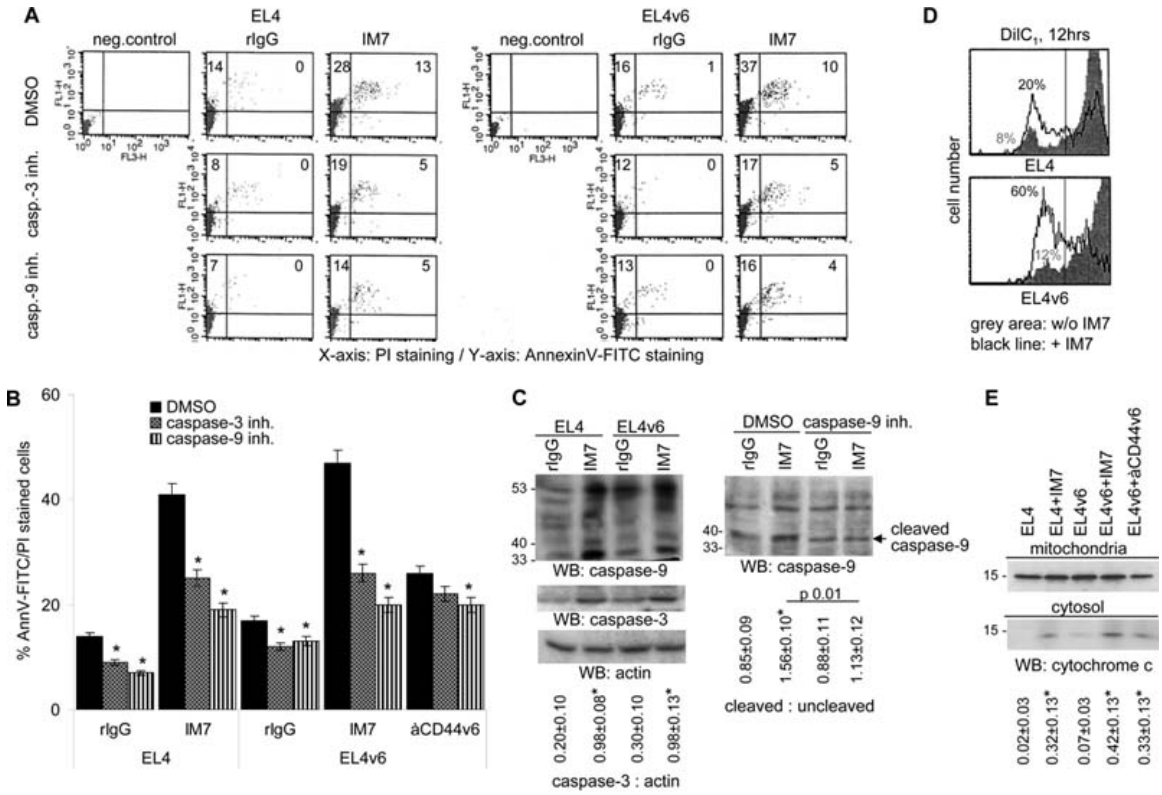


Figure 4: CD44 ligation induces apoptosis via the mitochondrial pathway: (A and B) EL4 and EL4v6 cells were cultured o/n in the presence of rIgG, IM7 or anti-CD44v6. Cultures contained in addition DMSO (control) or 2µM caspase-3 or caspase-9 inhibitor. Apoptosis was measured by annexinV-FITC/PI staining. (A) A representative example and (B) mean values±SD of triplicates are shown. Significant differences in the percentage of apoptotic cells in the presence of caspase inhibitors are indicated by *. (C) WB of caspase-3 and caspase-9 cleavage in lysates of EL4 cells cultured o/n in the presence of rIgG or IM7 or in the presence of a caspase-9 inhibitor. Significant differences by IM7 or a caspase-9 inhibitor are indicated by an asterisk. Caspase-3 and capase-9 cleavage is enhanced in IM7 treated cells and caspase-9 cleavage is reduced in the presence of the caspase-9 inhibitor. (D) EL4 cells were stained with the mitochondrial dye DilC1 and incubated with 10µg/ml IM7 for 12hrs. DilC1 staining was evaluated by flow cytometry. (E) EL4 cells were incubated with 10µg/ml IM7 for 12h. Mitochondria were separated from the cytosol. Lysates were separated by SDS-PAGE, proteins were transferred to a nitrocellulose membrane and blotted with anti-cytochrome c. The mean values±SD of the ratio of cytochrome c in the cytosol : mitochondria is shwon. Significant differences by IM7 or anti-CD44v6 are indicated by an asterisk. (D and E) Mitochondria integrity is strongly affected after 12hrs of culture in the presence of IM7.

These results indicate that CD44-induced apoptosis proceeds via caspase-9 and caspase-3 activation through the mitochondrial pathway.

2.4 Anti-CD44 induces ERK inhibition through PP2A:

To describe the molecular pathway initiated downstream of CD44 engagement, we checked for early activation signaling events which might be involved. EL4 cells stimulated with IM7 for up to 2hrs did not reveal any change in the phosphotyrosine profile (Fig.5A), and Akt phosphorylation (Fig.5B). Instead, ERK phosphorylation became strongly inhibited. Inhibition of ERK phosphorylation started 15min after IM7 addition and lasted for at least 1h in EL4 and EL4v6 cells (Fig 5C). Since the total amount of ERK remained stable, this result rules out an effect of CD44 on ERK degradation. Next we evaluated, whether a blockade of CD44 leads to ERK1/2 dephosphorylation via activation of a phosphatase. Addition of okadaic acid, an inhibitor of the protein phosphatases 1 (PP1) and 2A

(PP2A), to the culture stabilized ERK1/2 phosphorylation and reversed the effect of anti-CD44 (Fig 5D). These results suggesting that CD44 engagement leads to ERK inhibition through the activation of PP1 or PP2A.We did not observe PP1 phosphorylation concomitantly with ERK1/2 dephosphorylation (Fig.5E). In fact, PP2A, but not PP1 associates with CD44 upon CD44 binding. EL4 cells, cultured in the presence of IM7 for different time points, were lysed and immunoprecipitated with anti-CD44 and the immunoprecipitate was blotted with anti-PP1 and -PP2A. PP1 was not detected in the immunoprecipitates. PP2A was absent from the IM7 precipitates at time 0, but was detected after 15min of activation. Immunoprecipitating PP2A and blotting with anti-CD44 confirmed the association between CD44 and PP2A upon CD44 ligation, the association remaining stable for at least 1h (Fig.5F). The association of PP2A is accompanied by PP2A activation as revealed by blotting the PP2A immunoprecipitates with anti-pThr (Fig.5G) and evaluating phosphatase activity of PP2A immunoprecipitates after CD44 ligation. (Fig.5H).

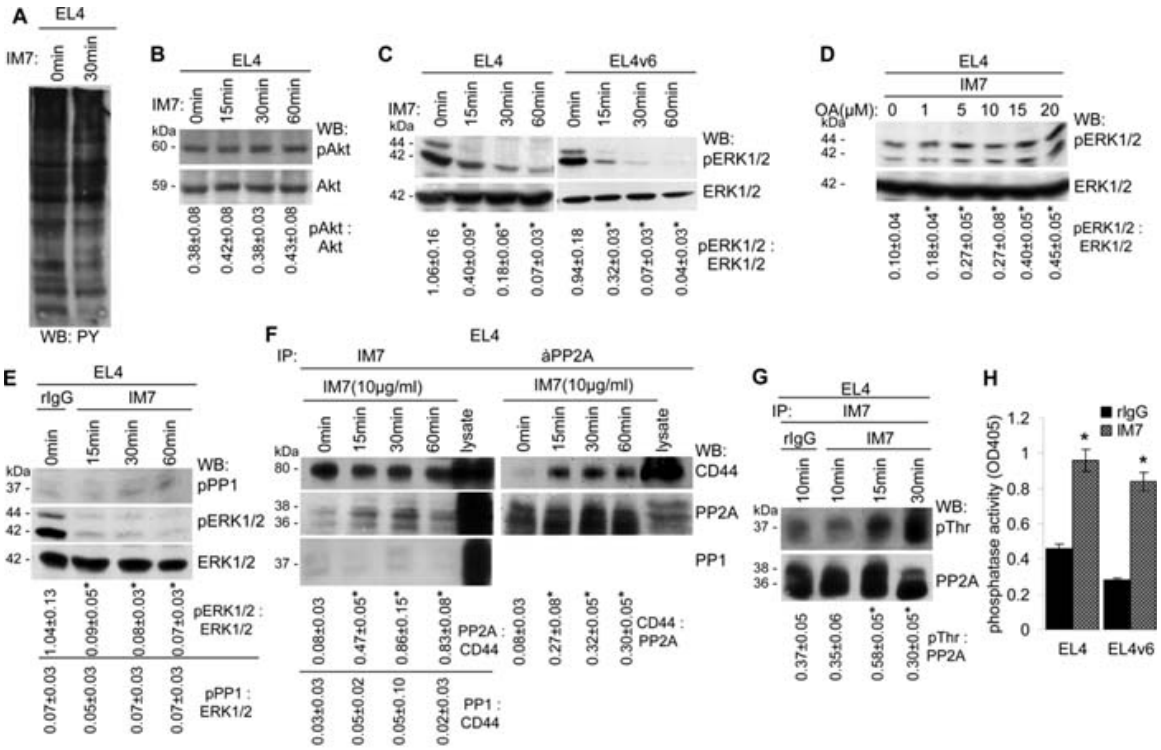


Figure 5: CD44 ligation induces PP2A activation and ERK1/2 dephosphorylation: (A) EL4 cells were cultured for 30min in the presence of IM7. Cells were lysed and proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-phosphotyrosine. (B and C) EL4 and EL4v6 cells were cultured for 15min - 60min in the presence of IM7. After lysis, SDS-PAGE and transfer, membrane were incubated with (B) anti-Akt and anti-pAkt and (C) anti-ERK1/2 and anti-pERK1/2. The mean values±SD of the ratio of pAkt : Akt and pERK1/2 : ERK1/2 are shown. Significant differences by IM7 are indicated by an asterisk. Tyrosine and Akt phosphorylation remained unaltered. ERK1/2 phosphorylation of EL4 and EL4v6 cells cultured in the presence of IM7 becomes strikingly reduced. (D) EL4 cells were cultured in the presence of rIgG or IM7 and increasing amounts of OA. Cells were lysed, lysates were separated by SDS-PAGE, transferred and blotted with anti-ERK1/2 and antipERK1/2. The mean values±SD of the ratio of pERK1/2 : ERK1/2 are shown. Significant differences by OA are indicated by an asterisk. In the presence of the phosphatase inhibitor, the IM7-induced reduction of ERK1/2 phosphorylation was prevented. (E) EL4 cells were cultured in the presence of rIgG or IM7. Cells were lysed, lysates were separated by SDS-PAGE, transferred and blotted with antipPP1, anti-ERK1/2 and anti-pERK1/2. The mean values±SD of the ratio of pPP1 : ERK1/2 and for comparison of pERK1/2 : ERK1/2 are shown. Significant differences by IM7 are indicated by an asterisk. PP1 did not become phosphorylated concomitantly with ERK1/2 dephosphorylation. (F) EL4 cells were cultured in the presence of IM7. Cells were lysed and immunoprecipitated with IM7 or anti-PP2A. Precipitates were separated by SDS-PAGE, transferred and blotted with IM7, anti-PP2A and anti-PP1. The mean values±SD of the ratio of PP2A : CD44, PP1 : CD44, respectively, of CD44 : PP2A are shown. Significant differences by IM7 are indicated by an asterisk. PP2A, but not PP1 co-immunoprecipitated with CD44 and vice versa. (G) EL4 cells were cultured in the presence of rIgG or IM7. Cells were lysed and lysates were immunoprecipitated with IM7. Lysates were separated by SDS-PAGE, transferred and blotted with anti-PP2A and anti-pThr. The mean values±SD of the ratio of pThr : PP2A are shown. Significant differences by IM7 are indicated by an asterisk. Threonine phosphorylation of PP2A became strengthened in the

presence of IM7. (H) Phosphatase activity of the anti-CD44 immunoprecipitate was tested by ELISA. Phosphatase activity was strongly increased after culture of EL4 and EL4v6 cells in the presence of IM7.

To unravel the mechanism responsible for PP2A activation, MALDI-TOF analysis of proteins coimmunoprecipitating with CD44 revealed the presence of Casein Kinase 2 (CK2), a constitutively active ser/thr kinase, which has a wide range of substrates including PP2A. There was a weak association between CK2 and CD44 by WB, which was increased after CD44 engagement (Fig.6A). To strengthen the assumption that CK2 and PP2A concomitantly associate with CD44, EL4 cells, cultured in the presence of IM7, were lysed and light and dense membrane fractions were separated by sucrose gradient centrifugation. Light, medium dense and heavy fractions were pooled and immunoprecipitated with IM7. After SDS-PAGE and transfer, membranes were blotted with IM7, anti-PP2A and anti-CK2. CD44 was recovered in all three fractions. Instead, both PP2A and CK2 coimmunoprecipitated with CD44 exclusively in the medium dense fractions of 1.15-1.21 (Fig.6B). To see, whether CK2 accounts for PP2A activation, EL4 cells, cultured in the presence of anti-CD44, were pretreated with a CK2 inhibitor to block its catalytic activity. This treatment resulted in a mild reduction in PP2A phosphorylation (Fig.6C) and in a reduction of the basal level of pERK1/2 (Fig.6D). Instead, the CK2 inhibitor strongly interfered with spontaneous and IM7-promoted apoptosis (Fig.6E). These findings suggest that CK2 does not exclusively account for PP2A activation, despite that both molecules can become associated with CD44.

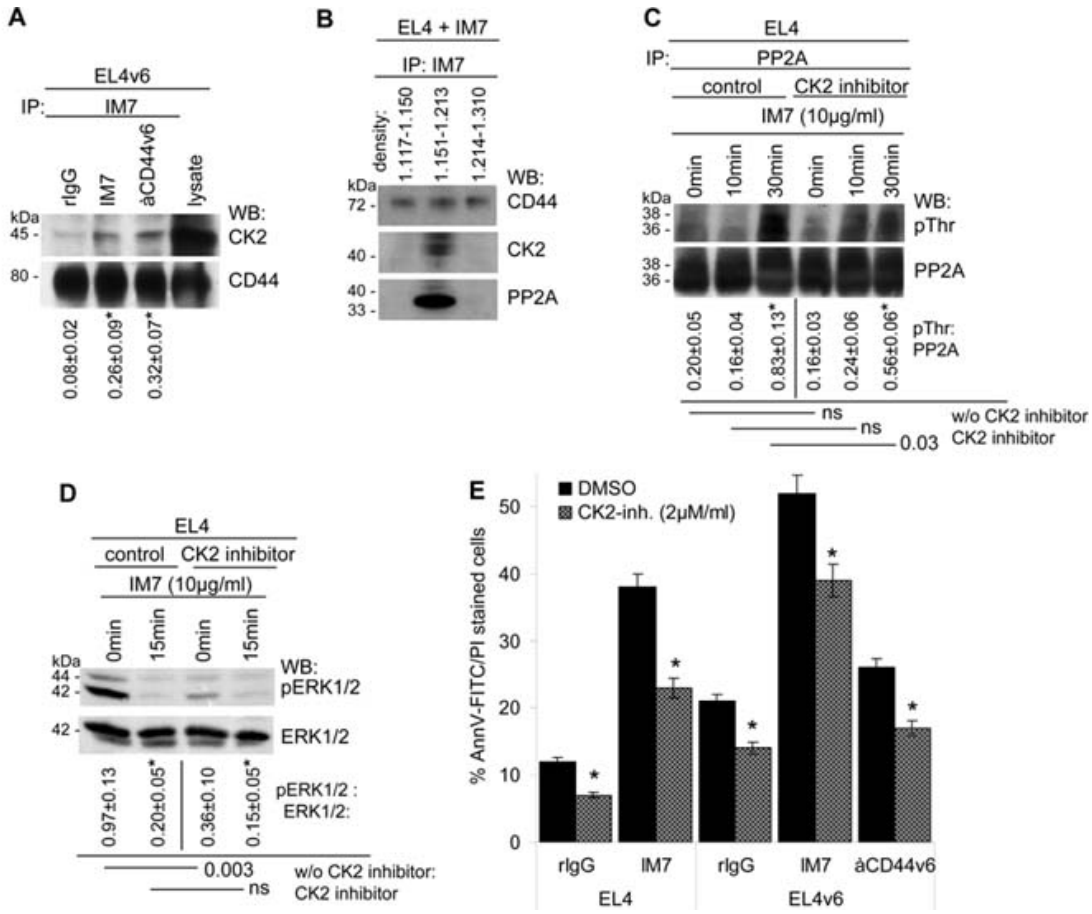


Figure 6: CK2 is associated with CD44 and promotes partial PP2A activation: (A) EL4 cells were cultured in the presence of rIgG or IM7. Cells were lysed and lysates were immunoprecipitated with IM7. After SDS-PAGE of the precipitate, proteins were transferred and blotted with anti-CD44 and anti-CK2. The mean values±SD of the ratio of CK2 : CD44 are shown. Significant differences by IM7 and anti-CD44v6 are indicated by an asterisk. The CD44 precipitate contained low amounts of CK2. (B) EL4 cells, cultured in the presence of IM7, were lysed and light and dense membrane fractions were separated by sucrose gradient centrifugation. Light, medium dense and heavy fractions were pooled and immunoprecipitated with IM7. After SDS-PAGE and transfer, membranes were blotted with IM7, anti-PP2A and anti-CK2. PP2A and CK2 coimmunoprecipitated with CD44 in the pooled fractions of density 1.15-1.21. (C) EL4 cells were cultured in the presence of IM7 and, where indicated a CK2 inhibitor (2µM/ml). Cells were lysed and the lysates were precipitated with anti- PP2A, separated by SDS-PAGE, transferred and blotted with anti-PP2A and antipThr. The mean values±SD of the ratio of pThr : PP2A are shown. Significant differences by IM7 and the CK2 inhibitor are indicated. The CK2 inhibitor partly inhibited PP2A phosphorylation. (D) EL4 cells were cultured in the presence of IM7 and, where indicated the CK2 inhibitor, lysates were precipitated by SDSPAGE, transferred and blotted with anti-ERK1/2 and anti-pERK1/2. The mean values±SD of the ratio of pERK1/2 : ERK1/2 are shown. Significant differences by IM7 and the CK2 inhibitor are indicated. ERK1/2 phosphorylation was reduced in the presence of the CK2 inhibitor. (E) EL4 and EL4v6 cells were cultured in the presence of rIgG, IM7 or anti-CD44v6. Where indicated the cultures contained a CK2 inhibitor. Apoptosis was evaluated after 24h by AnnexinV-FITC / PI staining. The CK2 inhibitor interfered with apoptosis induction independent of the presence of IM7 or anti-CD44v6.

Taken together, CD44 ligation is accompanied by apoptosis induction which becomes initiated by its association with PP2A. PP2A activation proceeds towards ERK1/2 dephosphorylation. CK2, also associated with CD44, promotes PP2A activation. However, in view of the low impact of a CK2 inhibitor on PP2A activation, an involvement of additional molecules in PP2A activation becomes likely.

2.5 ERK1/2 inhibition promotes apoptosis and caspase-9 cleavage:

Our data so far suggest that PP2A activation-induced apoptosis proceeds through ERK1/2 inhibition. IKB phosphorylation was not affected (Fig.7A) which suggest that the low level of nuclear NFkB in EL4 cells remained unaltered. IM7-mediated PP2A activation, at least predominantly, inducing apoptosis through ERK1/2 inhibition, we evaluated whether an ERK1/2 inhibitor would induce apoptosis and whether apoptosis induction would proceed via caspase-9. EL4 / EL4v6 cells show a high basic level of ERK activation, which was significantly inhibited in the presence of a MEK1,2 inhibitor (Fig.7B). In fact, when EL4 and EL4v6 cells were cultured in the presence of a MEK1,2 inhibitor, apoptosis became increased (Fig.7C), mitochondrial membrane depolarization was strengthened, as shown by the higher percentage of cells displaying reduced DilC1 staining (Fig.7D), and caspase-9 cleavage was pronounced (Fig.7E). Concomitantly, Bcl-2 expression and BAD phosphorylated was reduced and BAX expression was up-regulated. The same findings accounted for IM7-treated EL4 and EL4v6 cells (Fig.7F).

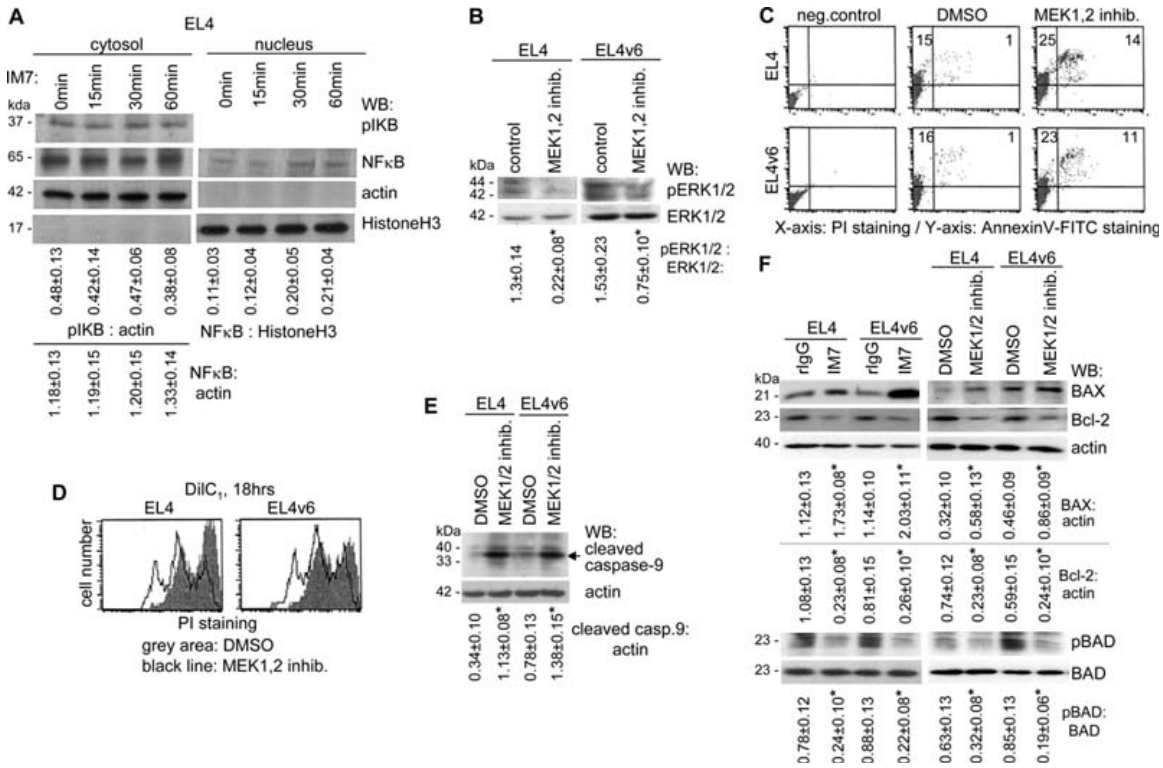


Figure 7: ERK1/2 dephosphorylation promotes mitochondrial membrane disintegration and caspase-9 cleavage: (A) EL4 cells were cultured for 15min - 60min in the presence of IM7. After separation of the cytosolic from the nuclear fraction, lysates were separated by SDS-PAGE, transferred and blotted with anti-pIKB and anti-NFkB. IM7 treatment did not influence IKB phosphorylation and did not significantly alter NFkB liberation. (B-F) EL4 and EL4v6 cells were cultured o/n in the presence of a MEK1,2 inhibitor. (B) WB and the mean values±SD of the ratio of pERK1/2 : ERK1/2 are shown. Significant differences by the MEK1,2 inhibitor are indicated by an asterisk. ERK1/2 phosphorylation in EL4 and EL4v6 cells was inhibited in the presence of MEK1,2 inhibitor. (C) Apoptosis (AnnexinV-FITC / PI staining) was significantly increased in the presence of the MEK1,2 inhibitor. (D) Cells were stained with DilC1 and mitochondrial membrane integrity was determined after 18h incubation by flow cytometry. In the presence of the MEK1,2 inhibitor, mitochondrial membrane integrity was decreased. (E) Cells were lysed, proteins were separated by SDS-PAGE, transferred and blotted with anti-caspase-9. The mean values±SD of the ratio of cleaved caspase-9 : actin are shown. Significant differences by IM7 and the MEK1,2 inhibitor are indicated by an asterisk. Caspase-9 cleavage was pronounced in the presence of the MEK1,2 inhibitor. (F) Cells were cultured o/n in the presence of the MEK1,2 inhibitor or in the presence of IM7. Cells were lysed and proteins were separated by SDS-PAGE, transferred and blotted with anti-BAX, anti-Bcl-2, anti-Bad and anti-pBad. The mean values±SD of the ratio of BAX and Bcl-2 : actin and of pBAD : BAD are shown. Significant differences by IM7 and the MEK1, 2 inhibitor are indicated by an asterisk. The MEK1, 2 inhibitor and IM7 promoted BAX activation and prohibited Bcl-2 expression and Bad phosphorylation.

Taken together, occupancy of CD44 drives leukemic T cells into apoptosis. This is accompanied by PP2A associating with CD44 and PP2A activation, which strongly promotes ERK1/2 dephosphorylation, mitochondrial membrane depolarization and caspase-9 cleavage.

4: Discussion

CD44 plays an important role in HSC homing and embedding in the bone marrow niche, which is required to support HSC survival. Interference of anti-CD44 with bone marrow cell homing has been studied widely. Avigador *et al.*, have nicely shown how CD34+ progenitors home efficiently into bone marrow and spleen and that homing is inhibited with anti-CD44. Homing is mediated in association with stromal cell derived factor-1 and results in adhesion of cells in the niches of BM [140]. CD44 has also been shown to mediate adhesive interactions of stem cells with bone marrow vasculature [141]. Khaldoyanidi *et al.* have elegantly shown the requirement of CD44 in BM homing as well as proliferation of hematopoietic cells by establishing long term bone marrow cultures [142]. Bone marrow stromal cell CD44v7 contributes to progenitor settlement [139].

4.1 CD44 expression in HSC and bone marrow stroma cells

In this work, I characterized bone marrow cells and hematopoietic stem cells and as a surrogate for the osteogenic niche, long term bone marrow culture stroma and S17 cells. CD44 and CD44v isoforms expression clearly showed that bone marrow stroma predominantly expressed CD44v7 while HSC and committed progenitors of the erythroid and myeloid lineages showed high expression of CD44v6, CD44v7 and CD44v10. RT-PCR analysis also revealed that HSC mostly express CD44v7 and CD44v10. Committed progenitors showed CD44v10 and combinations of CD44v3-v10.

Based on this analysis I focussed (i) on associating molecules as it has been shown that CD44 frequently interacts with adhesion molecules and mature leukocyte and progenitor markers, and (ii) on the interaction of HSC with bone marrow stroma, which provides an essential trigger for HSC survival. Besides the involvement of CD44 not much is known on the basic mechanism of this interaction. The importance of CD44v7 expression on stroma cells for progenitor cell homing and seeding has been demonstrated by the transfer of BMC from CD44v7-competent mice into CD44v7-deficient mice and vice versa [139].

4.2 CD44 associating molecule in HSC and bone marrow stroma cells

It has been described before that CD44 showed co-expression with stem cell and progenitor markers like CD43 and CD117, chemokine receptors mainly CD184 (SDF-1 receptor) and adhesion molecules like CD49d, CD49e and CD54 on BMC and LTBMC-stroma cells. We could confirm this with co localization and co-immunoprecipitation of integrins and cell adhesion molecules in HSC and bone marrow stroma cells.

With co-immunoprecipitation data, it has been confirmed that CD44 has association with CD49d and CD54 in HSC and LTBMC-stroma. Due to CD44-CD49d complex formation, CD44 gain access to FAK and CD49d gain access to CD44-associated lck and ezrin, such that downstream kinases become activated via CD44 or CD49d engagement. Thus, by their association, CD44 and CD49d mutually avail themselves of the partner's signalling pathways and the ligand binding of each one triggers signalling pathways of both. This strongly influences the leukocytes activation state and function, [126] which may also account for hematopoietic progenitors and HSC.

Notably, it was preferentially CD44v7 that showed association with CD49d and CD54 in the HSC and LTBMC-stroma cells. The importance of CD184 a chemokine marker (SDF-1 receptor) for marrow homing in hematologic malignancies, such as B-cell chronic lymphocytic leukemia [143], multiple myeloma [144], or acute myelogenous leukemia [145] has already been characterized.

4.3 Functional consequences of HSC CD44 interaction with bone marrow stroma cells

The interaction of HSC with the niche is important for the maintenance of quiescence. It has been confirmed with the results that the slower cell cycling of HSC than of bulk BMC is more strikingly affected by the crosstalk with LTBMC-stroma. It is widely known that the stem cell in general is in a quiescent state (G0 phase in cell cycle) and this quiescence prevents the stem cells from

entering into the cell cycle and differentiation. Arai *et al.* demonstrated that angiopoietin-1 (Ang-1) expressed in the osteoblast interacts with Tie-2, a type of receptor tyrosine kinase, expressed in HSC in bone marrow, and this enhanced adhesion between the niche cell and the stem cell contributes to the maintenance of the quiescence of the stem cell [63].

HSC relatively showed higher apoptosis resistance than bulk BMC. It became more pronounced when cultured on LTBMC-stroma as well as in the presence of cisplatin. Cisplatin is the most commonly used chemotherapeutic drug. The currently accepted mechanism of action for cisplatin is that it binds cellular DNA through the formation of inter-strand cross-links [146]. If the DNA adducts are not efficiently processed by the cell machinery, the eventual result is activation of apoptosis and cell death. Apoptosis resistance is promoted by the crosstalk of HSC with LTBMC-stroma. This resistance mainly relied on high level expression of anti-apoptotic proteins. Interaction of HA and CD44 with various signalling pathways is being increasingly examined as a possible mechanism mediating chemoresistance.

Taken together, HSC express CD44 at a high level. CD44 mostly associated with CD49d and CD54. CD44 expression contributes to the maintenance of quiescence and apoptosis resistance in HSC. LTBMC-stroma provides a niche to promote these functional activities.

4.4 CD44 activities in leukemic cells

There is strong evidence that leukemia initiating cells may also require CD44 for niche embedding and that an antibody blockade of CD44 can hamper leukemia growth such that leukemic stem cell survival becomes impaired and leukemic stem cells are driven into differentiation [147-152]. Thus, anti-CD44 treatment could be considered as a therapeutic option in leukemia. However, because CD44 is also required during haematopoiesis, it becomes important to elaborate possible differences in CD44 activities in hematopoiesis versus leukemia growth.

4.5 T lymphoma cells are less CD44-dependent than hematopoietic progenitors

Anti-CD44 retards T lymphoma growth, particularly at a subcutaneous site. This corresponds, although far less pronounced, to the effect of anti-CD44 treatment in myeloid leukemia [148, 149]. It has been demonstrated convincingly that there is a balance between CD44 isoform expression, hyaluronan binding and settlement by lymphoma cells in lymphoid organs. Accordingly, anti-CD44 inhibits metastasis formation in selective organs, but may be ineffective in other organs. We would suggest, in addition, that preventing settlement of lymphoma cells in one organ may actively support lymphoma cell settlement in others, which stroma provides ligands, including addition adhesion molecules, that allow lymphoma cell adhesion.

Though differing from studies on the impact of anti-CD44 on myeloid leukaemia homing, the finding that a CD44 blockade can more efficiently interfere with progenitor than T lymphoma cell homing is of clinical relevance, taking into account that BMC / HSC reconstitution is frequently considered as a therapeutic option in leukemia. In clinical settings also the possibility of anti-CD44-promoted metastasis formation should be taken into account.

4.6 A CD44 antibody blockade promotes death receptor-independent leukemic T cell apoptosis

In the non-reconstituted mouse anti-CD44 treatment retarded lymphoma growth. As a first step towards a therapeutic exploitation, we aimed to clarify the underlying mechanism. In line with several reports [147, 150-152], our in vivo findings provided evidence that a blockade of CD44 can drive leukemic T cells into apoptosis.

In the presence of anti-panCD44, anti-CD44v6 or HA the rate of apoptotic EL4 or EL4v6 cells increased 2-3 fold within 48hrs of culture. We interpret the observation that a CD44-specific antibody, that does not recognize the HA binding site as well as an antibody specific for a variant isoform and HA supported apoptosis induction as an indication that apoptotic signaling likely

proceeds directly via CD44 and not via associating transmembrane molecules or a feedback via ligand binding. The finding does not exclude apoptosis by neglect due to the absence of survival supporting signals. Nonetheless, co-operative activity of CD44 with receptor-mediated apoptosis was excluded by unaltered apoptosis induction upon death receptor cross-linking, the failure to observe cooperative activity in apoptosis induction by anti-CD44 plus death receptor cross-linking and the unaltered death receptor expression in EL4 cells cultured in the presence of anti-CD44.

4.7 Anti-CD44-promoted apoptosis proceeds via CD44 redistribution and PP2A association

PP2A, which mostly resides in the cytosol, but can attach to the plasma membrane depending on the activation state [154], activates pro-apoptotic and inhibits anti-apoptotic proteins of the Bcl-2 family [155-157]. PP2A associated with CD44 only in cultures containing anti-CD44, and became activated upon association. PP2A regulation is a complex mechanism and the impact of PP2A phosphorylation on its activation is debated [158-160]. However, several publications showed that PP2A is a substrate for CK2 and can become activated through CK2 [153, 158 and 159]. Because we observed that CD44 antibody occupancy was accompanied by both PP2A and CK2 associating with CD44, we hypothesize that by the induced proximity between CK2 and PP2A, CK2 may account for PP2A activation. In fact, PP2A phosphorylation was slightly reduced in the presence of a CK2 inhibitor. On the other hand, spontaneous and CD44-induced apoptosis was strongly inhibited in the presence of a CK2 inhibitor. Therefore we hypothesize that PP2A activation does not exclusively rely on CK2. In line with this hypothesis, the CK2α association with PP2A can be disrupted by activated raf [160], which could provide an explanation for the low impact of CK2 on PP2A activation.

4.8 CD44-associated PP2A promotes mitochondrial membrane destabilization via ERK1/2 dephosphorylation

EL4 cells show a high level of activated ERK1/2 in the absence of an external stimulus. Upon IM7 treatment, ERK1/2 phosphorylation becomes strikingly

diminished without evidence for ERK1/2 degradation. ERK activation mainly proceeding via ras activation, we first evaluated whether anti-CD44 treatment may alter ras activation. This has not been the case. Ras is highly activated in EL4 cells and a pull down of activated ras by a raf-GST fusion protein provided no evidence for altered ras activity in IM7-treated EL4 cells.

Alternatively, PP2A may account for ERK1/2 dephosphorylation [161, 162]. The antibody blockade of CD44 and the blockade of ERK1/2 phosphorylation was accompanied by low level of Bad phosphorylation and up-regulation of pro-apoptotic BAX, of mitochondria depolarization and caspase-9 cleavage. Both inhibition of ERK1/2 phosphorylation and PP2Aactivation promoting mitochondrial depolarization, caspase activation and apoptosis induction, it becomes most likely that in EL4 cells pERK1/2 is the preferential target of PP2A. However, anti-CD44-initiated PP2A activation did not display significant effects. Thus, in leukemic T cells PPA2-mediated ERK1/2 dephosphorylation appears to be the dominating theme in anti-CD44-induced apoptosis.

Taken together, the finding that anti-CD44 can interfere more efficiently with HSC than leukemic T cell homing into the bone marrow is clinically important for patients with T cell leukemia that receive a HSC transplant. In addition, anti-CD44 may more effectively block leukemic cell settlement in the bone marrow than in the liver and the thymus, which could promote metastatic growth. As both these drawbacks likely can be circumvented by the use of selected CD44 antibodies, it became important to evaluate the consequences of CD44 occupancy on leukemic T cells. Anti-CD44 actively induces apoptosis in leukemic T cells. By PP2A relocation in the proximity of CD44 and PP2A activation, the basic level of ERK1/2 phosphorylation cannot be maintained, which drives leukemic T cells into apoptosis by up-regulation of pro- and down-regulation of anti-apoptotic proteins, mitochondrial membrane destabilization and caspase activation. Unraveling this new pathway of anti-CD44-initiated apoptosis in leukemic T cells strengthens the clinical relevance of anti-CD44 in T leukemia and should allow for well targeted therapeutic interference.

5: Summary

CD44 expression on hematopoietic stem cells (HSC) has been shown to be required for homing into the osteogenic niche and maintenance of quiescence. It is not known, whether expression of the hematopoietic or variant CD44 isoforms (CD44s and CD44v) is required and a potential contribution of stroma cell CD44 has not been elucidated.

An analysis of CD44 expression in HSC and LTBMC-stroma cells revealed besides predominant CD44s expression, low level of CD44v6, CD44v7 and CD44v10 expression in $CD117^{+}SCA1^{+}$ HSC and mostly CD44v7 expression in LTBMC-stroma. Other adhesion molecules like CD49d and CD54, some committed progenitor markers like CD16, CD24 and cytokine receptors like CD184 (SDF-1 receptor) are also expressed on HSC and LTBMC-stroma.

HSC and LTBMC-stroma CD44 associate with distinct molecule. In HSC and LTBMC-stroma, CD44 co-immunoprecipitates with CD49d and CD54. In LTBMC-stroma CD44v7 selectively associates with CD49d. Functional studies provided evidence that the interaction of CD44-CD49d complex on HSC with the niche may account for the maintenance of quiescence. HSC CD44 also plays an important role in the apoptosis resistance and this apoptosis protection relies on high level expression of anti-apoptotic proteins.

Taken together, CD44 expression in HSC and LTBMC-stroma co-ordinately contribute to the maintenance of quiescence and apoptosis resistance. Such functional activities mediated by the crosstalk between the bone marrow stroma and HSC CD44 may allow for selective interference with HSC versus LSC interactions with the osteogenic niche. Infact, a blockade of CD44 can not only interfere with hematopoietic stem cell and leukemic cell homing, but also has been described that anti-CD44 can drive leukemic cells into differentiation and apoptosis.

An analysis of a blockade of CD44 on the growth of T cell lymphoma EL4 revealed that it retarded growth and promoted *in vivo* apoptosis of leukemic T cells. However, in bone marrow cell-reconstituted mice anti-CD44 drives progenitor T cells more efficiently than leukemic cell into apoptosis, such that

the survival time is shortened and the incidence of metastasis increases. *In vitro*, CD44 occupancy results in a 2-4-fold increase in apoptotic EL4 cells. Death receptor expression (CD95, TRAIL, and TNFRI) remains unaltered and CD95 cross-linking-mediated apoptosis is not affected. Instead, CD44 ligation promotes mitochondrial depolarization that is accompanied by caspase-9 cleavage and is inhibited in the presence of a caspase-9 inhibitor. Apoptosis becomes initiated by activation of CD44-associated phosphatase 2A (PP2A) and proceeds via ERK1/2 dephosphorylation without ERK1/2 degradation. Thus, anti-CD44 drives leukemic T cell into apoptosis via the mitochondrial death pathway by CD44 associating with PP2A.

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July 1995- June 1996 12th Standard, passed with honours 80%, Subjects includes Hindi, English, Physics, Chemistry, Biology

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Publications

1. Schubert M, Herbert N, Taubert I, Ran D, **Singh R**, Eckstein V, Vitacolonna M, Ho AD, Zöller M. Differential survival of AML subpopulations in NOD/SCID mice. Experimental Hematology, 2011 Feb;39(2):250-263

2. Vitacolonna M, Schubert M, Herbert N, Taubert I, **Singh R**, Ho A, Zöller M. Improved T and B cell recovery by the transfer of slowly dividing human hematopoietic stem cells. Leukemia Research, 2010 May;34(5):622-30

3. Rajasagi M, von Au A, **Singh R**, Hartmann N, Zöller M, Marhaba R. Anti-CD44 induces apoptosis in T lymphoma via mitochondrial depolarization. Journal of Cellular and Molecular Medicine, 2010 Jun;14(6B):1453-67

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Abstracts

1. **Singh R**, Rajasagi M, von Au A, Zöller M, Marhaba R. Anti-CD44 induces apoptosis in T lymphoma via mitochondrial depolarization. 2nd European Congress of Immunology, Berlin from 13th to 16th September 2009.