Allogeneic bone marrow transplantation and Role of CD44 in T cell maturation

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Abbreviations

Ag: Antigen
Ab: Antibody
APC: Antigen presenting cell
BSA: Bovine serum albumin
BMT: Bone marrow transplantation
BMC: Bone marrow cell
BMCT: Bone marrow cell transplantation
BM: Bone marrow
CD44s: CD44 standard isoform
CD44v: CD44 variant isoform
CFSE: Carboxyfluorescein succinimidyl ester
CLP: Common lymphoid progenitor
CMP: Common myeloid progenitor
Comp: Competent (wild type)
CTLs: Cytotoxic T cells
DC: Dendritic cells
DN: Double negative
d: Days
DP: Double positive
Depl: depleted
ETP: Early T lineage progenitor
ER: Endoplasmic reticulum
ECM: Extracellular matrix
ERM: Ezrin radixin moesin
Egr: Early growth response
ERK: Extracellular regulated kinase
FCS: Fetal calf serum
FACS: Fluorescent activated cell sorting
gm: Gram
GAGs: glycoseaminoglycans
GVHD: Graft vs host disease

GVT: Graft vs Tumor

HLA: Human leukocyte antogen

HSA: Heat stable antigen

HA: Hyaluronic acid

i.v.: Intravenous

Ig: Immunoglobulin

IL: Interleukin

IFN: Interferon

ITAMS: Immunoreceptor tyrosine based activation motif

ICAM: Inter-cellular adhesion molecule

JAM: Just another method

KO: Knock out

Kb: kilo basepair

Lin: Lineage

LSK: Lin^-, $Sca\text{-}1^+$, $c\text{-}kit^+$

MMP: Matrix metalloproteinase

MAPK: Mitogen activated protein kinase

MHC: Mono histocompatibility complex

ml: Millilitre

M: Molar

NTG: non-transgenic

NK cells: Natural killer cells

PSGL: P-selectin and p-selectin ligand

PKC: Protein kinase C

PI: Propidium iodide

PKB: Protein kinase B

PIP2: Phosphatidyl inositol 3,4 bisphosphate

PIP3: Phosphatidyl inositol 3,4,5- triphosphate

PDK1: phosphatidyl inositol kinase

PTKs: Phospho tyrosine kinase

RT-PCR: Reverse transcriptase polymerase chain reaction

SC: Spleen cells

$Sca\text{-}1^+$: Stem cell antigen-1

SP: Single positive

SLP-76: SH2-domain-containing leukocyte protein of 76 kDa

s.c.: Subcutaneous

TNF: Tumor necrosis factor

TC: Thymocytes

TG: Transgenic

TCR: T cell receptor

TGF: Transforming growth factor

T_{regs}: T regulatory cells

VLA: Very late antigen

VCAM: Vascular cell adhesion molecule

wk: Weeks

ZAP-70: Zeta associated protein

1. Introduction

1.1 Allogeneic Bone Marrow Cell Transplantation

Over the past years there has been significant improvement in conventional therapies for cancer, but still mortality due to this disease remains high and thus calls for a need of a combination of conventional and biological therapeutic approaches. Several approaches such as blockade of neoangiogenesis, modulation of matrix degrading enzymes, cancer gene therapy and immunotherapy procedures aiming at targeting tumor antigens are in practice [1-3]. It is only recently that we have gained better and deeper insight towards the molecular basis of immunology approach and still a lot has to be done to use immunotherapy alongside with surgery, chemotherapy, and radiotherapy as an equally efficient and at the same time a less harmful therapeutic strategy [4].

Allogeneic bone marrow transplantation (BMT) is one of the tools for immunotherapy of cancer. It involves transfer of bone marrow or hematopoietic stem cells from donor to host with matched histocompatibility [5]. Allogeneity is defined by a multigene system known as major histocompatibility complex (MHC). The gene products are called HLA in humans and H-2 in mice. HLA complex in humans is located on the 6^{th} chromosome. H-2 complex in mice is located on the 17^{th} chromosome [6]. These molecules are central in T cell activation. HLA and H-2 class I molecules present peptides to $CD8^+$ T cells whereas, class II present peptides to $CD4^+$ T cells [7, 8]. During the process of maturation of the immune system tolerance develops against these peptides such that, self peptides are tolerated by the immune system. Rejection of transplanted tissues and organs within a species is caused by recognition of proteins encoded by MHC genes according to the allelic polymorphism. Differences in the MHC Ags between a transplant donor and recipient lead to rapid elimination of the graft; however, polymorphisms in other genes can also cause rejection of tissues in MHC-matched individuals. These minor histocompatibility Ags (mHAgs) are presented as peptide fragments derived from proteins with more than

one allele that are presented by class I MHC molecules, initiating a $CD8^+$ CTL response [9]. The outcome of this is the probability of graft vs host disease (GVHD), graft rejection as well as differentiation between graft vs host disease and graft vs tumor activity [5].

Earlier it was believed that bone marrow cell transplantation (BMCT) could only be employed for hematological malignancies to replace patient's hematopoiesis after chemotherapy or radiotherapy [10]. Allogeneic BMT indeed provides a cell graft that is tumor free, as well as cells that have not been damaged by prior chemotherapy or radiotherapy and most importantly stem cells that can differentiate and constitute a functional hematopoietic system in the patient. However, until recently allogeneic bone marrow transplantation could be given only to younger patients due to myeloablative conditioning where the host relies completely on the donor bone marrow cells for total hematopoietic reconstitution. There is high toxicity involved in this process because myeloablation requires high doses of chemotherapeutic drugs and /or radiation dose [11, 12]. Thus, older patients develop high rate non relapse mortality probably due to poor engraftment and high susceptibility to infections as a consequence of particularly impaired reconstitution of immune cells form the allogeneic graft. The poor recovery of functionally active immune cells is atleast, partly due to thymus involution with age where the thymus is essentially required for T cell maturation. Since the median age of people with hematological malignances is >50, there was a need to develop a protocol that would suit the elderly. Recent evidence in animal models and first clinical trials suggests that non-myeloablative conditioning before allogeneic bone marrow transplantation (BMCT) could be tolerated by elderly patients as well as those in poor health [11, 12]. The non-myeloablative or myeloreductive conditioning is less toxic to patients and hence has a better outcome because it is less intensive and involves lower doses of chemotherapeutic drugs or radiation dose. These regimens typically use combinations of chemotherapy drugs such as fludarabine, busulfan, ATG and melphalan, with or without low-dose radiation. It became evident that this approach has an efficient therapeutic effect of inducing an immune response for the tumor that

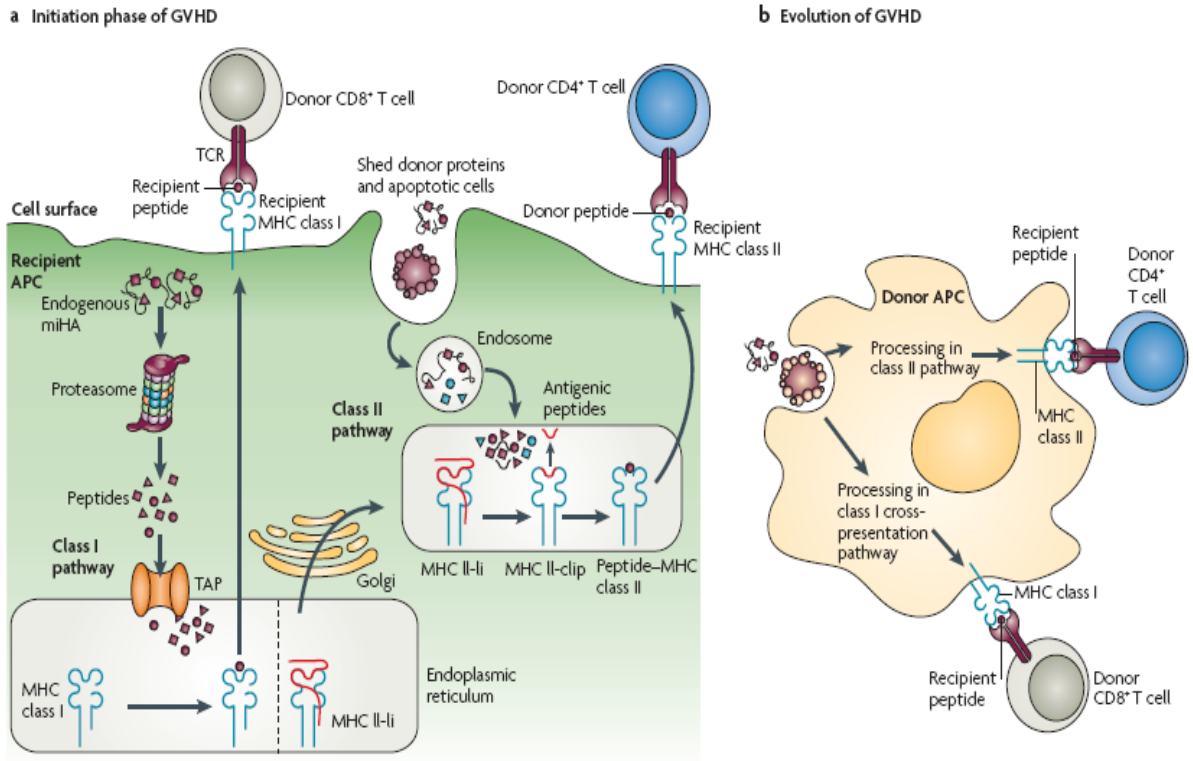
is allogeneic for the grafted cells. However there are also some problems connected to it such as how to reduce graft versus host disease without affecting anti-tumor activity, T cell dysfunction and delayed immune response [13]. In theory allogeneic bone marrow transplantation could serve as an ultimate curative therapy for patients with hematological malignancies as well as solid tumors which has been reported for renal cell carcinoma [14].

1.1.1 Graft versus host disease (GVHD) after allogeneic BMCT

The danger of GVHD development is an important consideration in allogeneic BMCT and is irrespective of the conditioning regimen i.e. myeloablative or non-myeloablative. It is a condition in which immune cells or precursors of immune cells of the immunocompetent donor develop a strong immune response against the immunodeficient host. The host being immunodeficient allows the engraftment of the donor. Clinical GVHD has an acute and a chronic form. The acute form develops within 100 days and is characterized by damage to skin, liver and gastrointestinal tract. The chronic form of GVHD develops after 100 days and with problems like autoimmune syndromes such as eosinophilic fasciitis [15].

So, we need to design tools that can inhibit GVHD but at the same time retain graft versus tumor (GVT) activity. Several approaches are being proposed and studied but till date no approach has proved to be ideal in solving the problem completely. Patients receive immune suppressive drugs so that the T cells accompanying the graft do not react with host cells. However, immune suppressive drugs impair immune reconstitution of the host to a large extent. Another approach is to deplete T cells from the graft [16]. αβ T cells are one of the main inducers of GVHD. Both CD4 and CD8 T cells can induce GVHD corresponding to MHC mismatch. In all minor HLA mismatched models CD8 T cells are most likely involved in mediating GVHD. But, CD4 T cells can also help inducing GVHD and in addition they support CD8 T cell mediated effects [15].

Blockade of co-stimulatory molecules would be an ideal tool since T cells require signals from co-stimulatory cells for their activation and function. Many concepts deal with blocking T cell activation directly or through co-stimulatory molecules such as application of anti-TCR, anti-CD40L or anti-CTLA-4Ig resulting in significant decrease in GVHD. Depletion of CD4 T cells which are thought to favor GVHD and addition of small number of CD8 T cells could favor engraftment [17-20]. Infusion of regulatory T cells (T_{regs}) is also being widely accepted. T_{regs} are a group of T cells that suppress the function of other immune cells, thus regulating homeostasis, tolerance and preventing over response against antigens. It has been shown that T_{regs} are involved in controlling GVHD and can also differentiate between GVHD and GVT. This feature of T_{regs} could be manipulated during BMC transplantation such that they can suppress GVHD and retain GVT reactivity [21]. The exact mechanisms by which T_{regs} mediate these effects are not fully understood. Some studies speculate that they suppress expansion of alloreactive donor T cells as well as the IL-2 receptor α-chain and do not interfere with GVT reactivity that is primarily mediated via perforin lysis pathway mediated by CD8 cytotoxic T cells [22].



Scheme A. Model of Graft vs Host disease *adopted from*[15]

1.1.2 Tumor vaccination after allogeneic bone marrow transplantation

Tumor antigens are molecules on a tumor cell that can be recognized by immune cells. They are either tumor specific (expressed only on tumor cells and not in normal cells) or tumor associated antigens (expressed at lower levels in normal cells) [23]. We know that patients bearing tumors don't mount an efficient immune response. So there is a need for an external stimulus to trigger anti-tumor response. For this purpose vaccination could be a useful tool to boost the immune system.

Allogeneic BMT along with vaccination including whole tumor cell vaccine from the recipient produces a good graft vs tumor (GVT) response but it comes along with high GVHD. High GVHD is due to minor histocompatibility antigens on the whole tumor cell vaccine. Donor T cells immunized against a specific tumor antigen and not against minor histocompatibility antigens (mHAgs) would be efficient in attacking the tumor without GVHD. For this purpose vaccines directed against a tumor specific antigen and delivered in the form of DNA or RNA coding for tumor antigens or proteins have evolved that would not immunize T cells against mHAgs and thus only mediate GVT efficiently without causing GVHD [24]. One such example is the murine ubiquitin gene fused to murine melanoma peptide epitopes gp100 (25-35) and TRP-2 (181-188) when administered (oral DNA vaccine), leads to rejection of a murine melanoma line. Immunity against the tumor was mediated by MHC class I restricted CD8 cells along with the help of CD4 T cells. The anti-tumor mechanisms involved high secretion of TNF-α and IFN-γ, increased expression of co-stimulatory molecules CD28 on T cells and B7.1 and CD48 on antigen presenting cells (APCs) *Ex vivo* primed dendritic cells have emerged as a good source for antigen presentation and triggering T cell activation [25-27].

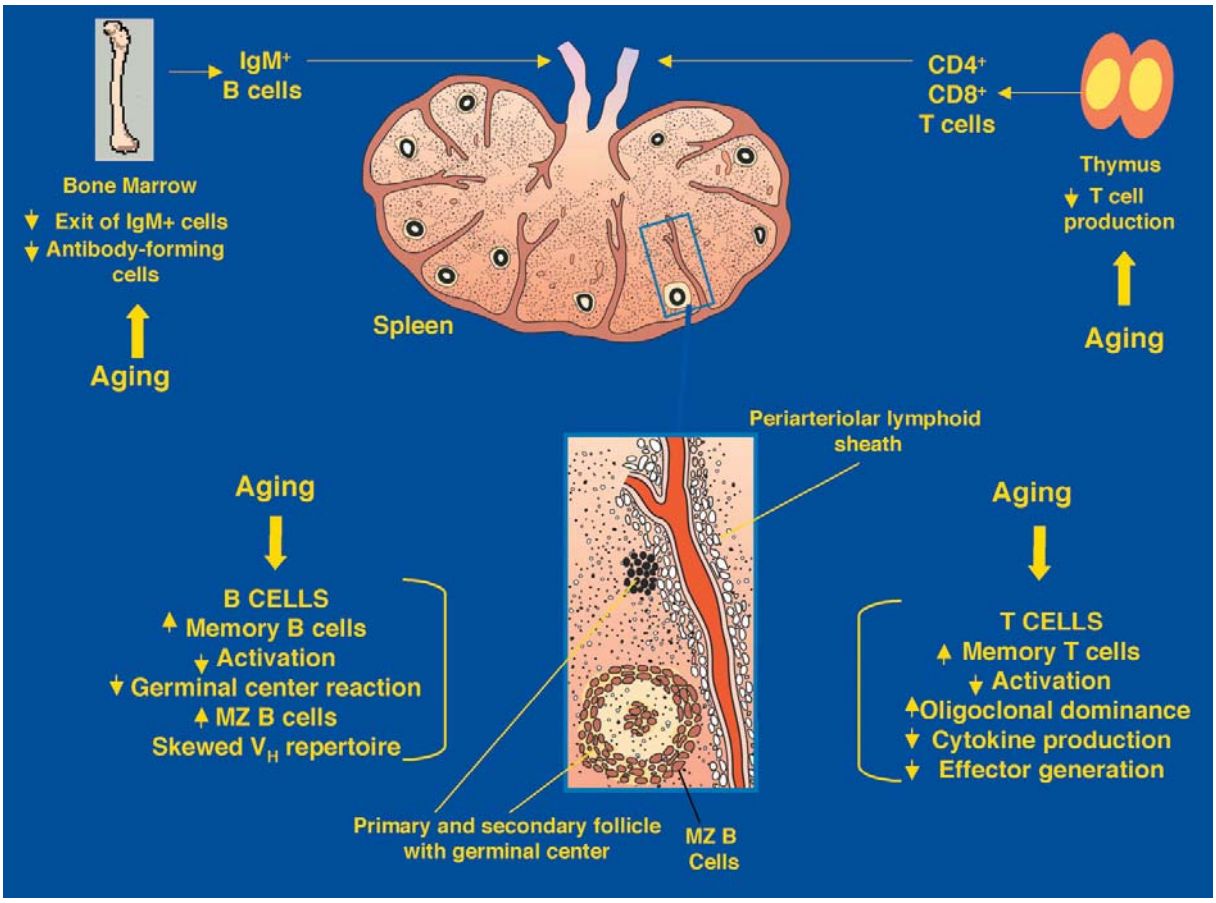
When vaccination is given against a tumor antigen the cytotoxic T lymphocytes (CTLs) become activated and kill the tumor cells. However, for a sufficient immune response T helper cells also have to be activated since they release cytokines that are necessary for activating CTLs [28]. A major drawback associated with tumor vaccination is inefficiency of the immune system to respond actively. Vaccination can

fail when the immune system becomes tolerant towards the tumor antigens or effector cells induced in due course of vaccination can attack tumor as well differentiating cells on the system leading to autoimmune response and GVHD [29, 30].

However, before considering vaccination protocols in allogeneically reconstituted host, the question on how to re-establish a competent immune system needs to be solved. Because intra thymic maturation of T cells is a prerequisite to reach this goal, we have to come back to the chapter on the inherent difficulties accompanied by allogeneic hematopoietic stem cell transplantation, which includes the problem of thymus involution with age.

1.1.3 Thymus involution

Thymus is the central organ for T cell development and in adults there is a confrontation of the problem of thymus involution which is of grave concern in allogeneic bone marrow transplantation setup [31]. Involution of thymus (thymic atrophy) starts at a young age, epithelial space starts to shrink by year one in humans and by middle age post parenchymal tissue is replaced by fat; nevertheless thymus is functional till the age of 60 although maturation of T cells and emigration into periphery is about 5% of that in younger individuals [31-33]. In the normal scenario thymic atrophy does not affect people since the peripheral T cell pool still has a high capacity to proliferate and contains many memory T cells [34].



Scheme B. Age related B and T cell development *adopted from* [35]

Now the question arises on how efficient is T cell maturation after allogeneic BMT. Allogeneic bone marrow transplantation relies on naive T cells for immune functions. In younger patients that receive BMT, T cells can mature in the thymus efficiently. But a major problem arises in adults where T cell reconstitution is hampered by thymic atrophy [36]. Thus, how to regenerate the thymus and promote T cell maturation in allogeneic BMT is a major question in the field of BMT.

Many approaches have been proposed to increase to some extent thymic reconstitution and thymopoiesis. One approach is implanting thymic grafts, however they are rarely available [37]. One of the main causes of thymic involution is said to be increased production of sex steroids (androgen, oestrogen and progesterone) after puberty. Decreasing the level of these hormones through castration helps to regain thymic cellularity and reformation of thymic architecture to some extent [38]. In addition to their role in regulating metabolism, growth hormone and insulin like growth

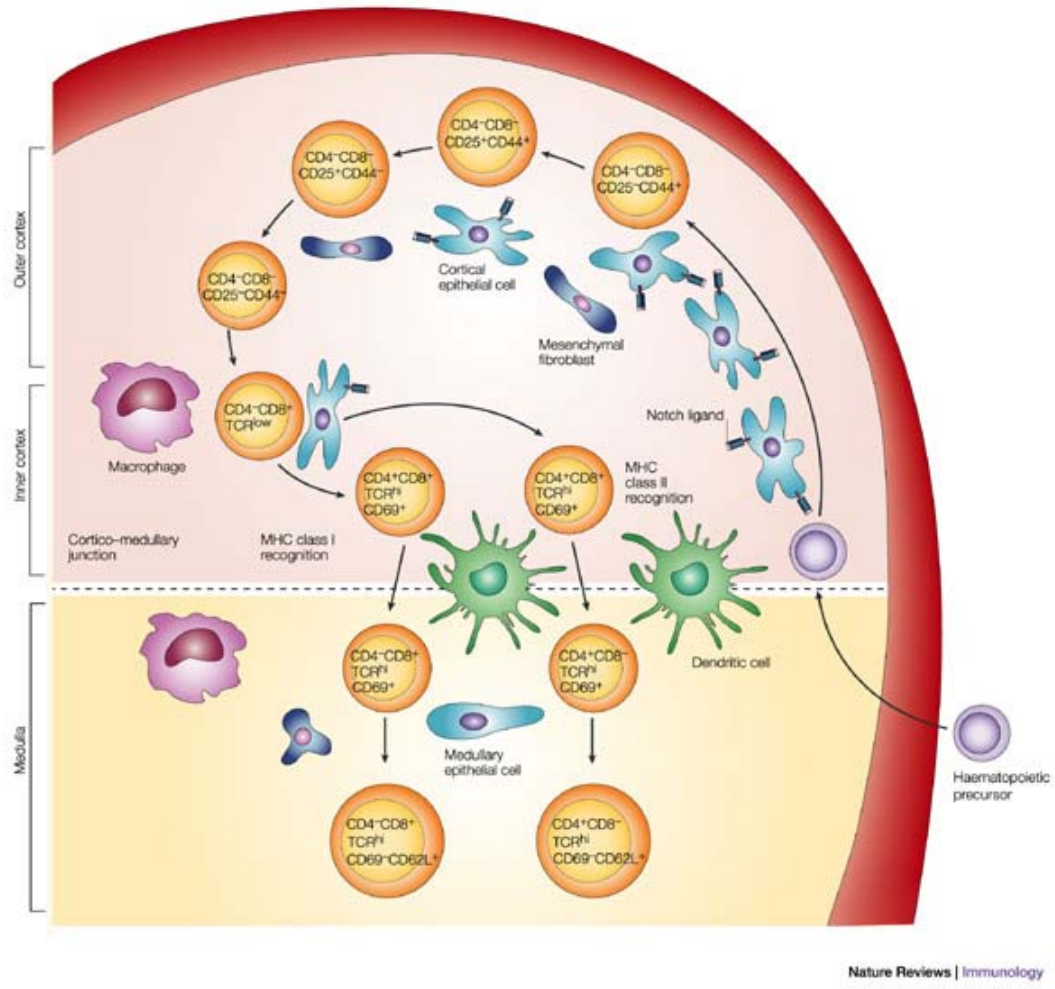
factor also play a role in hematopoiesis, lymphopoiesis, T and B cell development. These features can also be exploited to enhance T cell repopulation [39]. Administration of IL-7 has many stimulatory effects on T cell development such as increased thymopoiesis [40], increased peripheral $CD4^+$ and $CD8^+$ T cells [41] and increased numbers of cytotoxic T cells [42]. Inspite of several approaches being studied till date thymus regeneration still seems to be a major concern in allogeneic bone marrow cell reconstitution.

1.2 Thymocyte maturation

Thymus is a primary lymphoid organ that essentially is required for maturation of self tolerant T lymphocytes from incoming bone marrow derived progenitors. It is found in all vertebrates with the exception of jawless fish. Its emergence came in parallel with the identification of VDJ gene rearrangements as a means for diversifying antigen receptors. Thymopoesis is a complex process involving three main steps, first thymus as a site for incoming and outgoing thymocytes, second, nature of thymic progenitors that enter the thymus and third, development of thymocytes through different stages to become T lymphocytes ready to exit to the periphery. This process is mediated through several signaling mechanisms and interactions involving thymocytes and stromal environment including epithelial cells, dendritic cells, and fibroblasts [43].

Thymocytes undergo a defined differentiation process that is characterized by the expression of CD4 and CD8. Double negative (DN) thymocytes, are the earliest in the differentiation process at which T cell progenitors lack CD4 and CD8 expression. They are further subdivided into DN1 through DN4 depending on the expression of CD44 and CD25 as follows, DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$). The next step is double positive (DP) stage ($CD4^+CD8^+$) and finally the single positive (SP) stage ($CD4^+$ or $CD8^+$). Double negative thymocytes compose 5% of the whole population, double positive thymocytes compose 80% and single positive thymocytes compose 10%,

respectively [44, 45]. DN1 cells move very slow and remain near the site of thymic entry (cortico medullary junction) for 9-11 days after entry. This is the time when they proliferate and undergo lineage commitment losing their capacity to differentiate into B and natural killer (NK) cells. Cells now move out of the cortico medullary junction to the middle cortex where they can still give rise to T or dendritic cells (DN2 stage). They remain there for 2 days after which they move to the outer cortex where they transform into DN3 cells. In approximately 13 days the transition to DP stage occurs in the capsule. After which they start moving towards the medulla, undergoing positive and negative selection and get committed towards CD4 and CD8 T cells and finally exit to the periphery [46-48].



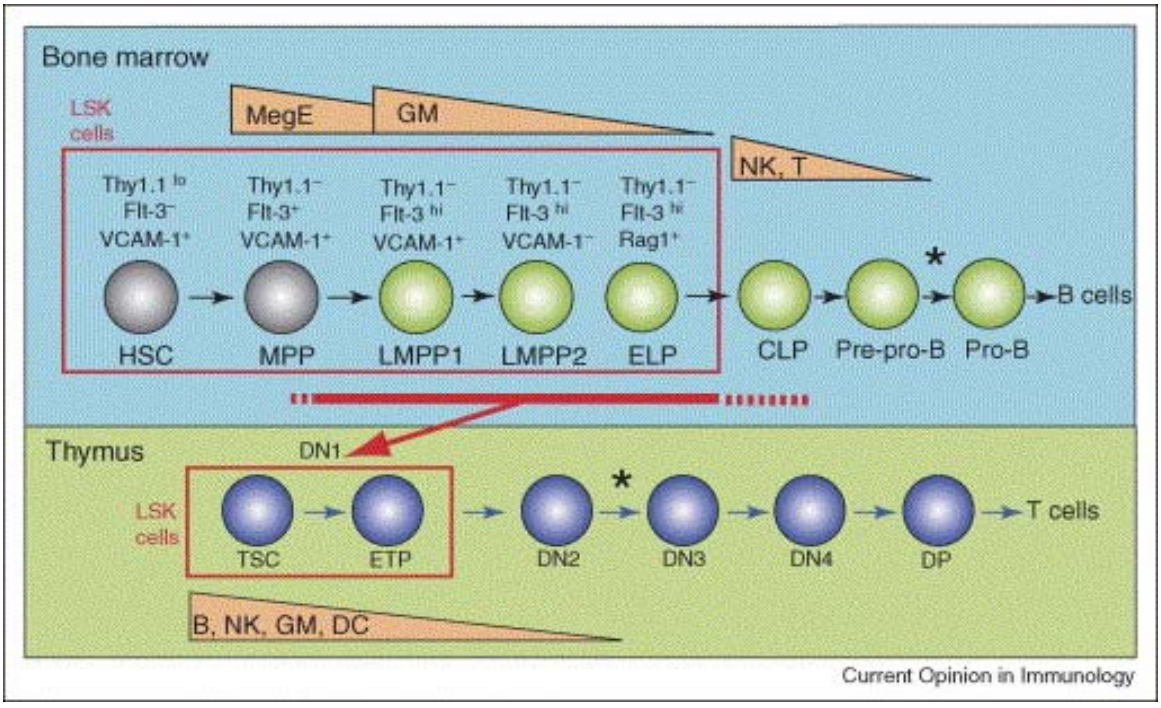
Scheme C. Stages in thymocyte development and involvement of thymic environment *adopted from* [49]

1.2.1 Thymocyte homing and migration

Thymocyte migration can be subdivided as follows, entry of bone marrow derived progenitors into thymus, migration of thymocytes from the cortex through the medulla, and exit to periphery [45].

1.2.1.1 Thymic progenitors

Hematopoetic stem cells reside in the fetal liver or adult bone marrow. They have self renewing capacity and are able to generate cells of all blood lineages. They are the source of precursors such as common lymphoid (CLP) or common myeloid progenitors (CMP). The most primitive progenitors in the thymus are DN1 and they are $CD4^{low}$ and $ckit^{+}$. More detailed analysis of this population has revealed early T lineage progenitor (ETP) population that is $lin^{-}ckit^{+}CD25^{-}IL\text{-}7R\alpha^{low}$. These cells have T cell potential but limited B and myeloid cell potential [50]. Another study has shown two subsets of DN1 a and b using subfractionation of DN1 cells depending on $c\text{-}kit^{+}$, $HSA^{low/-}$ and $c\text{-}kit^{+}/HSA^{+}$, resulting in precursor progeny relationship ETP being precursor and DN being progeny. ETPs were defined as a pool of DN1 a and b. ETPs can further be subdivided depending on expression of fms like tyrosine kinase 3. ETPs expressing Flt3 form an immature subset and loss of Flt3 leads to loss of B cell potential. ETPs are said to be similar to bone marrow LSK ($lin^{-}Sca\text{-}1^{+}cKit^{+}$) cells that are composed of a heterogenous population of hematopoetic cells with long and short term self renewal capacity. Recent LSK cells with T cell potential have been identified. The phenotypic similarity between ETPs and LSKs and also the presence of LSKs in circulating blood has led to the suggestion that a subfraction of LSK home into the thymus as progenitors further giving rise to thymocytes and T cells [51]. Another study has identified two subpopulations of bone marrow cells known as CLP1 ($lin^{-}c\text{-}kit^{+}IL\text{-}7R\alpha^{+}$) and another population designated as CLP2 ($lin^{-}c\text{-}kit^{low}$ $B220^{+}$) that originates from CLP1 cells *in vitro.* CLP2 population efficiently homes into thymus upon intravenous transfer and gives rise to T cells indicating that they are lineage committed [52].



Scheme D. Thymic progenitors and T lineage commitment *adopted from* [53]

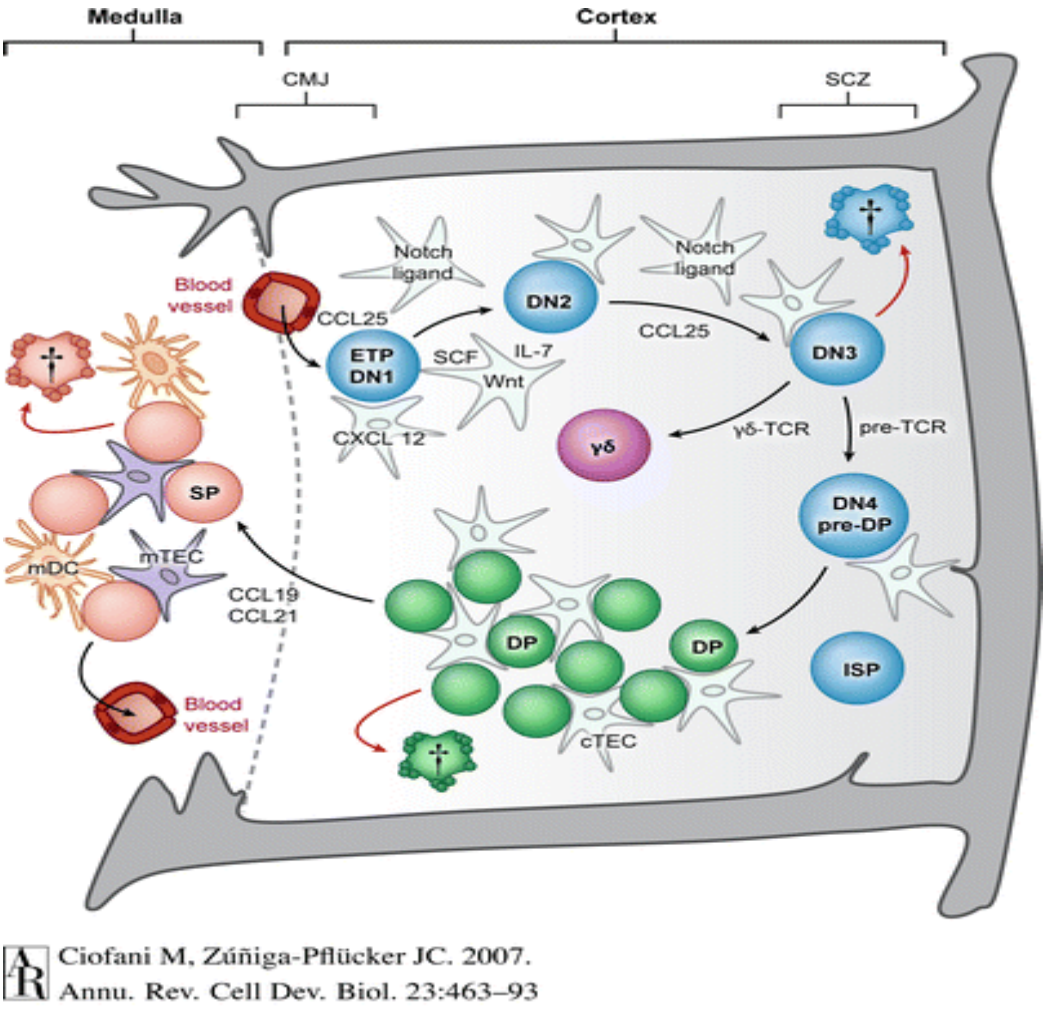
1.2.1.2 Chemokines

Chemokines and extracellular matrix are involved in driving the thymocytes through the thymic region during development. They attract thymocytes at different stages into different regions of the organ. Thymocytes differentially express chemokine receptors at different stages of development. CXCL-12 is expressed in subcapsular and medullary region and attracts double negative and double positive thymocytes that express CXCR4. However previous data showed that CXCR4 or CXCL12 knock out (KO) mice still have normal thymocyte development indicating other receptors and chemokines that might act in parallel [45].

The chemokine CCL25 also known as TECK is expressed by thymic epithelial cells and dendritic cells. Its receptor CCR9 is expressed in all subsets of thymocytes with maximum expression on double positive thymocytes [54]. Since CCR9 is down regulated in mature thymocytes and the fact that there is lack of responsiveness to CCL25 before exit to the periphery, it is speculated that CCR9/CCL25 interactions might be necessary for retention of thymocytes in the thymus [45]. Lack of CCR9 reduces but does not abolish homing and migration completely and this indicates an

involvement of other factors responsible for attracting as well as guiding the homing and migration of thymocytes such as CCL21 [43, 55].

CCL19/CCR7 mediate thymocyte exit. Higher number of thymocytes accumulate in the thymus in CCL19/CCR7 knockout (KO) mice leading to lesser T cells in the periphery suggesting a role for this chemokine/receptor in mediating thymocyte emigration [56].



Ciofani M, Zúñiga-Pflücker JC. 2007.
Annu. Rev. Cell Dev. Biol. 23:463–93

Scheme E. Chemokines and growth factors involved in thymocyte development

adopted from [57]

1.2.1.3 Adhesion molecules

Thymic microenvironment expresses many adhesion molecules that have also been proposed to be involved in thymus homing and migration. Thymocyte adhesion to the matrix is blocked when antibodies against fibronectin receptor (α5β1 integrin) and laminin receoptor (α6β1 integrin) are added. Adhesion of immature thymocytes (DN) is impaired when anti-VLA-4 (α4β1 integrin) and/or antagonists of α4β1 are added. In addition it has been suggested that moderate interactions of thymocytes with fibronectin are responsible for thymocyte migration. Thymocyte exit to the periphery is also mediated by α4β1 mediated ECM interactions [45, 58].

CD44 is very highly expressed on early thymocytes as well as on progenitors and plays a role in binding to the extracellular matrix. It was shown that adding antibodies against the adhesion molecules of CD44, α4β1 integrin and lymphocyte function associated antigen-1 in thymic organ cultures where T cell development was induced from BM progenitors inhibited the T cell development to a large extent. However these antibodies did not inhibit the development when fetal thymic cells were used. Hence this shows a prerequisite for adhesion molecules especially in the early stages on T cell development [59].

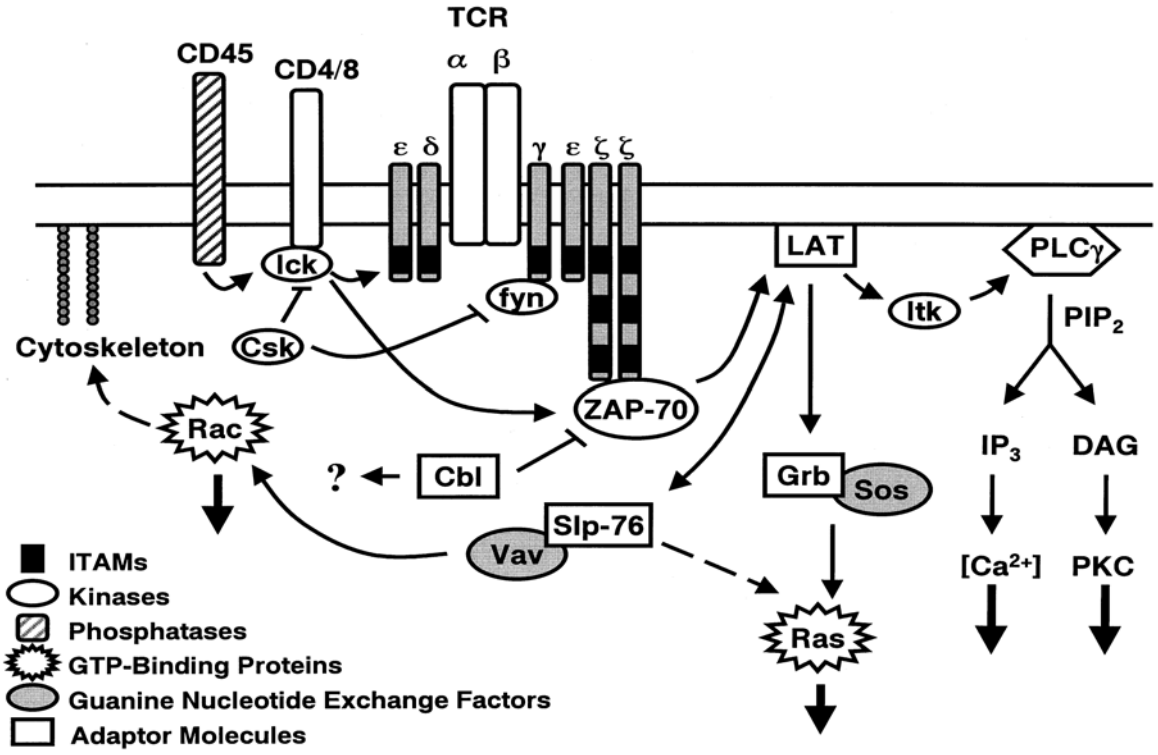
P-selectin and P-selectin ligand (PSGL) have been proposed to be involved in the recruitment of progenitors into thymus. P-selectin is expressed on thymic vessels and PSGL is expressed on circulating thymic progenitors. Homing capacity of progenitors is strongly reduced in the absence of either P-selectin or PSGL. Hence P-selectin is the first molecule that fulfils the requirements of recruiting progenitors into thymus [60].

DN1 cells express integrins α4β1, α5β1, α6β1 heterodimers that potentially bind to VCAM-1 (vascular cell adhesion molecule), fibronectin and laminin1 respectively. DN2 cells express α4β1, α5β1, α6β1, α6β4 and α4β7 that potentially bind to fibronectin, collagen and VCAM that have been identified in the cortex. DN3 cells express the same integrins as DN2 cells with the exception of β7; hence they are not

able to interact with VCAM-1. DN4 cells express all the same α integrins as DN3. The expression of β integrins has not yet been investigated [46, 48, 61].

1.2.2 Signaling in T cell development

Thymocyte development from double negative to single positive $CD4^+$ or $CD8^+$ cells involves many signal transduction events which can broadly be grouped into three main stages involving pre-T cell receptor and αβTCR receptors expression as follows.



Scheme F. Signaling mediating signaling events at the TCR complex *adopted from* **[62]**

1.2.2.1 β selection checkpoint

The first checkpoint termed as β selection checkpoint occurs at the DN3 stage where the cells undergo screening processes for successful arrangements of TCRβ genes. TCRβ chain is expressed in concert with the invariant preT-α chain and CD3 signal transduction moleclues. Saito and co-workers have proposed that specific residues in

the pre-T α membrane proximal sequence induce pre-TCR oligomerisation and confer β-selection in vivo [63].

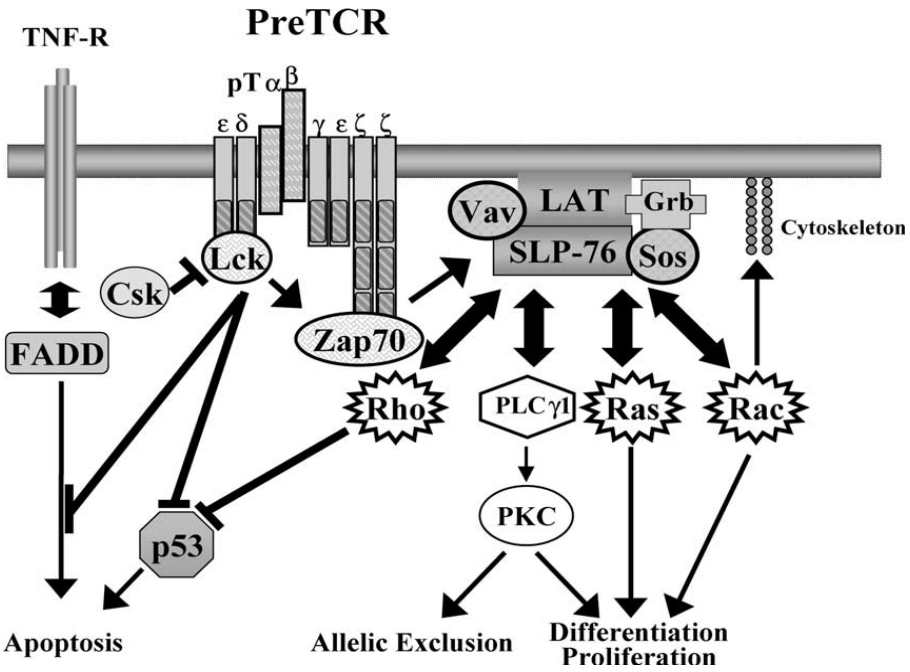
At this step various signaling events take place inducing proliferation as well as co-expression of the co-receptors CD4 and CD8. Various signaling molecules have been proposed to be involved at this step. Small GTPases such as Ras, Rap1A, RhoA, Rac and cdc42 promote survival and proliferation of DN cells but not allelic exclusion [64]. pLCK (Y505A) and PKC (Protein Kinase C) transgene mutants suggest the role of PKC and Lck in allelic exclusion and in addition cell survival and proliferation. A membrane targeted form of PKD is able to promote proliferation as well as expression of CD4 and CD8. In contrast the cytoplasmic form suppresses Vβ and DJβ recombination. HDAC, a transcriptional repressor has been shown as a target for the cytoplasmic domain of PKD and HDAC includes transcription factor Nur77, involved in regulating apoptosis in thymocytes as one of its targets [65].

Interaction between transcription factors Egr3, RORγt and E proteins also regulate transition from DN to DP stage involving rapid proliferation before the TCRα gene rearrangement and differentiation. In accordance with the preTCR signals Egr3 expression induces proliferation with down regulation of RORγt by inducing the expression of its inhibitor Id3 and vice-versa. RORγt promotes cell cycle arrest by inducing CpeB4, TCRα rearrangement by inducing Rag-2 and cell survival by increasing Bcl-x_L expression [66].

PI3K/Akt Survival pathway

Thymocyte development requires integration from several signaling events through pre-TCR, Notch, IL-7 for a programmed lineage commitment from DN to SP stage. The PI3K/Akt signaling acts at this point to translate these signals towards a functional outcome such as survival, proliferation, differentiation and allelic exclusion at the β-selection check point. PIP2 (phosphatidyl inositol 3,4 bisphosphate) and PIP3 (phosphatidyl inositol 3,4,5- triphosphate) are the two products of PI3K that serve as recruiting molecules for Akt and its activating kinase PDK1 [67-69]. PI3K is

divided into four classes, IA, IB, II and III [70]. Mice lacking the class IB subunit p110γ have decreased numbers of DP followed by decrease in thymic cellularity. Mice lacking both p110 γ and δ show increased apoptosis, decreased proliferation at the β selection checkpoint and DP stages [70]. PI3K activation leads to accumulation of phosphatidyl inositol kinase (PDK1) at the plasma membrane and activation of Akt. Mice with conditional deletion of PDK1 have a block at the DN4 stage of development [71]. Akt1 deletion leads to decrease in thymic cellularity, but Akt2 deletion alone has no effect. However a double deletion of Akt 1 and 2 leads to a higher number of DN3 thymocytes and less DPs, suggesting that these Akt isoforms may be involved in β-selection checkpoint [72]. Cell survival through Akt pathway is supposed to be mediated through Bcl-xL, p-Bad and A1 anti-apoptotic proteins [73].



Scheme G. Signaling events mediating β selection *adopted from* **[64]**

1.2.2.2 Positive versus Negative selection

Many theories have been proposed over the years regarding signals transmitted through αβ-TCR leading to positive or negative selection. Receptor activity and

duration of receptor binding are most likely to influence this selection process. The details are discussed below. Double positive thymocytes whose TCR binds with weak avidity towards MHC I or II receive signals from the TCR whereby they are selected positively and the rest undergo apoptosis. Elimination of autoreactive SP thymocytes in the medulla is termed as negative selection.

TCR CD3 components

Antigen recognition by TCR is mediated by TCR αβ or γδ which are products derived from DNA recombination events. Additional molecules of the TCR complex include CD3γδε and ζ chain and this together forms the T-cell receptor complex that mediates signal transduction. The subunits of the TCR are assembled at the ER and then transported to the cell surface. The CD3 components are able to transduce signals through shared functional sequence termed immunoreceptor tyrosine based activation motif (ITAM) [74].

Several studies in the past suggest that mutations of either TCR or associated CD3 components highly influence thymocytes selection. Deletion of TCRα chain connecting peptide domain (CPM) could inhibit positive selection without affecting negative selection [75]. Deletion of CD3δ component suggests a role for CD3δ in mediating thymic selection process from DP to SP thymocytes. [76]. CD3ζ chain influences both positive and negative selection. Multiple ITAMS are involved in modulating the TCR signals and thus influencing selection. The ζ chain functions to amplify the signals initiated by the TCR [62, 77].

TCR proximal kinases, phosphatases and adaptor molecules

Induction of Lck and Fyn, Src kinases and recruitment at the TCR is one of the earliest events following TCR stimulation. The Src kinases once activated further phosphorylate ITAMS in the CD3 complex. Tyrosine phosphotylation of ITAMS provides docking sites for recruitment of syk protein family kinase ZAP-70 (ζ

associated protein-70). Lck and fyn phosphorylate Zap-70 [78-80]. Zap70 in turn phosphorylates SLP-76 (SH2 domain containing leukocyte protein) and LAT (linker for activation of T cells). These adapter proteins lack intrinsic kinase domains, but phosphorylation of these proteins facilitates interaction with other signaling molecules [81, 82]. LAT and SLP-76 function in concert to provide docking sites for enzymes such as PLCγ and VAV-95 [83]. These two are responsible for further down stream signaling involving small G proteins of Ras family and Rho GTPases leading to ERK phosphorylation. T cell activation also involves signals mediating lipid kinase activation such as PI3K [84]. Defects in any of the molecules mentioned can lead to the following consequences in thymocyte development.

When the catalytically active form of Lck was over expressed positive selection was inhibited [85]. TCR transgenic mice that don't express Fyn showed impaired maturation of selected thymocytes as well as a slight reduction in negative selection. Therefore both Src kinases Lck and Fyn are crucial for positive and negative selection [62, 86].

CD45 and Csk positively and negatively regulate Src family tyrosine kinases. CD45 is a transmembrane tyrosine phosphatase that is essential for positive selection. Csk is also an important regulator of Lck and Fyn since it inactivates them by phosphorylating the regulaltory C-terminal tyrosine residue. On the whole Csk regulates the sensitivity of thymocyte stimulation whereas, CD45 contributes to both positive and negative selection [87]

Zap-70 and Syk belong to the Syk family of tyrosine kinases. Zap-70 deficiency leads to inhibition of development of CD8 T cells and also non-functional CD4 cells. In mice Zap-70 deficiency shows a block in both CD4 and CD8 T cells. Syk on the contrary can compensate for some functions of Zap-70 but cannot substitute for the development of single positive T cells [88, 89].

Cbl and SLP-76 serve as adaptor molecules that lack intrinsic enzymatic activity but can mediate protein-protein interactions with signaling molecules influencing T cell

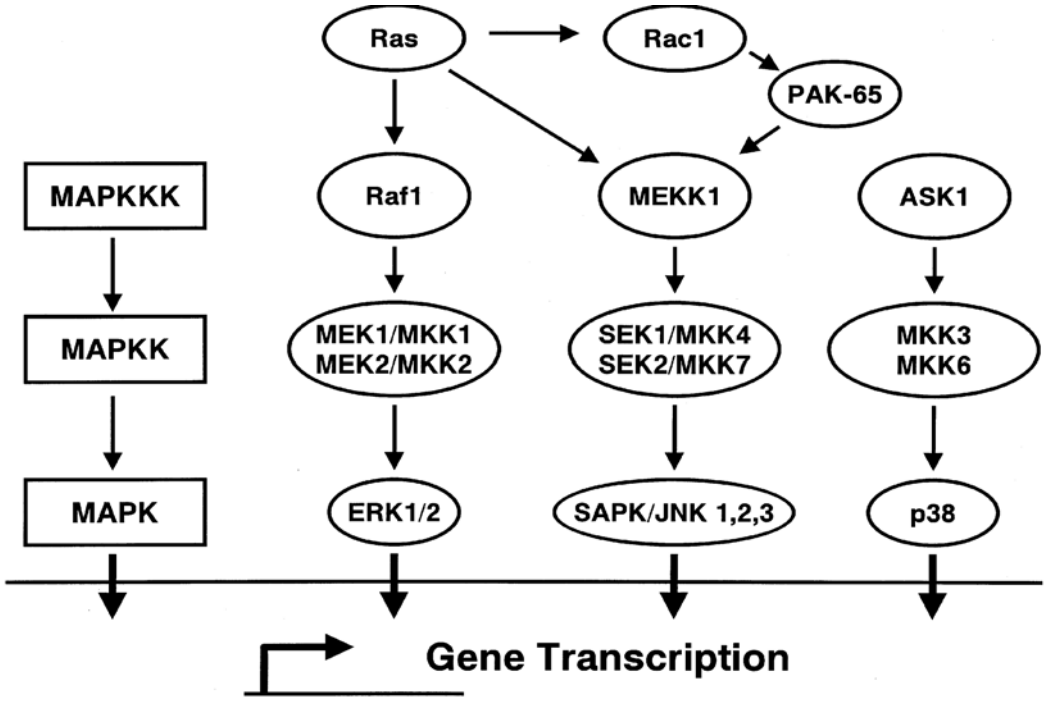
activation. Cbl deficient mice exhibit enhanced positive selection of thymocytes whereas SLP-76 deficient mice show a block in thymocyte development at the DN stage [90, 91].

Vav is a Rho-family guanine nucleotide exchange factor that is required for efficient TCR signaling and thymopoiesis. Vav deficient mice show inhibition in both positive and negative selection of thymocytes. It was proposed that this could be due to inefficient TCR multimerization and internalization [92].

MAPK pathways

The extracellular-regulated kinase (ERK) pathway that includes Ras, Raf-1, MEK1,2 and ERK1,2 is activated upon TCR engagement and plays a role in pre-TCR signaling, positive and negative selection and lineage commitment. Transgenic mice over expressing Ras, Raf-1 and MEK1 can inhibit positive selection but leaves negative selection intact [93]. The JNK SAPK group of MAPKs consists of three members JNK1, 2 and 3. Targeted disruption of these individual genes did not alter T cell development nor CD4 and CD8 numbers. Dominant negative form of JNK1 however led to increased resistance to anti-CD3 mediated apoptotic death of thymocytes [94].

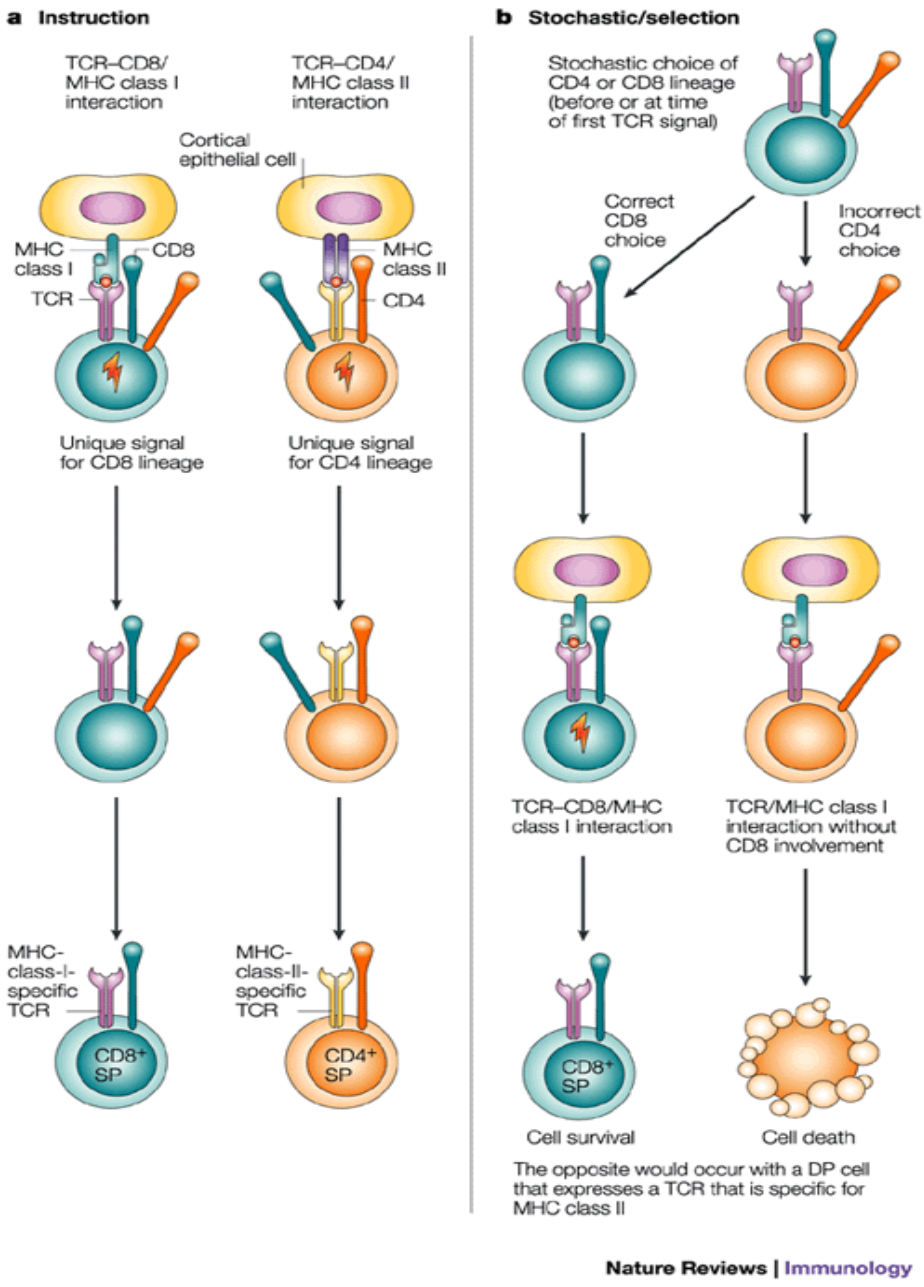
P38 is another MAPK member and is also reported to play an essential role in mediating intrathymic signals *in vivo.* It has been shown that P38 pathway is critical for negative selection of thymocytes but not positive selection [95]. Taken together these studies suggest that ERK is essential for positive selection whereas JNK and p38 mediated negative selection leading to opposite fates in immature thymocytes. (Sakata, Patel et al. 1995)



Scheme H. MAP Kinase pathway *adopted from* [62]

1.2.2.3 Lineage commitment

Cell fate decisions are usually controlled by conserved receptors that interact with evolved ligands. Hence, the lineage fate decision of thymocytes to form CD4 or CD8 positive cells is unusual since it is regulated by clonally expressed, somatically generated T-cell receptors whose specificity cannot be defined. At the end each mature T cells retains only one receptor CD4 that binds to MHC-II molecule or CD8 that binds to MHC-I molecules. Two models have been proposed to explain this fate one was '*instruction'* model that depends on initial signaling events initiated on binding of TCR complex to MHCI or MHCII along with CD4 and CD8 coreceptor or a random '*Stochastic'* model where the decision takes place depending on which MHC molecule is interacting with the DP thymocyte. If the MHC-II interacts first with CD4 then the CD8 co-receptor is automatically down regulated and vice-versa. Recent evidence suggests that the instruction model holds true [96, 97].



Scheme I. CD4/CD8 lineage commitment during thymocyte development *adopted from* [96]

Studies support both strength and kinetic signaling models to explain the fate choice between CD4 and CD8 T cells. CD4 and CD8 co-receptors have different abilities to associate with Lck and this in turn instructs fate choice when DP thymocytes bind to MHCI or MHCII. Studies have shown that greater Lck activity favored CD4 lineage fate and vice-versa [98].

Erk is another likely candidate that influences fate choice between CD4 and CD8. Complete deletion of ERK1 and ERK2 in DP thymocytes has been shown to affect CD4 maturation more than CD8. RasGRP1 a nucleotide guanine exchange factor had a greater impact on CD4 than CD8 T cell development. This possibly indicates that Ras/MAPK pathway is crucial for CD4 lineage fate reviewed in [99].

1.3 CD44 Molecule

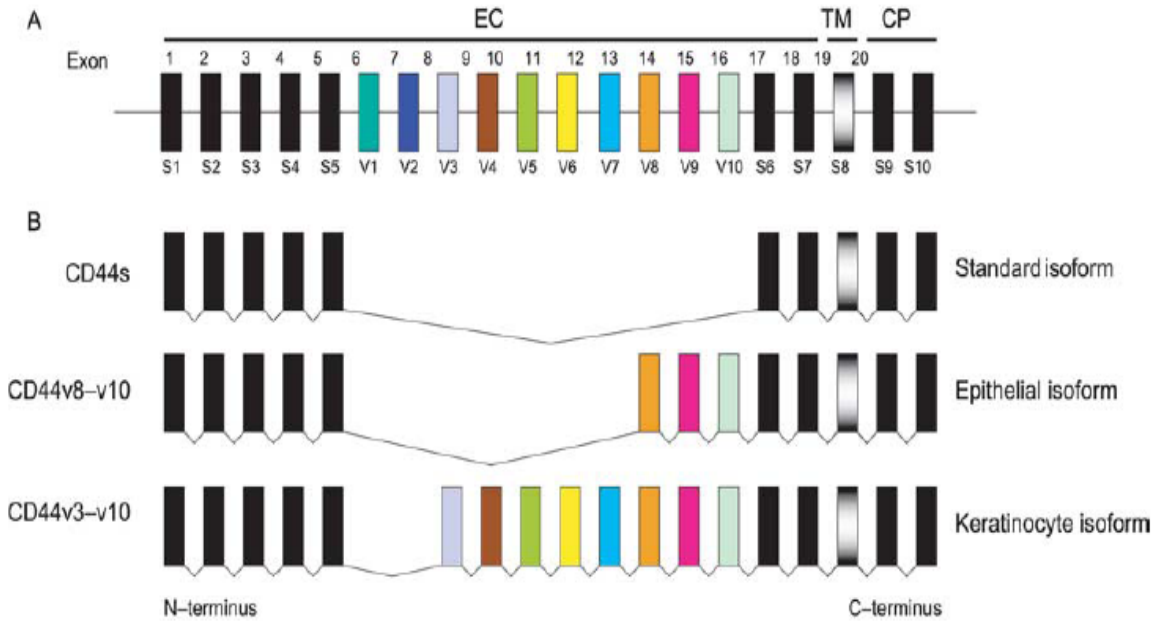
Adhesion molecules are cell surface glycoproteins that have a large extracellular domain, a transmembrane domain and an intracellular cytoplasmic domain. These molecules are termed adhesion since they bind strongly to specific ligands. This interaction is more complex in terms of sensing the extracellular environment and sending signals to adjacent cells. Cell adhesion molecules are becoming increasingly important in relation to disease processes and as targets for use in diagnostic pathology [100].

CD44 glycoproteins are one of the very well characterized members of the hyaluronate receptor family of adhesion molecules. CD44 is a Type-I family transmembrane protein that mediates interaction of cells to their microenvironment. CD44 family of adhesion molecules bind to hyaluronic acid as the major ligand. HA is an extracellular polysaccharide present abundantly in extracelullar cell matrix (ECM) [101]. The CD44 epitope was first discovered as an antigen using a monoclonal antibody that was raised against human white blood cells [102]. It was then identified that a group of polymorphic proteins had this epitope in common (80-200KD in size).

1.3.1 Structure

CD44 is encoded by a single gene spanning a 50-60 kb region [103, 104]. It has 20 exons from which various protein products are further generated through 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. The CD44 isoform without the variant exons is termed as CD44s or hematopoietic isoform [105]. CD44 gene is highly conserved among mammals. Between the human and mice genomic sequence there is 85% and 87% homology in the N and C-terminus respectively [104].



Scheme J. Structure of CD44 Molecule *adopted from* [106]

Hyaluron binding, amino terminal domain

This site contains motifs that provide as docking sites for several components of the extra cellular matrix. *In vitro* studies have revealed that interaction of CD44 with hyaluronic acid (HA), collagen, laminin and fibronectin promote matrix dependent cell migration [102, 107]. 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) [108, 109] 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 [110].

Stem structure

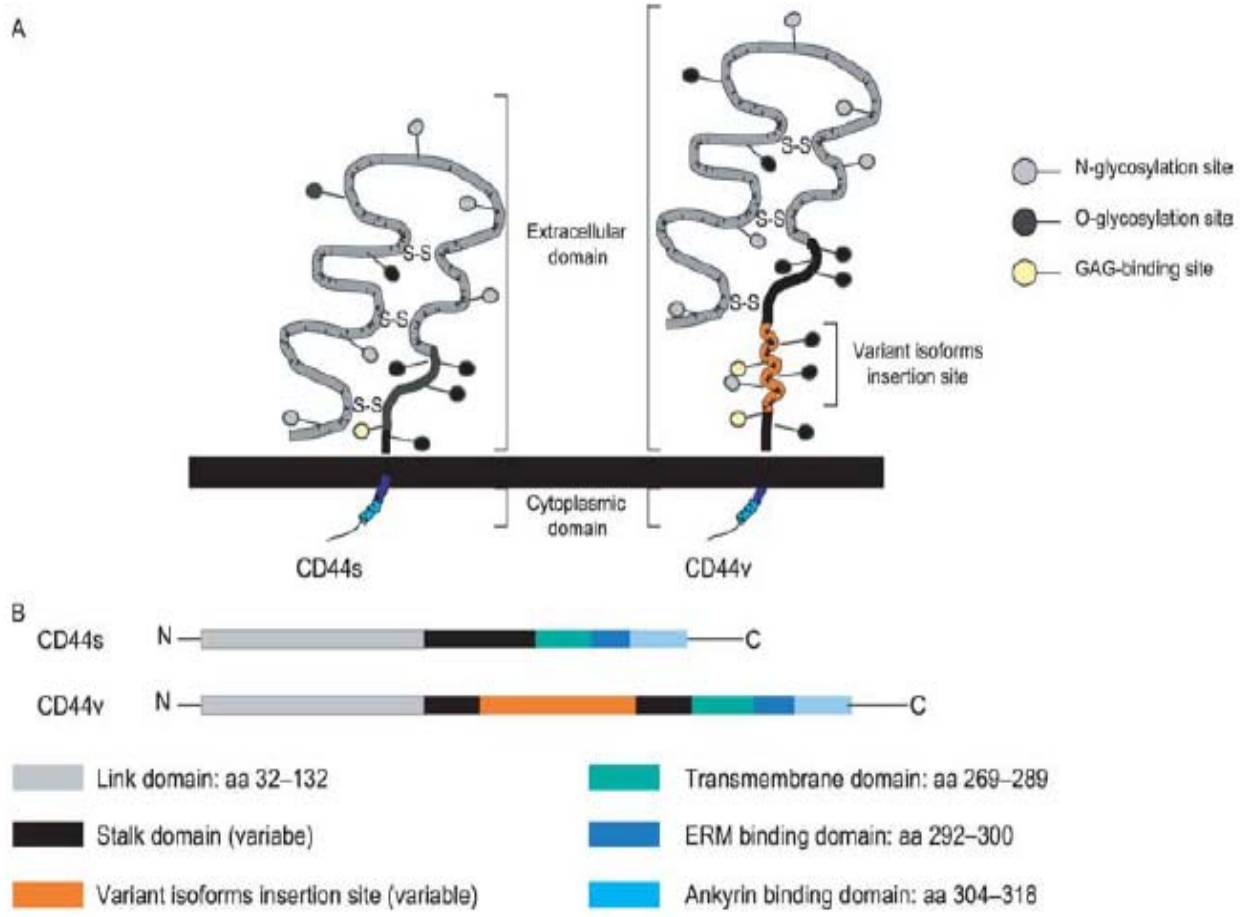
The amino terminal globular domain of the smallest CD44 isoform (CD44 standard) is separated from the plasma membrane by a 46 amino acids stem structure. It contains proteolytic cleavage sites. It also contains the variant exon region of CD44. The inclusion of several combinations of variant exons depends on mitogenic signals including stimulation of Ras-MEK-ERK pathway that regulate splicing events [111, 112] Some variant exon segments include motifs for post translational modifications but less is known about the exact structure of these variants [102].

Transmembrane and cytoplasmic domain

The transmembrane and cytoplasmic domain are required for efficient ligand binding [113]. 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 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 association in lipid rafts is still not clearly validated [114].

The cytoplasmic domain is encoded by exon 19 or 20. Inclusion of one or the other of these exons by alternative splicing generates a tailless CD44 ending at Arg_{294} or a 73 amino acid long cytoplasmic tail. The cytoplasmic tail is responsible for interacting with other intracellular signaling molecules and for this process Ser_{325} and Ser_{291} residues are important [115]. 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 [102]. The cytoplasmic domain is known to promote Hyaluron internalization and thus migration of cells [116].



Scheme K. CD44 domains *adopted from* **[106]**

1.3.2 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.

1.3.2.1 Cell adhesion and migration

Cell adhesion and migration are crucial for leukocytes and tumor cells in case of inflammation and metastasis, respectively. CD44 primarily plays a role in maintaining

the three dimensional structure of organs and tissues [117]. 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 [102]. 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 [113].

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 [118]. Rolling of cells is generally mediated by selectins and carbohydrate ligands where they promote leukocyte entry into sites of inflammation [119]. 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) and cdc42/Rac. In addition intracellular events such as serine proteases might also support CD44-mediated cellular mobility by cleavage of the molecule [120-123].

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 [124]. 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 [125].

1.3.2.2 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 also merlin [126].

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 the cytoskeleton [127]. CD44 binds to ankyrin cytoskeletal protein linking the plasma membrane to underlying cytoskeleton. The binding affinity is further enhanced through GTP binding proteins [128-130].

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 [126]. 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 [131-133]. Thr_{567} Ezrin, Thr_{564} Radixin and Thr_{558} Moesin phosphorylations result in rearrangement of the cytoskeleton [134].

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. 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 [115].

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 [135]. Merlin does not have an actin binding site and merlin-CD44 complex cannot bind to cytoskeleton [136]. The ability of ERM/melrin 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 [115]. 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 [137, 138].

1.3.2.3 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 [139]. CD44v3 has been shown to immobilize chemokines such as osteopontin, scatter factor, endothelial growth factor, MIP-I β and RANTES, reviewed in [106].

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) [140]. 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. [141]

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 [142].

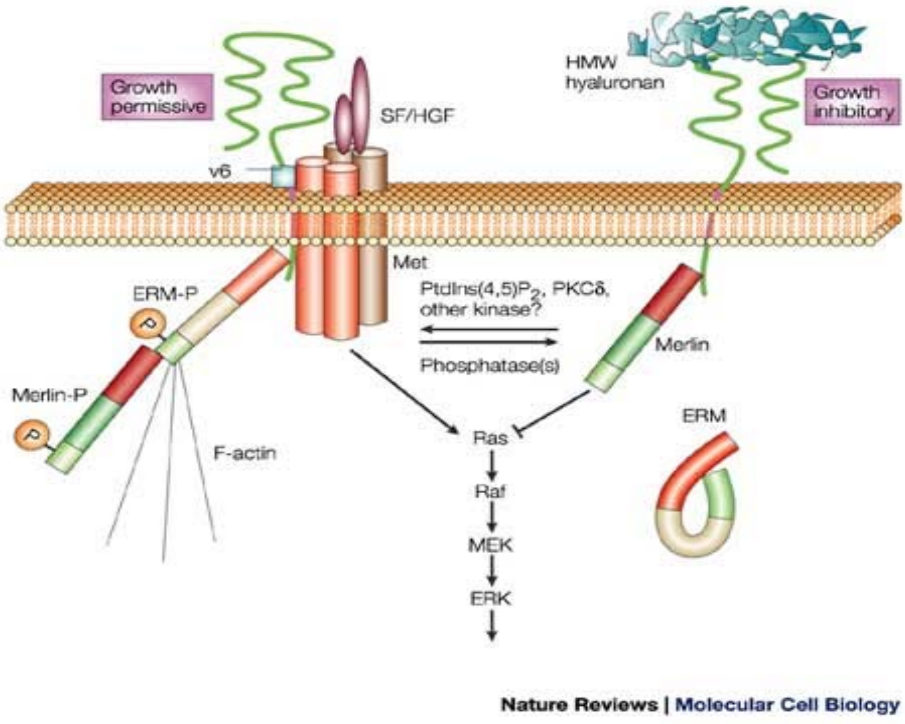
CD44v3 heparan sulphate proteoglycan and v6 bind to epidermal growth factor, heparin binding epidermal growth factor and fibroblast growth factor and present in an efficient way to their receptors for survival and proliferation of leukocytes and tumor cells. One example is CD44v3 heparan sulphate proteoglycan 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 [143]. 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 [144].

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

1.3.2.4 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. 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 [145] and CD44 is one of them. The multifunctional CD44 molecules are known to be involved in various signaling cascades starting from growth and proliferation, survival, apoptosis, cytoskeletal organization, cell motility and adhesion.

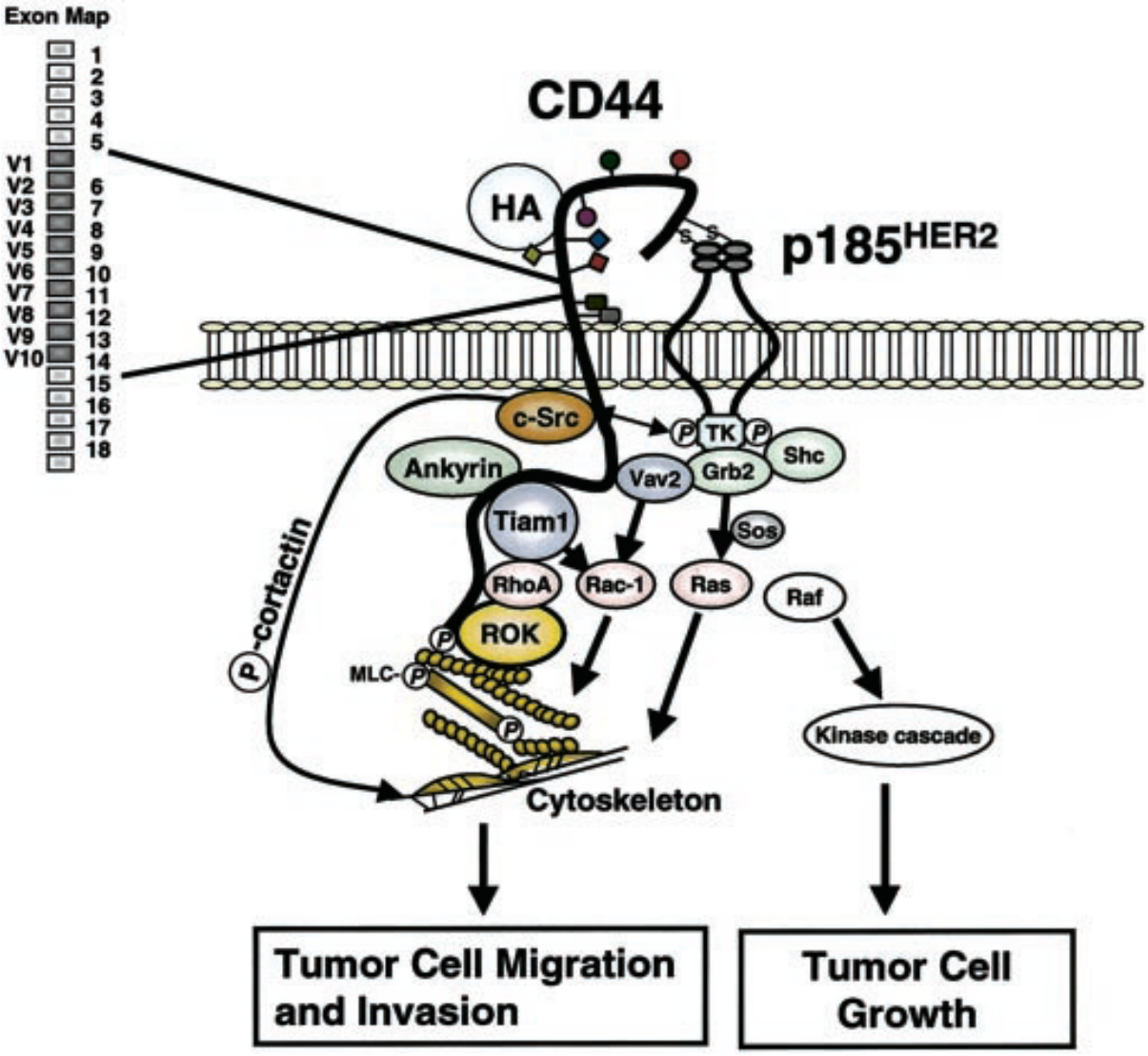
Since cytoskeletal organization and cell migration have been dealt with in the previous sections this section will mainly focus on cell growth proliferation, survival, apoptosis and anti-apoptosis.



Scheme L. Signaling mediated by CD44 *adopted from* [101]

One main reason for the involvement of CD44 in multiple signaling events is its association with the Src family of phospho tyrosine 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 [106]. When activated they mediate several signaling events including activation 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 upregulation and down regulation of several signaling pathways involved in cell activation.



Scheme M. Accessory functions and signaling via CD44 *adopted from* [146]

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 [147]. Src kinases cotranslocate 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 [148]. 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 [149].

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 [150]. 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 [150, 151]. 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. In the T helper line apoptosis was mediated through CD95 and CD95L upregulation. 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 [150].

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 [150]. Some studies have shown that CD44 by itself is capable of activating human resting T cells and mouse cytotoxic T cells 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 [152].

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 [153]. 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 [153].

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 [154].

CD44 signaling also mediates proliferation and apoptosis in several tumor lines depending on the cell type and engagement of CD44 isoform as discussed below.
The Src-kinase Lyn, was found to be activated via CD44v6 in a colorectal cancer cell line. The functional relevance was, increased chemoresistence against the drug 1,3-bis (2-chloroethyl)-1-nitrosurea and this was mediated through the PI3K/Akt survival pathway [155]. 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 [156]. 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 IKappa B was phosphorylated suggesting NFkB activation. Indeed, NFκβ was found to be translocated to the nucleus. Consequently, IL-2 production was increased [157].

On the other hand, CD44 was also shown to strengthen dexamethasone induced apoptosis of a lymphoma line that was mediated through upregulation 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 [158]. CD44 cross-linking in fibroblast derived cells drives them into apoptosis suggesting a role for CD44 in fibroblasts [159]. CD44 ligation induced apoptosis of a promyelocytic leukemia line. Apoptosis was initiated via caspase 8 and in addition effector caspase 3. Serine protease dependent pathway was also proposed to be involved in mediating apoptosis of these cells [160].

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 [161]. 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 [162].

One of the main events mediated by CD44 is cytoskeletal organization which is energy dependent and requires an intact actin and microtubulin system. Small GTPase Rac1 activation is required for this process. Rac1 is also known to co-localise with ezrin [163]. These events along with phosphotyrosine kinase activations are responsible for cytoskeletal re-organizations in the cell. In T lymphocytes cross-linking anti-CD44 leads to CD44 dependent spreading through F-actin polymerization, accompanied by T cell adhesion, flattening and spreading [151]. 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. This could be a possible explanation for CD44 mediated effects on the cytoskeleton

[151, 164, 165]. 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 [166].

1.3.2.5 CD44 in the context of allogeneic bone marrow transplantation

CD44v7 isoform present on the bone marrow stroma supports homing of progenitors into the bone marrow and this was shown by injecting $CD44v7^{+/+}$ BMC into the $CD44v7^{-/-}$ mice and vice versa [167]. CD44v10 isoform was proposed to play a role in maturation of hematopoietic cells using *in vivo* and *in vitro* blocking studies with a receptor globulin as well as monoclonal antibody specific for v10 isoform. It also plays a role in efficient mobilization of progenitors and this could be exploited for use in bone marrow transplantation [168]. When mice with a transgene for rat CD44v4-v7 were used in comparison to the wild type for syngeneic and allogeneic bone marrow transplantation it was observed that the former had an advantage for repopulation. The total number of thymocytes recovered in this case was more and in this regard more T cells were obtained from spleen and lymph nodes. The T cells were also more functional compared to the non transgenic mice (NTG). Another observation made was that host reactive T cells were significantly reduced in the case of TG mice that could be due to tolerance induction [169]. Hence a more detailed investigation of the role of CD44 and its mechanism with regard to allogeneic bone marrow cell reconstitution (BMT) could prove this molecule as a convincing therapeutic target during allogeneic BMT.

Aims of study

Active vaccination in the allogeneically reconstituted tumor bearing host essentially requires donor T cell tolerance. To create a basis for vaccination in the allogeneically reconstituted, lymphoma-bearing host and for improving homing of progenitors into thymus, **A)** we aimed to elaborate a reconstitution protocol where progenitor T cells were injected along with bone marrow cells depleted of T cells. We were interested to investigate the potential role of these thymocytes in homing into the thymus, anti-host reactivity as well as suitability for vaccination with this experimental setup. Based on the results of this study and in view of the fact that CD44 has been suggested to facilitate progenitor homing and settlement in the bone marrow as well as being a T progenitor marker **B)** we explored whether CD44 standard (CD44s) and/or variant isoforms CD44v6 and CD44v7 contribute to thymus repopulation and thymocyte maturation.

2.1 Materials

2.1.1 Animals

Mice	Type	Origin
129SVEV	Wild type	Charles River, Suzfeld, Germany
CD44v6 intercross	(CD44v7 exon KO)	[170]
CD44v6-28	(CD44v6/7 KO)	[170]
Balb/c	Wild type	Charles River, Suzfeld, Germany
Balb/c transgenic rat (CD44v4-7)	Transgenic- rat meta-1	[171]

2.1.2 Cell Lines

Cell Line	Origin
EL-4 EL-4 transfected with CD44v6	American Type Tissue Culture (ATCC) [157]
IM7.8.1	mAb IM7.8.1 producing hybridoma line (ATCC)
A2.6	mAb A2.6 (rat-CD44v6) producing hybridoma line (ATCC)
145-2C11	mAb (CD3) (ATCC)

2.1.3 Primers

CD44s	**F** 5'-GATCCATGAGTCACAGTGCG-3'
	R 5'-GCCTACTGGAGATCAGGATG-3'
CD44v6	**F** 5'-CTCCTAATAGTACAGCAGAA-3'
	R 5'-GCCTACTGGAGATCAGGATG-3'
CD44v7	**F** 5'-CTTCGGCCCACAACAACCAT-3'

	R 5'-GCCTACTGGAGATCAGGATG-3'
CCR9	**F** 5'-ATTGCACAA GAGTGAAGACC-3'
	R 5'-GTCAACAGCCTGCACTACAA-3'
CCL25	**F**5'-TGGAATGTTCTCCGGCATGCTAGG-3'
	R5'-TGGCACTGGCATGCCTAGAAGACG-3
GAPDH	**F** 5'-GACCCCTTCATTGACCTCAAC-3'
	R 5'-CTTCTCCATGGTGGTGAAGAC-3'

2.1.4 Primary antibodies

Antibody	**Species**
Anti-CD3 (145-2C11)	ATCC
Anti –myc	ATCC
Anti -rCD44v6 (A2.6)	Matzku et al. 1989
Anti -pan CD44 (IM7.8.1)	ATCC
Anti-Bcl2	Becton Dickinson
Anti-CD11c	Becton Dickinson
Anti-CD18	Becton Dickinson
Anti-CD29	Becton Dickinson
Anti-CD34	Becton Dickinson
Anti-CD44v6	Bender Medsystems
Anti-CD49d (PS/2)	ATCC
Anti-CD51	Becton Dickinson
Anti-CD54 (YN1/1.7.4)	ATCC
Anti-CD62E	Becton Dickinson
Anti-CD62L	Immunotools
Anti-CD62P	Becton Dickinson
Anti-CD117	Becton Dickinson
Anti-CD123	Becton Dickinson
Anti-CD127	Becton Dickinson
Anti-IL-3	Becton Dickinson
Anti-IL-7	Bio Trend

Anti-IL-3R	Becton Dickinson
Anti-IL-7R	Becton Dickinson
Anti-pAkt	Becton Dickinson
Anti-akt	Becton Dickinson
Anti-pERK1/2	Santacruz Laboratory
Anti-ERK1/2	Becton Dickinson
Anti-phosphotyrosine (py20)	Becton Dickinson
Anti-pLck	Becton Dickinson
Anti-Lck	Becton Dickinson
Anti-Ly6	Becton Dickinson
Anti-Ter119	Becton Dickinson
Anti-pZAP70	Cell signaling
Anti-Zap70	Becton Dickinson

2.1.5 Secondary antibodies

Name	**Company**
Anti-mouse IgG HRP	Amersham
Anti-rabbit IgG HRP	Amersham
Anti-rat IgG HRP	Amersham
Anti-mouse IgG PE	Jackson Laboratories
Anti-hamster IgG FITC	Jackson Laboratories
Anti-mouse IgG FITC	Jackson Laboratories
Anti-mouse IgG APC	Jackson Laboratories
Anti-rat IgG FITC	Jackson Laboratories
Anti-rat IgG PE	Jackson Laboratories
Anti-rat IgG APC	Becton Dickinson
Streptavidin FITC	Jackson Laboratories
Streptavidin PE	Jackson Laboratories
Streptavidin APC	Jackson Laboratories
Streptavidin HRP	Rockland, PA

2.1.6 Inhibitors

MEK1/2 Inhibitor (SL327) - Calbiochem

PI3K Inhibitor (Ly249002) - Calbiochem

2.1.7 Instruments

Common Name	Company	Company name
Agitator for Bacterial cultures	Infors HT	
Beta-counter harvester	Tomtec/Perkins Elmer	
Beta-counter reader	LKB Wallace	1205 betaplate
Developer(Autoradiography)	Amersham	Hyper processor
Eagle eye	Herolab	Mididoc
ELISA plate reader	Anthos labtec	Reader 2001
FACS	Becton-Dickinson	FACS Calibur
Microscope	Zeiss	
Microscope (Inverse)	Leica	
PCR cycler	Eppendorf	Master cycler
pH meter	Knick	F61-calimatic
Spectrophotometer	Spectronic unicam	Genesis 10UV
Sonicator	Bandelin	Sonoplus
Tabletop centrifuge	Hereaus	Biofµge 15
Centrifuge	Sorval	RC 5B Plus
Centrifuge	Beckman	J6B
Water bath (Cell culture)	Julabo	Eco temp TW8

2.1.8 Miscellaneous Materials

Material	Company
Autoradiography films	Amarsham Biosciences
Culture flasks 25, 75 cm^3	Cellstar
Filters (0.2um)	Renner GMBH
Nitrocellulose membrane	Amarsham Biosciences
96-well microtitre plates (flat & round bottomed)	Grainer bio-one
Tissue culture plates (6 & 24 well)	Grainer bio-one
Whattmann paper	Sartorius
CFSE	Invitrogen
Magnetic beads	Miltenyi Biotec

2.1.9 Chemicals

Product	Origin
Acetic acid min.99.8%	Riedle-de-Haen
Annexin V- FITC	Becton Dickinson
Acrylamide/Bisacrylamide	Roth
Agarose-low grade	Sigma
Ammoniumperoxidisulphate (APS)	Roth
Biorad Protein assay reagent	Biorad
Bovine serum albumin	PAA
Bromophenol blue sodium salt	e.a. Thomas GMBH
Copper (II) sulphate	Merck
Coomassie brilliant blue	Merck
Diethyl pyrocarbonate (DEPC)	SIGMA
Dimethylsulphoxide (DMSO)	Merck
Deoxynucleotidyltriphosphate(dNTP)	Invitrogen
ECL reagent	Amersham bioscience

Ethanol absolute	Riedle-de-Haen
Ethidium bromide ultrapure	BRF lifesciences
Fetal calf serum	PAA
Folin Ciocalteu phenol reagent	Merck
Formaldehyde 37%	Merck
Glucose	SIGMA
Glycerine	Roth
Isopropanol	Fluka
ImProm-II reverse transcriptase and buffer	Promega
Potassium Chloride	Applichem
Propidium Iodide	R & D Systems
Potassium Dihydrogen Phosphate (KH_2PO_4)	Merck
Ladder prestained protein	Fermantas
Ladder gene ruler (1kb and 100bp)	Fermantas
L-glutamine	SIGMA
Magnesium Chloride Hexahydrate($MgCl_2 6H_2O$)	Thomas Chemikalian GMBH
Methanol	Fluka
Milk Powder	Roth
Natrium Hydroxyphosphate (Na_2HPO_4)	Merck
Natrium Fluoride	SIGMA
RPMI-culture medium	SIGMA
Sodium Azide (NaN_3)	Applichem
Sodium Carbonate anhydrous (Na_2CO_3)	Fishcer Scientific
Sodium Hydrogen Carbonate ($NaHCO_3$)	Applichem
Sodium Chloride (NaCl)	J.T.Baker
Sodium Dodecyl Sulphate (SDS)	SIGMA
Streptomycin	Calbiochem
TEMED (N,N,N',N-Tetramethylethylene diamine)	SIGMA
Taq polymerase and buffer	SIGMA
Tri-reagent	SIGMA

Tris	Roth
Trypan Blue	Roche
Trypsin	SIGMA

2.1.10 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 RNA preparation, cDNA synthesis and amplification

Total RNA was prepared from 10^7 cells with Tridy reagent preparation 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

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, 52°C-30secs, 72°C-1min -32 cycles, 72°C-10min

CCR9 & CCL25 : 94°C-5min, 94°C-30secs, 69°C-30 secs, 72°C-1min -32cycles, 72°C-10min

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

2.2.1.2 DNA gel electrophoresis

PCR products or samples containing DNA of interest were checked by running an agarose gel of 1-2%, depending on the size of the product. When Red Taq polymerase was not used then DNA sample was mixed with 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

Hybridoma supernatants were purified by passing around 1-2 l of supernatant over Protein-G Sepharose column (Pharmacia). 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 Folin test.

2.2.2.2 Folin test (Protein concentration determination)

Based on the Lowry test, this method aims at detecting cuprous ions by Folin-Ciocalteu reagent. In this assay a protein (antibody) solution is mixed with an alkaline solution of copper salt. Under these conditions cupric ions chelate with peptide bonds resulting in reduction of cupric ions ($Cu2^{+}$) to cuprous ions (Cu^{+}). Cuprous ions can be detected by reduction of the Folin-Ciocalteu reagent which produces blue colour that can be read at 748 nm. A blank solution with only PBS and BSA standard dilutions of 0.5 mg/ml and 1mg/ml were used for calibration. Assays were done in duplicates. From each sample 50µl were mixed to 1ml alkaline solution containing $Cu2^{+}$ ions. Then 100µl of Folin Ciocalteu reagent was diluted 1:1 in water and added to each sample. The samples were agitated for 20 minutes at RT and read at 748 nm.

Sample concentration mg/ml: F*10'mean sample OD_{748}

F= A+B/2 A:500/(mean $OD_{standard\ 500\mu g/ml}$ $*10^3$)

B:250/(mean $OD_{standard\ 500\mu g/ml}$ $*10^3$)

2.2.2.3 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).

2.2.2.4 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 a ntibody 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.

2.2.3 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 DMEM 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.
Trypan blue (2x): 0.4% Trypan blue (4vol)
4.5% NaCl (1vol)

2.2.3.2 Magnetic Beads separation

BMC were T cell-depleted by panning on Petri dishes coated with a mixture of anti-CD4 and anti-CD8 collecting the non-adherent fraction (purity: <2% $CD4^+$ and $CD8^+$ cells). Lin^- cells were enriched by incubation with biotinylated anti-Ter119, DX5,

Ly6C/G, CD3, CD8, CD11c and CD19 followed by incubation with streptavidin-conjugated magnetic beads (Miltenyi) collecting the non-adherent fraction. CLP1 (c-kit$^+$B220$^-$) and CLP2 (c-kit$^-$B220$^+$) cells were derived from lin$^-$ cells by incubation with the respective magnetic bead-coated antibodies. DP (CD4$^+$CD8$^+$) and DN (CD4$^-$ CD8$^-$) thymocytes (TC) were enriched by magnetic bead sorting using magnetic-bead-coated anti-CD4, followed by magnetic bead-coated anti-CD8 (regain of DP TC ~60%, regain DN TC ~5%, purity ~95%-98%). DN TC were enriched for DN1 (CD44$^+$CD25$^-$), DN2 (CD44$^+$CD25$^+$), DN3 (CD44$^-$CD25$^+$) and DN4 (CD44$^-$CD25$^-$) by incubation with anti-CD44 followed by anti-rat IgG-coated magnetic beads. Adherent and non-adherent fractions were further separated by incubation with anti-CD25-PE and anti-PE-coated magnetic beads or biotinylated anti-CD25 and streptavidin-coated magnetic beads (regain: <1%, purity ~85%-95%). Where indicated, cells were labeled with CFSE (Molecular Probes).

Donor- and host-derived CD8$^+$ T cell from the spleen were prepared by negative selection. CD8$^+$ cells were subsequently incubated with biotinylated anti-H-2^b or anti-H-2^d and Streptavidin-coated magnetic beads (regain of donor- and host-derived CD8$^+$ SC 5%-10%, purity 90%-95%).

2.2.3.3 Immunoreactivity

Immunoreactivity was evaluated by the analysis of IFN-γ expression (flow cytometry) and cytotoxic T lymphocyte activity. The cytotoxicity assay was performed according to the JAM-Test (Just Another Method) method described by Matzinger [172]. The assay measures DNA fragmentation. Target cells were labeled with ^{3}H thymidine to a final concentration of 2.5 to 5 µCi/mL for 4 to 6 hours. They were then pelleted, washed once with culture medium, and distributed in triplicates (10^4 cells per well) into 96-well round-bottomed microwell plates. After adding effector cells in appropriate dilutions as mentioned in results, the plates were incubated in a humidified atmosphere at 5% CO_2 for 6 hours approximately. The cells and medium were than aspirated onto fiber glass filters using a cell harvester. After washing and drying the filters, they were placed in liquid scintillation fluid, sealed and counted

using a liquid scintillation β-counter. The radioactivity measured corresponds to intact DNA, since DNA from dead cells is degraded into small fragments that pass through the filter. For calculating % specific killing, the standard formula for the JAM test is: % specific killing = (S-E)/S 100, with E = experimentally retained DNA in the presence of effector cells (in cpm) and S = retained DNA in the absence of effector cells (spontaneous).

2.2.3.4 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 labeled 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 from the same species).

For cytokines, intracellular FACS was performed. The cells were first incubated with formalin 1% to fix them for 10 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.

2.2.3.5 Proliferation assay

^{3}H-Thymidine incorporation was used to measure DNA synthesis. Thymocytes ($2x10^{5}$) were seeded on flat bottomed 96 well plates that were pre-coated with anti-CD3 alone, anti-CD44/CD44v6 alone or both and isotype control antibody. All assays were run in triplicates. Cells were cultured in RPMI 1640 complete medium for 48 hours. ^{3}H-thymidine (10µCi/ml) was added after 48 hours and cultures were maintained for an additional 16 hours. Thereafter the cells were harvested by vacuum onto glass fibre filters. During this step free ^{3}H-thymidine was washed through the filters and incorporated ^{3}H-thymidine was retained in the DNA. This radioactivity retained in the filters was measured by liquid scintillation counting in a β-counter.

2.2.3.6 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. Thymocytes ($2x10^{5}$/well) were seeded on flat bottomed 96 well plates that were pre-coated with anti-CD3 alone, anti-CD44/CD44v6 alone or both and isotype control antibody. All assays were run in triplicates. Cells were cultured in the incubator for 12, 24 or 48 hours. After the desired time points 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.

2.2.3.7 Thymocytes Stimulation and Activation

Thymocytes were stimulated by antibody cross-linking. Flat bottomed-96 microwell plates or 60mm cell culture dishes were coated with indicated concentrations of antibodies overnight at 4°C. Bicarbonate buffer at pH 9.6 was used for coating. Anti-

CD3 (1 µg/ml), anti-CD44/CD44v6 (10µg/ml) or both and control IgG antibodies were used for coating. Plates were washed with PBS several times and blocked with PBS/1% BSA for 1 hour at 37°C, 5% CO_2. Thereafter, plates were again washed several times with PBS and kept at 4°C until use.

For short term signaling experiments (tyrosine phosphorylation, ERK1/2, MEK1/2, Lck, ZAP-70 and Akt activation) 40µl of $5x10^5$ -10^6 cells in RMPI1640 were added to control and antibody coated plates. Cells were kept at 37°C, 5% CO_2 for 18 minutes. Thereafter, 20µl of reducing Laemmli buffer was added to each well to stop the reaction and to lyse the cells followed by sonication to destroy the DNA. The samples were frozen till they were loaded onto the gel. Cells were incubated over night on antibody coated plates to detect apoptotic and anti-apoptotic proteins.

2.2.4 Animal Experiments

2.2.4.1 Preparation of hematopoietic cells

Mice were killed by cervical dislocation. Thymi and spleen were removed aseptically and placed in medium. They were then placed on a petri dish with fine gauze and meshed carefully to release the thymocytes. Bone marrow cells (BMC) were obtained by flushing femur and tibia with PBS and meshed like before. The cell suspension was washed several times before adding medium. For short term signaling experiments of thymocytes RPMI without FCS was used. For long term culture periods between 1-5 days complete RPMI medium was used.

2.2.4.2 Reconstitution, tumour implantation and vaccination

For experiments with allogeneic bone marrow transplantation, irradiated BALB/c mice received i.v. injections of anti-asialo GM1 every 2 wk for NK cell depletion. Mice were reconstituted with $2x10^6$ T_{depl} SVEV BMC 24 h after irradiation. Where indicated mice received $2x10^5$ DP TC from SVEV mice. IL-3 (1µg / mouse) and IL-7 (1.5µg /

mouse) were given i.v. twice weekly. Mice received s.c. application of RENCA ($5x10^4$), YC8 / YC8.lacZ ($1x10^2$) or Sp6 ($2x10^3$) cells 5 days after reconstitution. The tumor dose corresponds to 5x the minimal dose for 100 % tumor take in non-manipulated, syngeneic BALB/c mice. For vaccination, mice received 1 wk after tumor cell application s.c. injection of YCA. LacZ lysate plus ß-Gal (100µg) or of Sp6 lysate plus Sp6 IgM (200µg) in adjuvant. For short term reconstitution, T_{depl} BMC ($1x10^7$/mouse) and DP TC ($5x10^6$/mouse) were CFSE-labeled. Cells were labeled with 2-3 µM CFSE for 10-15 min, then washed several times with PBS and used for injections. CFSE (Carboxyfluorescein succinimidyl ester) is a fluorescent cell labeling dye which gets diluted through every cell division and intensity of the dye on cells can be measured by FACS to check the percentage of migrating cells into the bone marrow, spleen and thymus after intravenous (i.v.) injections into mice. Mice were sacrificed after 18h-120 h.

For experiments dealing with thymocyte maturation lethally irradiated BALB/c (8Gy) or SVEV (9.5Gy) mice were i.v. reconstituted with $5x10^6$ BMC or BMC/TC subpopulations, 24h after irradiation. Some mice in addition received i.v. injections of 100µg anti-CD44 (IM7) or rat IgG (control), twice per week starting at the day of reconstitution. For short term reconstitution, mice received an i.v. injection of $1x10^7$ CFSE-labeled BMC and/or 1-$5x10^6$ BMC or TC subpopulations, as indicated in the individual experiments. Mice were sacrificed at the indicated time points, SC, BMC and TC were isolated and the recovery of fluorescent cells was evaluated by flow cytometry. Depending on the percentage of fluorescent cells, up to 100,000 events were collected. Animal experimentations were approved by the governmental authorities of Baden-Wuerttemberg, Germany.

2.2.4.3 Graft vs Host Disease (GVHD), graft rejection and tumor growth

Evidence for GVHD was obtained by weight loss and macroscopic inspection of skin, gut and liver. Graft rejection was defined by flow cytometry of host and donor-derived cells in central and peripheral lymphoid organs and the absence of donor-derived cells. Tumor growth was evaluated twice per week (mean tumor diameter) and

surveying for signs of rejection. Animals with (bloody) diarrhoea, cachexy and >25% weight loss or with a tumor of 2 cm diameter were sacrificed (survival time).

2.2.5. Statistical analysis

Significance of differences was calculated according to the Wilcoxon rank sum test (*in vivo* assays) or the Student's T test (*in vitro* studies). Functional assays were repeated at least 3 times. Mean values and standard deviations of *in vivo* experiments are derived from 3-5 experiments with 8-10 mice / group in the individual experiments. Accordingly, mean ± SD are mostly derived from 24-50 mice / group. Mean ± SD of *in vitro* studies are based on 3-4 replicates.

3.Results

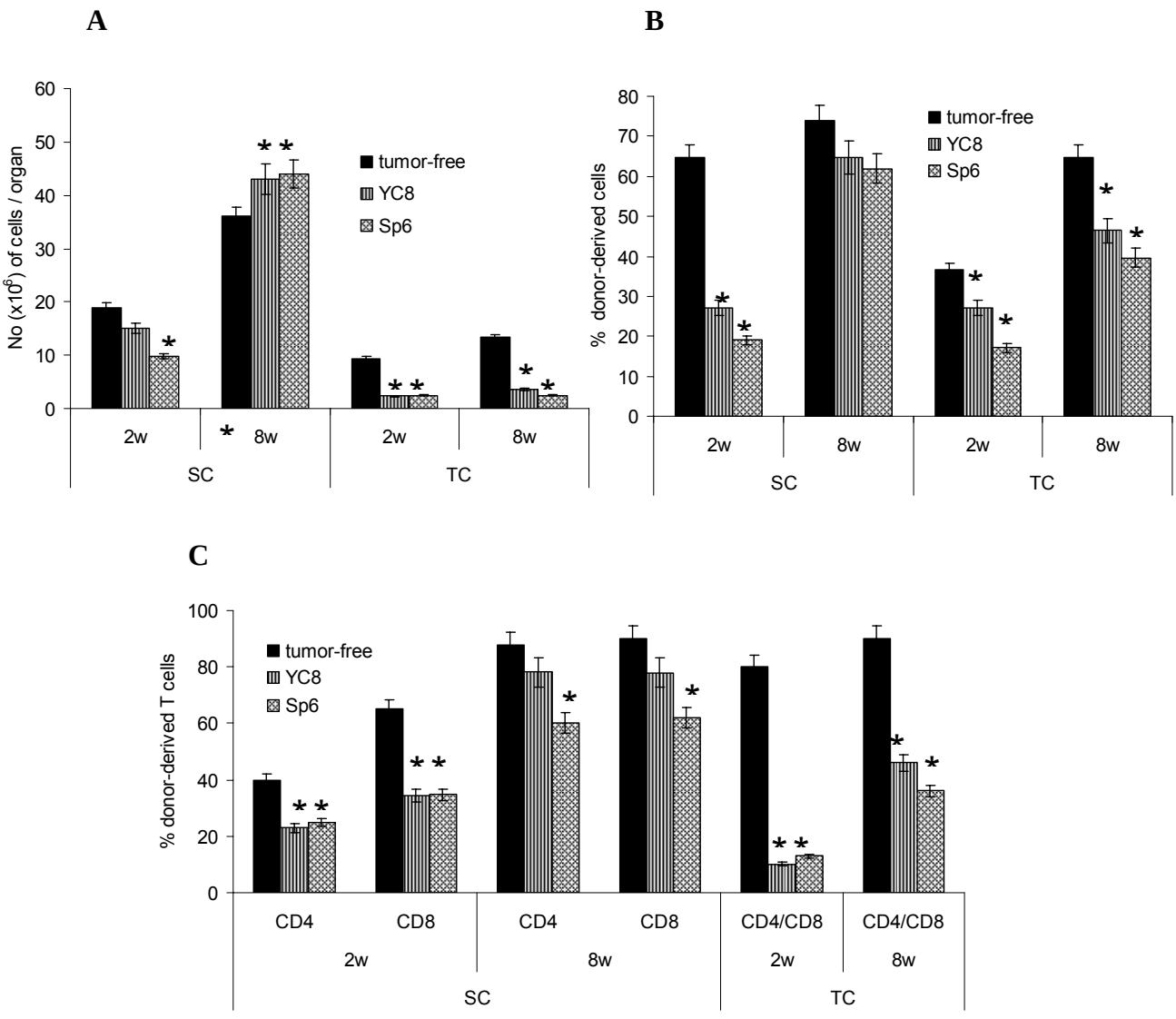
We are particularly concerned with improvement of thymic reconstitution including establishment of tolerance induction towards the host after allogeneic bone marrow cell reconstitution of tumor bearing host. For this purpose I first evaluated the importance of allogeneic bone marrow transplantation (BMT) on B and T lymphoma bearing mice as described in detail below.

3.1 T and B Lymphoma bearing mice show poor repopulation of thymus

It has been shown in our lab using a renal cell carcinoma model that host NK cell depletion favors acceptance of allogeneic T cell depleted (depl) BMCs in myeloreductively conditioned host. In the first few weeks after reconstitution, repopulation of the thymus was poor but in the later weeks donor-derived bone marrow cells dominated and were also host tolerant [173]. We checked whether these observations would also hold true for leukemia bearing mice. Irradiated (6Gy) and NK depleted Balb/c mice received T cell depleted (depl) bone marrow cells, from SV129 together with tumor application of YC8/YC8.LacZ (T lymphoma) or SP6 (B lymphoma) both being of Balb/c origin. It was observed that less spleen cells were recovered in tumor bearing mice after 2 weeks as compared to tumor free mice. The difference had vanished after 8 weeks. However, in the thymus much lesser cells were recovered in the tumor bearing mice as compared to tumor free host (Fig.1A). When the percentage of donor derived cells were checked it was seen that from the spleen in the first 2 weeks much lesser donor-derived cells were obtained as compared to tumor free mice and after 8 weeks (wks) the difference though less pronounced was still visible. In the thymus the number of donor derived cells remained low during the observation period of 2 and 8 weeks in lymphoma bearing mice as compared to tumor free mice (Fig.1B). Evaluating donor derived T cells revealed that both CD4 and CD8 T cells were lesser in spleen after 2 and 8 weeks in tumor bearing mice. In the thymus $CD4^+CD8^+$ (DP) cells were much lesser in the

lymphoma bearing mice than the tumor free mice (Fig.1C). A very high number of lymphoma bearing mice suffered from GVHD (Fig.1D).

Cytotoxic assay was performed to look for anti-donor, anti-host and anti-tumor activities using host CD8 T cells and donor CD8 T against donor spleen cells , host spleen cells and tumor lysates respectively. It was observed that anti-host activity was very high throughout and no significant anti-tumor activity was seen. No significant difference in anti-donor activity was observed (Fig.1E). There was no significant difference observed in the number of surviving lymphoma bearing mice, between lymphoma bearing mice receiving syngeneic reconstitution in comparison to allogeneic reconstitution (Fig.1F& G).



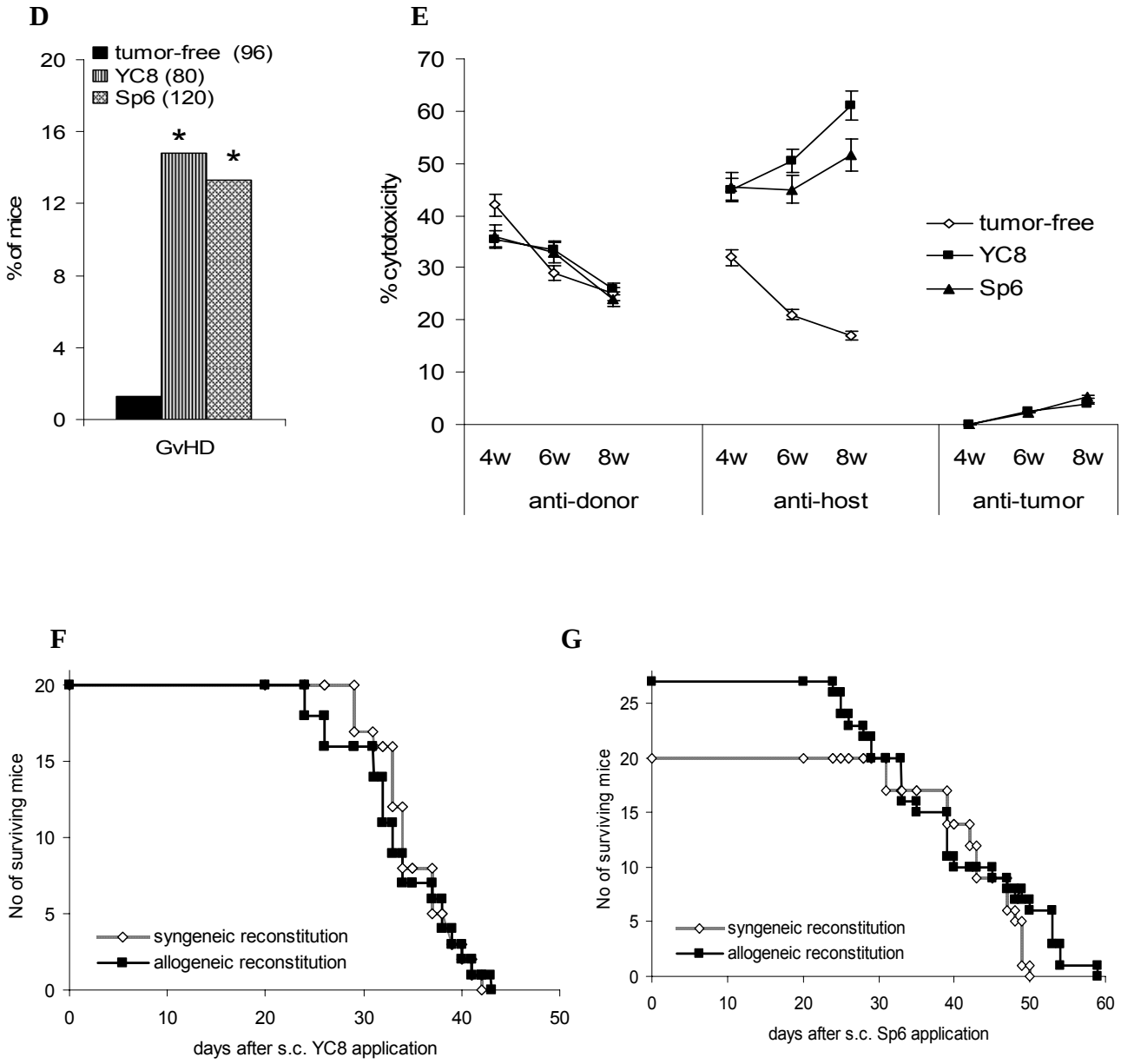


Fig. 1 Repopulation of the thymus in the lymphoma-bearing mouse

Irradiated (6Gy) and NK_{depl} BALB/c mice were reconstituted with $2x10^6$ T_{depl} SVEV BMC. Mice received a s.c. injection of $2x10^1$ YC8 or $4x10^2$ Sp6 cells, 5d after reconstitution (A) The number of SC and TC was evaluated 2 and 8 wk after tumor cell application; (B) The percentage of donor-derived SC and TC and (C) of donor-derived T cells was evaluated by flow cytometry; (A-C) Mean values±SD of 3 independently performed experiments; significant differences as compared to tumor-free mice are indicated by an asterisk (D) The percentage of mice that were sacrificed due to severe GVHD; The actual number of mice is given in brackets. Significant differences as compared to tumor-free mice are indicated by an asterisk; (E) SC were collected 4, 6 and 8 wk after tumor cell application. Cells were restimulated *in vitro* with irradiated donor or host lymphocytes or the corresponding tumor cells for 8d. Cytotoxic activity against the target used as stimulator during restimulation was evaluated at E:T = 25:1. For the evaluation of anti-tumor reactivity, host-derived lymphocytes were added as cold target (T:cT = 1:10). Values represent the mean±SD of triplicates; (F and G) BALB/c mice conditioned as described above and reconstituted with T_{depl} BMC from either

BALB/c or SVEV mice received $1x10^2$ YC8 or $2x10^3$ Sp6 cells. The survival time of mice developing tumors is shown (Mice sacrificed because of severe GVHD were excluded). Differences between syngeneically and allogeneically reconstituted mice were not significant.

Thus, T and B cell lineage derived malignancies hampered the process of thymus repopulation. The few donor-derived T cells in the periphery obviously were not tolerant towards the host, as a considerable percentage of mice succumbed with severe GVHD, and T cells of the remaining mice developed strong cytotoxic activity against host lymphoblasts. These unexpected results indicated i. that the thymus becomes repopulated rather late after reconstitution with T_{depl} BMC, and ii. that at such a late state, hematopoietic tumors may compete with T progenitor cells for thymus repopulation. The failure to achieve a timely appropriate repopulation of the thymus demanded an elaboration of alternative reconstitution protocols.

3.2 Continuous application of cytokines (IL-3 and IL-7) promotes expansion of donor and host cells in thymus and spleen

The cytokines IL-3 and IL-7 provide as major growth factors for B and T cells. Application of cytokines induces proliferation of thymocytes especially in the early stages as well as increases the peripheral T cell output [60, 174].

So, we included application of cytokines in our protocol, to check if they were able to improve reconstitution and increase the numbers of donor derived cells homing into thymus. Mice received i.v. injections of IL-3 and IL-7 after BMCT. It was seen that reconstitution improved in mice after receiving cytokines especially in spleen cells (SC) and to some extent in thymocytes (TC) (Fig.2A). However, the percentage of donor derived cells was still less in thymus even after receiving cytokine injections (Fig.2B). Anti-donor and anti-host cytotoxicity remained high through out the period observed (Fig.2C). The survival rate of mice also remained the same even after IL-3 and IL-7 application (Fig.2D & E).

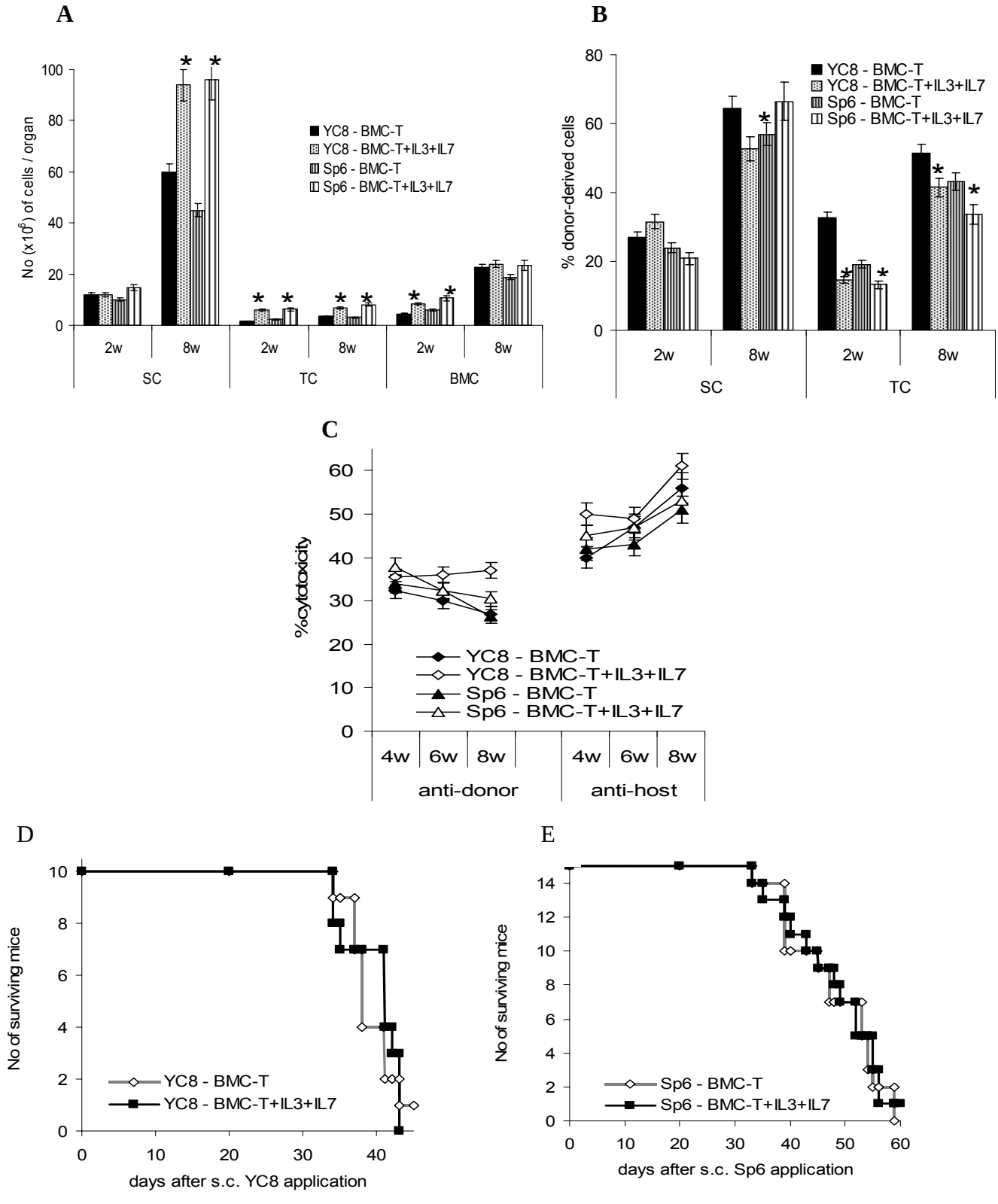


Fig. 2 IL-3 and IL-7 support maturation of host and donor progenitor cells

BALB/c mice were conditioned and reconstituted as described in Fig.1. Mice received, in addition, IL-3 plus IL-7, twice per week, starting at the day of reconstitution. Tumor cells ($2x10^1$ YC8 or $4x10^2$ Sp6). were injected 5d after reconstitution (A) Number of SC, TC and BMC at 2 and 8 weeks after tumor cell application; (B). Percentage of donor-derived SC and TC (A and B) Mean values±SD of 3

independently performed experiments; significant differences between mice receiving or not receiving IL-3 plus IL-7 are indicated by an asterisk; (C) Cytotoxic activity against donor and host cells was evaluated as described in Fig 1. Values represent the mean±SD of triplicates; (D and E) BALB/c mice conditioned and reconstituted as described above, received $1x10^2$ YC8 or $2x10^3$ Sp6 cells. The survival time and rate are shown (Mice sacrificed because of severe GVHD were excluded). Differences between mice receiving or not receiving IL-3 plus IL-7 were not significant.

We concluded that in the myeloreductively conditioned host, IL-3 and IL-7 provided a growth stimulus that, however, accounted for host and donor lymphoid progenitors. Therefore we next asked, whether weakening the host hematopoietic system may provide a growth advantage for donor BMC.

3.3 Donor derived hematopoietic progenitor cell expansion can be improved by strengthening the myeloreductive conditioning

Since strengthening the myeloreductive conditioning increases the number of donor cells in the host during reconstitution [175] we thought of increasing radiation dose to 7Gy hoping to see more donor cell expansion in the host. The theory behind it is, increasing the radiation dose kills more host cells and the host would rely more on the donor hematopoietic cells for expansion and differentiation.

Balb/c mice were irradiated with 6Gy or 7Gy and treated with anti-asialoGM1, for host NK cell depletion. In addition mice received BMT depleted of T cells from SVEV mice and Sp6 tumor cells. Where indicated injections of IL-3 and IL-7 were given twice per week. The percentage of donor derived cells recovered in host SC and TC was more after myeloreductive strengthening (7Gy) (Fig.3A). The percentage of donor derived T cells was then calculated. In the spleen a higher percentage of CD4 cells were recovered after 7Gy and IL-7 injections after 2 and 6 weeks. There was no change in CD8 T cells. In the thymus higher donor derived DP ($CD4^+8^+$) thymocytes were recovered, which could be distinguished clearly after 2 weeks of transfer (Fig.3B). IFN-γ expression was checked as a marker for cytotoxic T cell activity [176]. The expression of IFN-γ by donor T cells decreased in mice receiving 7Gy irradiation and in addition after receiving IL-7 injection significantly after 6 weeks (Fig.3C). There

was also lesser cytotoxicity against the donor and host observed especially after 6 to 8 weeks in the JAM cytotoxicity assay (Fig.3D).

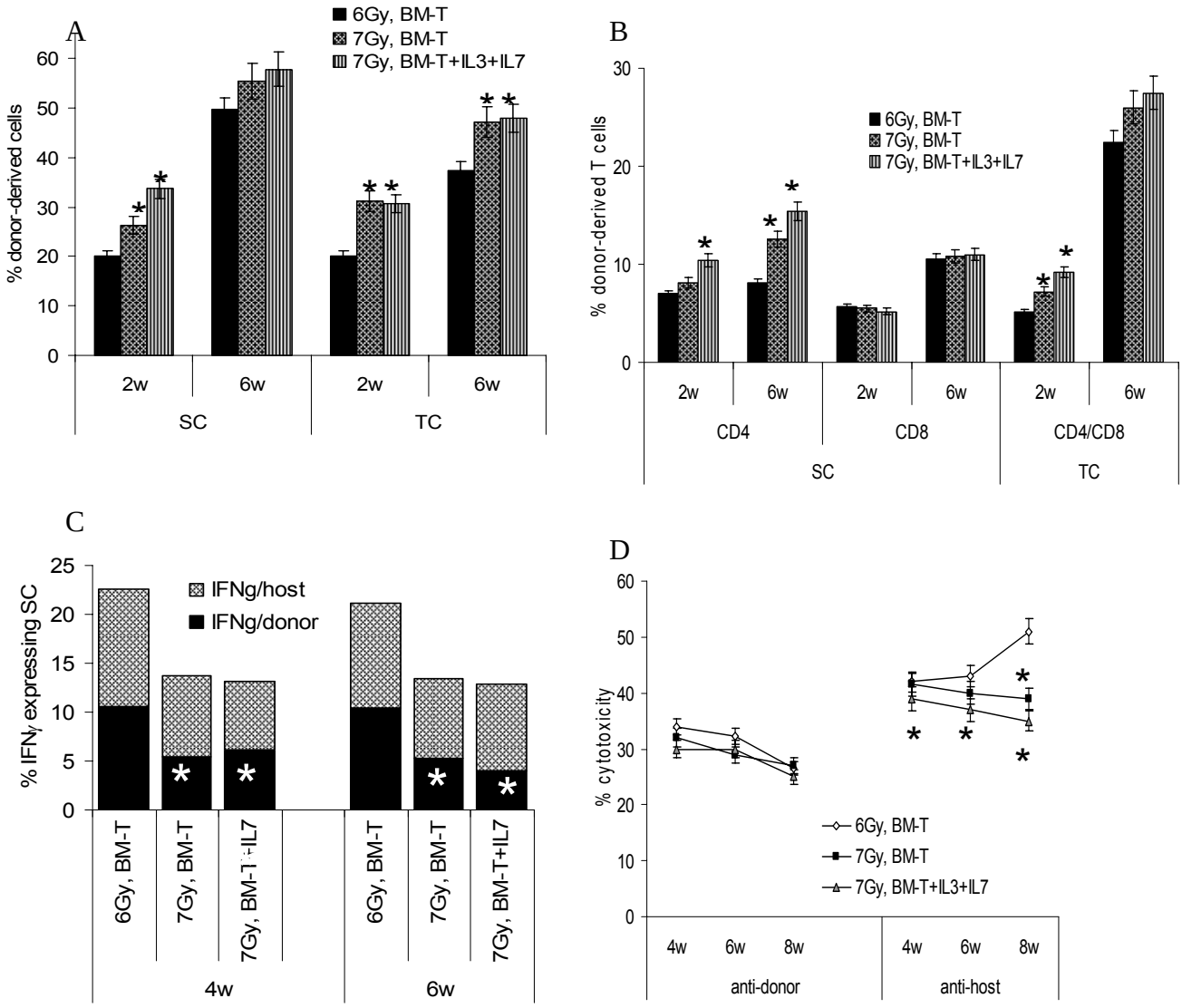


Fig. 3 Stronger myeloreduction improves donor cell expansion

NK_{depl} BALB/c mice were conditioned with 6Gy or 7Gy. Mice were reconstituted as described in Fig. 1 and received, in addition, IL-3 plus IL-7, twice per week, starting at the day of reconstitution (A) The percentage of donor-derived SC and TC, (B) the percentage of donor-derived splenic T cells and donor-derived DP TC and (C) the percentage of donor or host-derived IFNγ-expressing SC was evaluated by flow cytometry. Mean±SD of 3 independently performed experiments; significant differences between mice conditioned with 6Gy or 7Gy are indicated by an asterisk; (D) Splenic host- and donor-derived T cells were isolated by magnetic bead sorting (purity: >90%). Donor- and host-derived T cells were cultured with irradiated host or donor lymphocytes for 8d. Cytotoxic activity against donor or host cells (E:T = 25:1). was evaluated as described in Fig 1.

Values represent the mean±SD of triplicates; 8wk after reconstitution, anti-host reactivity was significantly lower in 7Gy than 6Gy conditioned mice (indicated by an asterisk).

Thus, even a minor increase in the percentage of donor-derived progenitor T cells in the thymus sufficed for a reduction in GVHD and host-directed cytotoxicity. Because 7Gy were not lethal, i.e. reconstituted mice survived, we proceeded with the stronger myeloreductive regimen to support repopulation of the thymus.

3.4 Immature double positive (DP) thymocytes from donor preferentially home into the host thymus

We hypothesized that the problem of GVHD could be partially solved by injecting early thymocytes along with donor BMCs. In this case the thymocytes would mature in the host thymus and hence would be host tolerant as well as have the ability to kill the tumor [4]. So we performed the experiments using DP thymocytes. In the following experiments Balb/c mice were NK depleted and conditioned with 7Gy followed by injections of 10^7 CFSE labeled T depleted BMC or 10^7 CFSE labeled T depleted BMC plus $5x10^6$ CFSE labeled DP TC or unlabeled T depleted BMC plus labeled DP TC. To avoid overlapping spectrum from labeled dyes, the same dye was used to label BMCs and TCs. TCs were differentiated from BMC by staining them in addition with CD4-APC and CD8-PE antibodies. The recovery of CFSE labeled cells was recorded by FACS during the following days. CFSE labeled T depleted BMCs were first recovered in the spleen and then became enriched in bone marrow after 48hrs. IL-3 and IL-7 in the absence of DP TCs had no major impact on homing into bone marrow and thymus. When mice received BMCs along with DP TCs a very high number was found to recover also in the thymus (Fig.4A). However, when different combinations of dye labeled BMC and DP TCs were injected it was observed that no CFSE labeled DP TC could be recovered from the host bone marrow (Fig.4B). In addition it was observed that about 34% of the cells in thymus were double positive for CFSE dye as well as DP TCs when CFSE labeled BMCs plus CFSE labeled DP TCs were used, suggesting that CFSE labeled DP TCs preferentially homed into the thymus. In addition when only dye labeled DP TCs were used and unlabeled BMCs it

was seen that the dye labeled DP TCs only homed into thymus and were not recovered in the bone marrow (Fig.4C). In the spleen there was no change in the ratio of dye labeled T depleted BMCs and dye labeled DP TCs for the period of 120 hrs observed. In the bone marrow very few dye labeled DP TCs almost negligible were recovered at 96 and 120 hours (later time points). In the thymus hardly any dye labeled T cell depleted BMCs were recovered. However the number of dye labeled DP TCs recovered in the thymus kept increasing constantly with the time (Fig.4D).

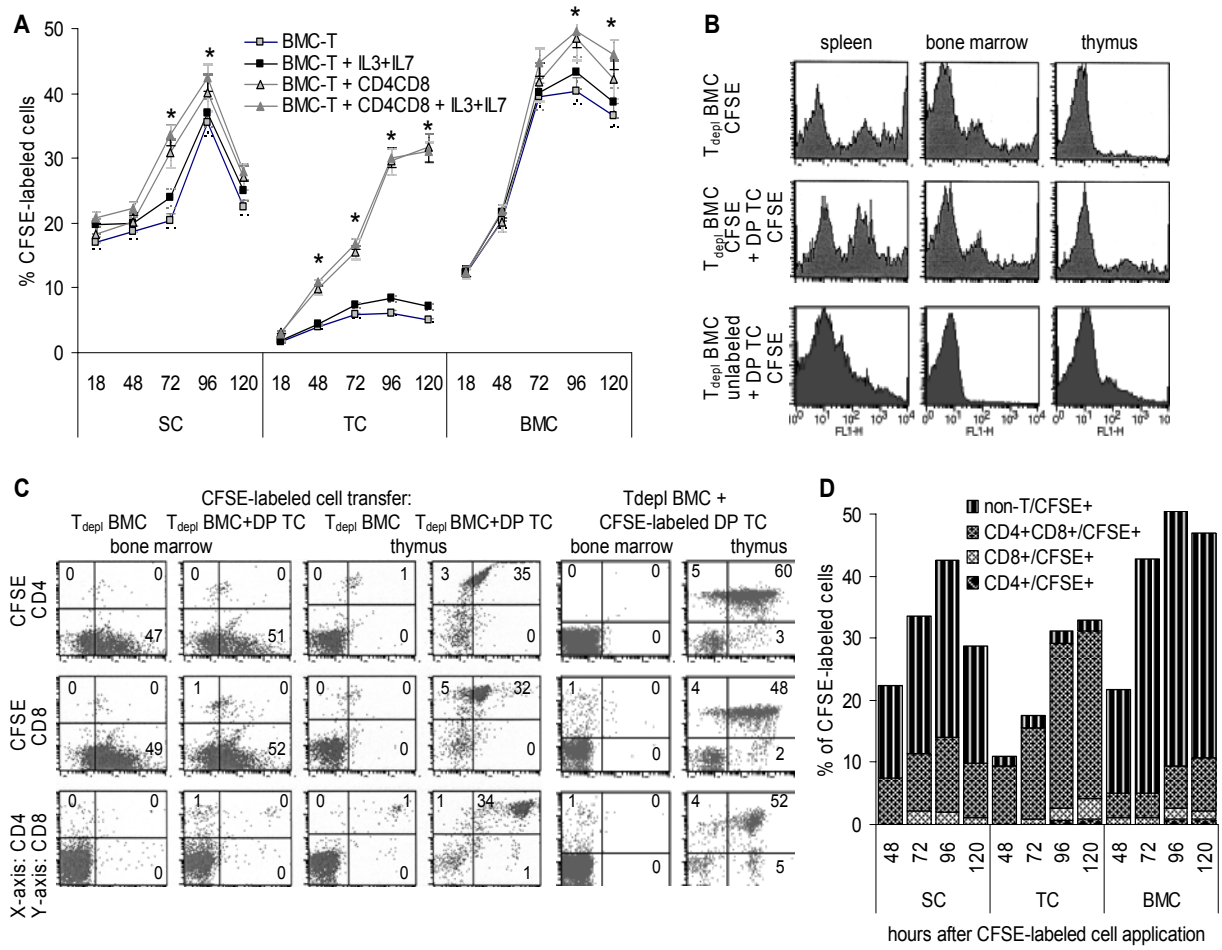


Fig. 4 Homing of T_{depl} BMC and of DP TC in the myeloreductively conditioned allogeneic host

NK_{depl} BALB/c mice were conditioned with 7Gy and received $2x10^7$ T_{depl} BMC or $2x10^7$ T_{depl} BMC plus $1x10^7$ DP TC from SVEV mice. TC or both BMC and TC had been CFSE labeled. Mice were sacrificed after 18h-120h (A) Both T_{depl} BMC and DP TC were CFSE-labeled. The percentage of CFSE-labeled SC, TC and BMC was evaluated by flow cytometry; (B) Examples of the recovery of CFSE-labeled cells in spleen, bone marrow and thymus and (C) of $CD4^+CD8^+$ cells 96h after application of CFSE-labeled T_{depl} BMC of CFSE-labeled T_{depl} BMC plus CFSE-labeled DP TC or of unlabeled T_{depl} BMC plus CFSE-labeled DP TC (D) The percentage of dye-labeled $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD8^+CD4^-$ SC,

TC and BMC in mice that had received dye-labeled T_{depl} BMC plus DP TC plus IL-7 has been evaluated by counterstaining with anti-CD4-APC and anti-CD8-PE (Mean values of 3 mice per group).

3.5 Reconstitution of myeloreductively conditioned mice with T_{depl} BMC plus DP TC accelerates repopulation of the periphery with donor-derived lymphocytes

7Gy and anti-asialo GM1 conditioned mice were reconstituted with $2x10^6$ T depleted BMC and $2x10^5$ DP TC with or without IL-3 and IL-7 application. In the spleen a slightly higher number was recovered after DP TC application after 6 weeks and with the application of cytokines the number increased a little more. In the host thymus after 2 wk number of cells recovered was much higher after receiving DP TC along with BMC. There was no effect on cytokine application. The effect was normalized after 6wk (Fig.5A). The percentage of donor derived cells was calculated in SC and the numbers were higher 2 weeks after DP injections along with BMC and IL-7 application in addition. In the thymus a very high percentage of donor cells were recovered after the addition of DP with BMC instead of BMC alone, with no effect on cytokine application (Fig.5B).

When cytotoxicity was measured against the donor and host we saw that when DP TCs were injected along with BMC there was a significant decrease in anti-donor as well as anti-host activity. Application of cytokines led to a further decrease in anti-host reactivity with no major effect on anti-donor activity (Fig.5C).

From this experiment onwards mice received vaccinations in addition, to strengthen the immune response against tumor. Two and four weeks after reconstitution with BMC plus DP TC, mice received in addition a subcutaneous (s.c.) injection of a mixture of tumor lysate ($1x10^7$ cells) plus tumor antigen (β- galactosidase plus YC8.LacZ lysate or SP6 IgM plus SP6 lysate) in adjuvant. SC were collected 1 wk after the challenge and cytotoxicity against host and tumor were measured using irradiated host and tumor cells respectively. There was a striking reduction in the activity against the host but the tumor activity was higher in mice that received T depleted BMC plus DP TC in both B and T lymphoma bearing mice (Fig.5D).

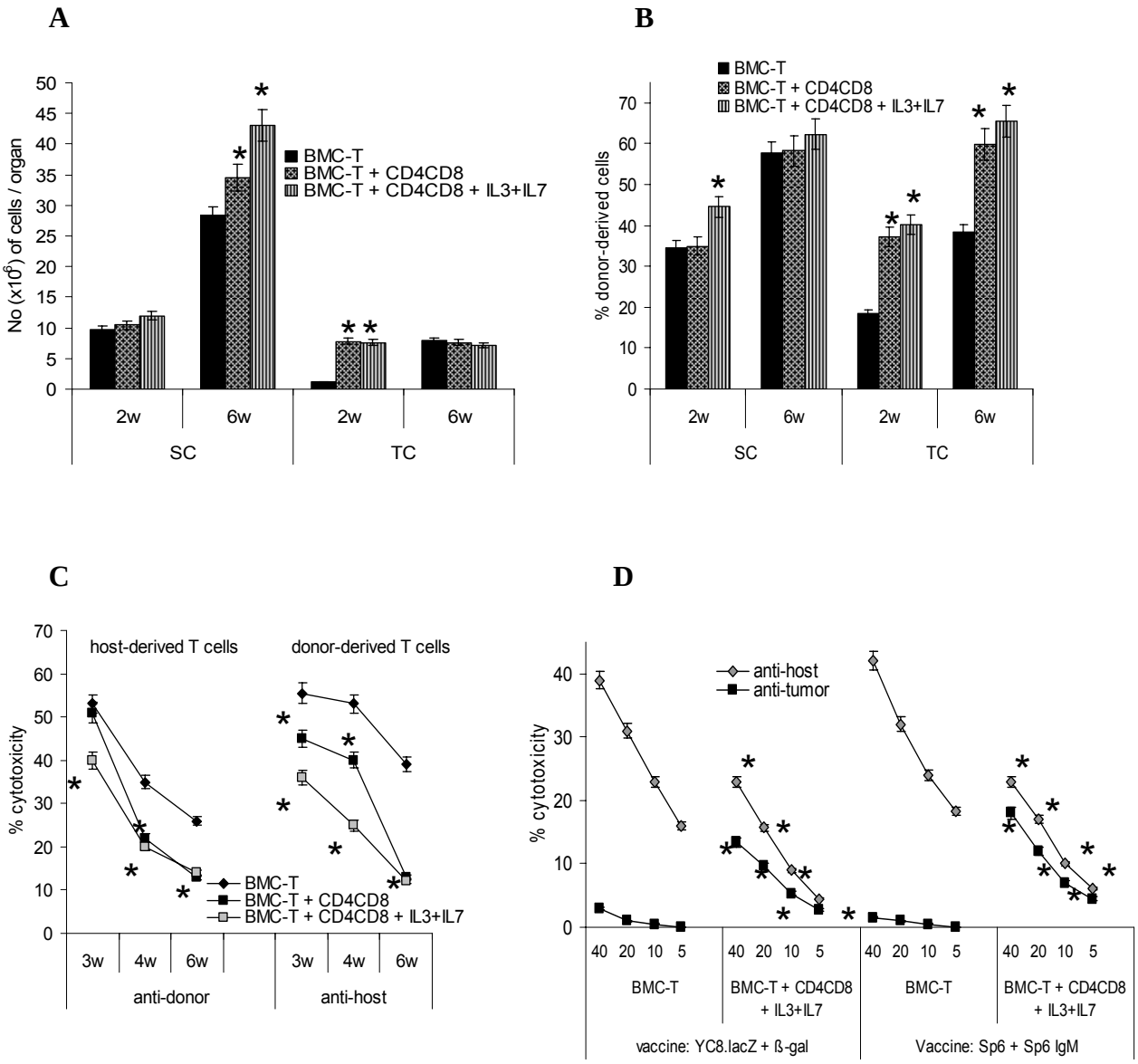


Fig. 5 Reconstitution of myeloreductively conditioned mice with T_{depl} BMC plus DP TC accelerates repopulation of the periphery with donor-derived lymphocytes

BALB/c mice were conditioned as described in Fig.3 and were reconstituted with T_{depl} BMC plus DP TC and received i.v. injections of IL-3 and IL-7 twice per week. Sp6 myeloma cells ($4x10^2$) were s.c. injected 5d after reconstitution (A) Number of SC and TC after 2wk and 6wk and (B) percentage of donor-derived cells (A and B). Data represent the mean±SD of 3 independently performed experiments; significant differences to mice receiving only T_{depl} BMC are indicated by an asterisk; (C) Donor- and host-derived T cells, separated as described in Fig 3 were cultured with irradiated host or donor lymphocytes for 8d. Cytotoxic activity against donor or host cells (E:T = 25:1) was evaluated as described in Fig 1. Values represent the mean±SD of triplicates. Significant differences in cytotoxic activity between SC from mice receiving T_{depl} BMC or T_{depl} BMC plus DP TC are indicated by an asterisk (D) Mice reconstituted as described in (A) received s.c. injections of YCA.lacZ lysate plus ß-Gal or Sp6 lysate plus Sp6 IgM in adjuvant 2wk and 4wk after

reconstitution. Mice were sacrificed after 5wk and unseparated SC were restimulated *in vitro* with irradiated tumor cells or host lymphocytes for 1wk. Cytotoxic activity against host lymphoblasts and YC8.lacZ or Sp6 at E:T = 40-5:1 is shown. Values represent the mean±SD of triplicates; significant differences between mice reconstituted with T_{depl} BMC or with T_{depl} BMC plus DP TC plus IL-3 and IL-7 are indicated by an asterisk.

Taken together, a considerable percentage of DP TC homed into the thymus. Distinct to peripheral donor-derived T cells from mice reconstituted with only T_{depl} BMC, donor-derived T cells from mice receiving DP TC, in addition, were tolerant towards the host, but still could respond against a tumor. Thus, this reconstitution protocol might allow for tumor vaccination.

3.6 Lymphoma growth retardation and rejection in the myeloreductively conditioned mouse reconstituted with T depleted BMC and DP TC

In order to check if BMC transplantation along with injection of DP TC supports a tumor vaccination protocol in lethally irradiated mice, animals were reconstituted and received along with tumor application of Sp6, an s.c. injection of SP6 cell lysate plus SP6 IgM in adjuvant. Vaccination was repeated every 2 wk.

From 21 mice receiving T depleted BMC plus DP TC only 1 mouse/group developed GVHD showing that DP TC do not induce high GVHD, which is always a threat in the reconstitution protocol. After vaccination 6/21 mice reconstituted with T depleted BMC died of GVHD and 2/21 reconstituted with T depl BMC plus DP TC died of GVHD (Fig.6A). The number of SC counted were lowest in T cell depleted BMC reconstitution followed by T cell depleted BMC plus vaccination, T cell depl BMC plus DP TC and the highest numbers in SC was recovered in mice receiving T cell depl BMC plus DP TC plus vaccination (Fig.6B). The percentage of CD4 T cells recovered in SC was highest after vaccinating mice receiving DP TC and BMC, followed by no vaccination, mice receiving vaccination and BMC alone without TC and lastly mice receiving no vaccination and BMC alone (Fig.6C). T cell depl BMC reconstituted mice that received vaccination had the highest expression of IFN-γ which decreased significantly in T cell depl BMC plus DP TC reconstituted mice

receiving vaccination (Fig.6D). As the next step anti-host and tumor activity were measured using SC from the host (effector) and host lymphoblasts and tumor cells as target. The anti-host activity was reduced to a great extent in mice receiving DP TC with or without vaccination. The anti-tumor activity was high only in the case of DP TC mice receiving vaccination (Fig.6E). The survival rate of mice was calculated. Mice receiving vaccination after reconstitution had higher survival rates in general. Mice reconstituted with DP TC had almost 80% survival rate after tumor vaccination (Fig.6F).

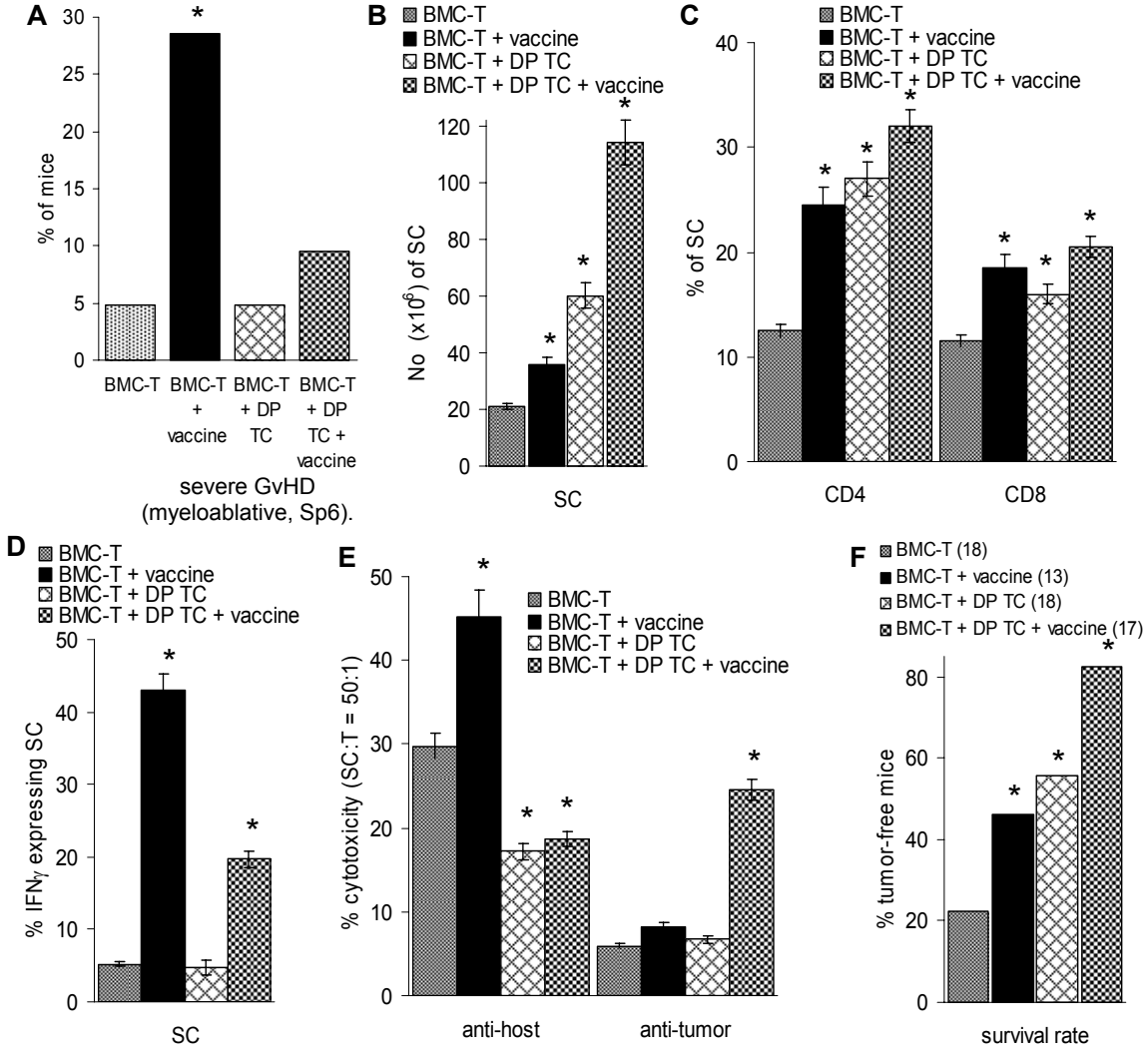


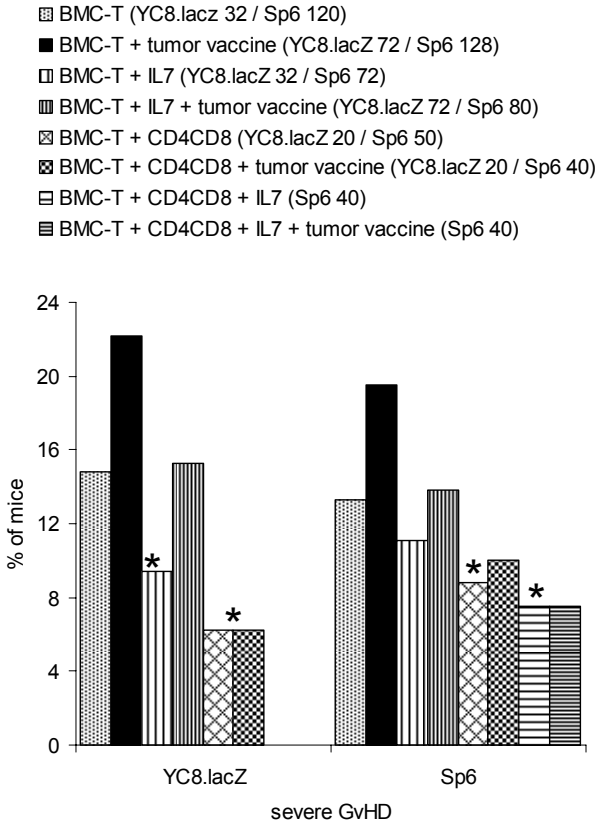
Fig. 6 GVHD and GvT reactivity in myeloablatively conditioned mice reconstituted with T_{depl} BMC plus DP TC and vaccinated with tumor cells

BALB/c mice received 8Gy and anti-asialoGM1 and were reconstituted as described in Fig.5. They received a s.c. injection of a mixture of Sp6 lysate (corresponding to $1x10^7$ cells) and Sp6 IgM (150µg / mouse). in adjuvant at the day of Sp6 cell inoculation. Vaccination was repeated after 2wk (A) The percentage of mice succumbing with severe GVHD is shown (B-E) The following parameters were evaluated ex vivo in 2 mice / group 1wk after the second vaccination (B) The number of SC, (C) the

percentage of $CD4^+$ and $CD8^+$ SC (flow cytometry).; (D) the percentage of IFNγ expressing SC (flow cytometry) and (E) cytotoxic activity of SC against host lymphoblasts and Sp6 tumor cells evaluated as described in Fig 1. The % cytotoxicity at an E:T ratio = 50:1 is shown (F) The percentage of tumor-free mice 80d after reconstitution is shown (A-F) Significant differences as compared to mice reconstituted with T_{depl} BMC are indicated by an asterisk.

It is known that only non-myeloablative or myeloreductive conditioning is comparably well tolerated by patients with hematological malignancies [177]. Hence, we repeated the experiment in a myeloreductively conditioned host. Mice that received T cell depleted BMC had high incidence of GVHD. This remained unaltered even when the mice received additional injections of IL-7. However the incidence of GVHD was reduced in mice receiving no vaccination. When mice received DP TC with or without IL-7; vaccination did not enhance the GVHD (Fig.7A). IL-7 application did not have a major impact on tumor take and survival of mice. But when mice were injected with DP TC along with BMC plus vaccination, tumor take was reduced and the survival rate was increased. Vaccinating the mice receiving BMC without DP, with or without IL-7 did not improve the survival time. However, the survival rate was improved significantly, almost doubled in mice reconstituted with DP TC and receiving vaccination (Fig.7B & C). The same observations accounted for mice receiving SP6 vaccination. The survival time improved in mice reconstituted with DP TC plus BMC as compared to only T depleted BMC. Also the tumor take was lesser in these mice (Fig.7 D & E), suggesting that DP TC injection along with BMC creates a room for vaccination, as well as increases the survival time of mice by decreasing the tumor take.

A


BMC-T (YC8.lacz 32 / Sp6 120)
BMC-T + tumor vaccine (YC8.lacZ 72 / Sp6 128)
BMC-T + IL7 (YC8.lacZ 32 / Sp6 72)
BMC-T + IL7 + tumor vaccine (YC8.lacZ 72 / Sp6 80)
BMC-T + CD4CD8 (YC8.lacZ 20 / Sp6 50)
BMC-T + CD4CD8 + tumor vaccine (YC8.lacZ 20 / Sp6 40)
BMC-T + CD4CD8 + IL7 (Sp6 40)
BMC-T + CD4CD8 + IL7 + tumor vaccine (Sp6 40)
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YC8.lacZ
Sp6
severe GvHD

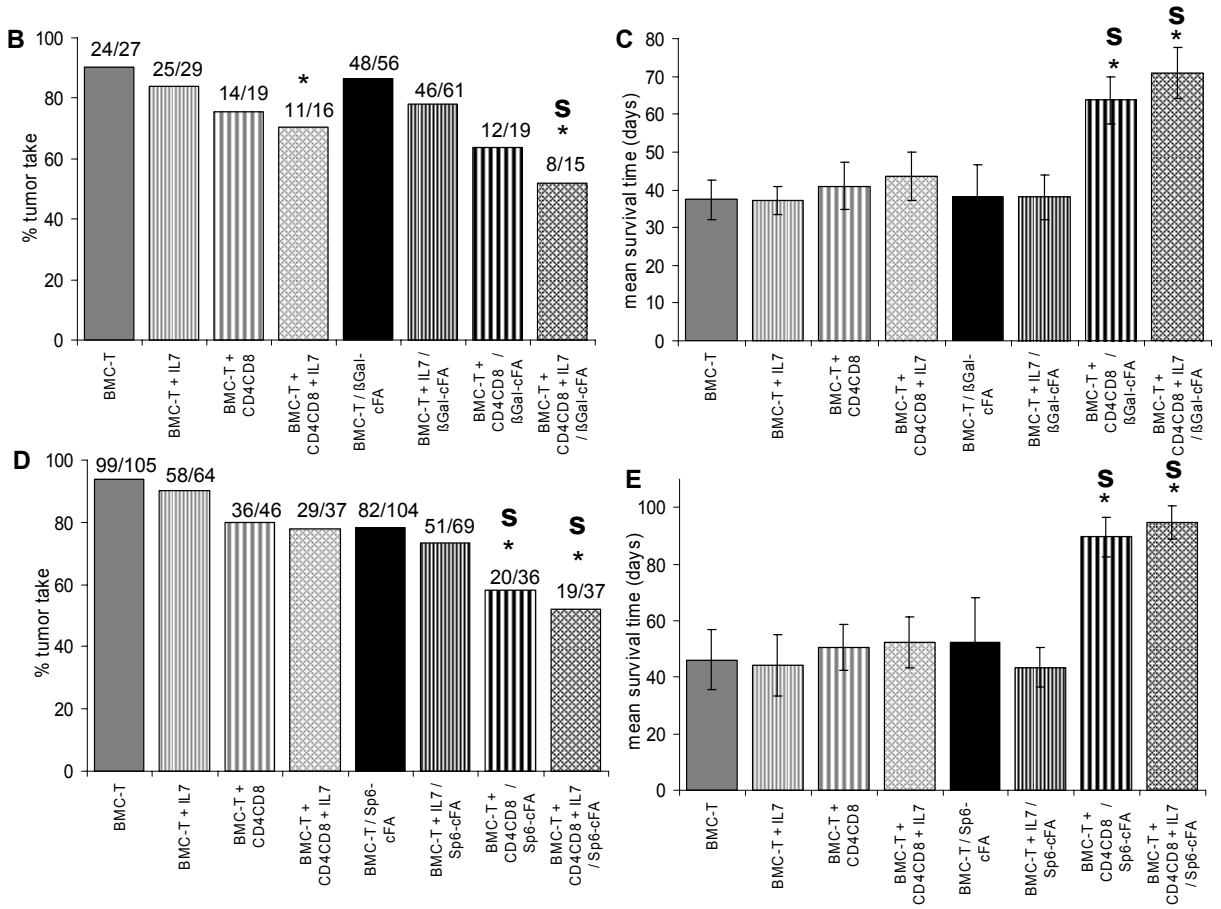


Fig. 7 GVHD and GvT reactivity in myeloreductively conditioned mice reconstituted with T_{depl} BMC plus DP TC and vaccinated with tumor cells

BALB/c mice conditioned and reconstituted as described in Fig.5 received a s.c. injection of a mixture of YC8.lacZ lysate (corresponding to $1x10^7$ cells) and β-Gal (100µg / mouse) or of a mixture of Sp6 lysate (corresponding to $1x10^7$ cells) and Sp6 IgM (150µg / mouse) in adjuvant at the day of tumor cell inoculation. Vaccination was repeated after 2wk (A) The percentage of mice succumbing with severe GVHD is shown. Significant differences as compared to mice reconstituted with T_{depl} BMC are indicated by an asterisk. Significant differences between vaccinated and non-vaccinated mice are indicated by s (B and D) The number of mice developing tumors and (C and E) the mean survival time are presented (B-E) Significant differences between mice receiving only T_{depl} BMC and the remaining groups are indicated by an asterisk. Significant differences between non-vaccinated and vaccinated groups are indicated by s.

Taken together, reconstitution of the myeloreductively conditioned host with T_{depl} BMC plus DP TC obviously supports donor T cell tolerance induction, such that tumor vaccination is not accompanied by an aggravation of GVHD. These donor-derived, host-tolerant T cells can still mount an anti-tumor response, while the survival rate and time become significantly improved.

Since transfer of DP TCs efficiently improved thymus reconstitution we wanted to further strengthen the protocol for allogeneic BMT with respect to thymus homing and maturation. Hence, it was important to study molecules that support thymus homing and maturation. CD44 has been proposed as a marker for T cell progenitors [44] and also facilitates thymus homing [178]. In addition, we reported previously that bone marrow cells from mice transgenic for the ratCD44v4-v7 variant isoforms had a clear advantage of repopulating the thymus over the wild type and in this case thymocytes also expanded earlier than the wild type mice. This effect could be blocked by an antibody against CD44v6 [169]. So we went on to explore the role of CD44 standard and variant isoforms v6 and v7 in thymocyte homing and maturation as stated below.

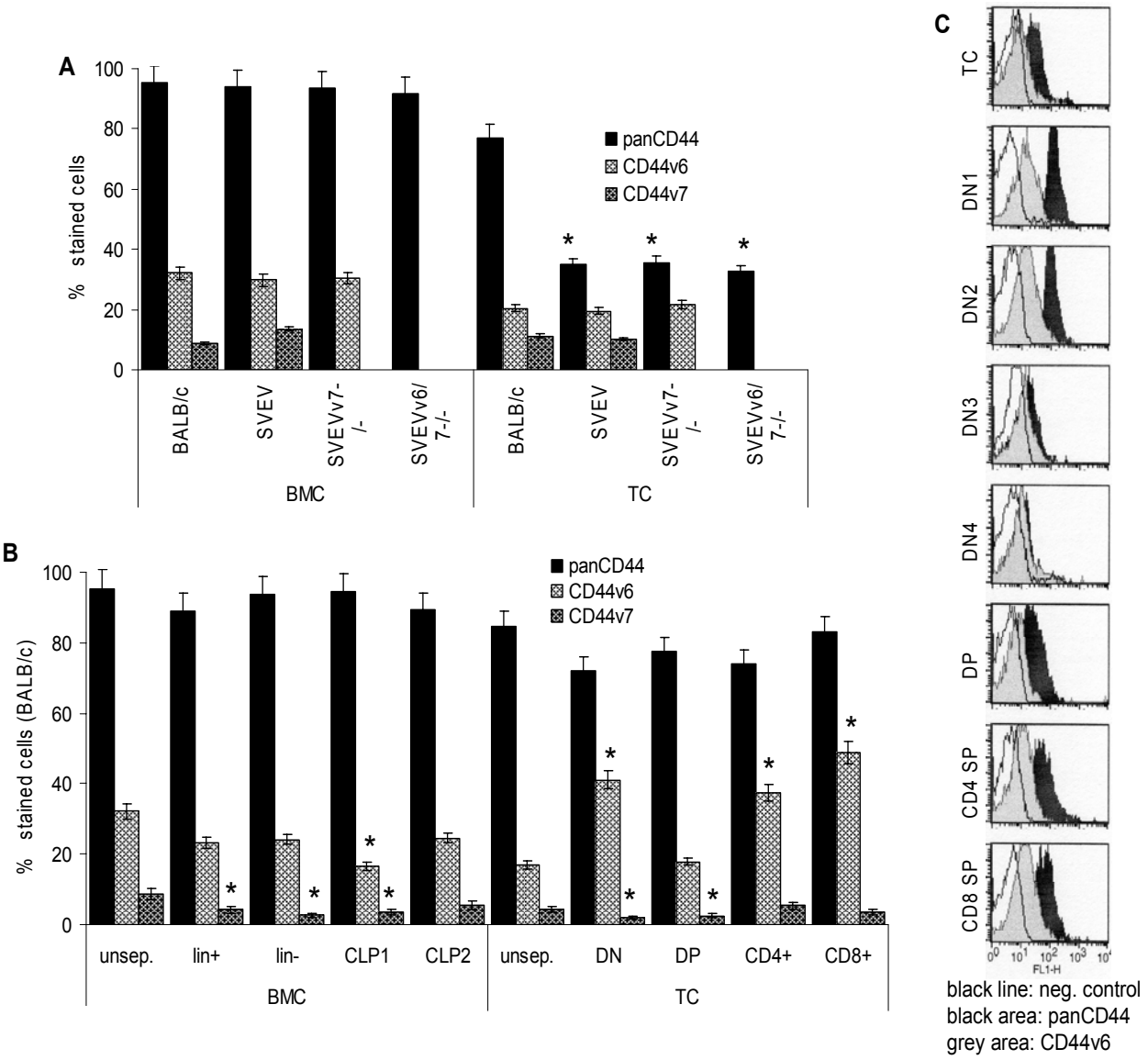
3.7 Expression of adhesion molecules, interleukins and chemokines on hematopoietic progenitors and thymocytes

Before exploring its role in thymus reconstitution, we looked for the expression of CD44 and its variants v6 and v7 in hematopoietic progenitors and thymocytes.
CD44 expression in the bone marrow was the same in all mice strains, however in the thymus; Balb/c had highest expression of CD44 (80%), which decreased in the SVEV strain to around 40%. CD44v6 and v7 expression were almost similar in all strains in BMC and TC (Fig.8A). In the individual progenitors the expression of CD44 and variants did not vary much except that CD44v6 was highest in double negative (DN), lower in double positive (DP) and comparatively higher in single positive (SP) populations of thymocytes. The intensity of CD44 expression in DP TC was lower than in other populations (Fig.8B and C).

Interaction of thymocytes with elements of the thymic microenvironment is very essential for the differentiation of thymocytes [59]. CD44 and CD49d interaction in peripheral T cells has been reported. 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 molecule of the other [154]. In this regard expression of additional adhesion molecules on thymocytes was checked. CD49d, CD18, CD29 were expressed by a

majority of thymocytes between 60-80%. The expression of these molecules decreased to a great extent in DP TC. CD54 and CD62L were expressed by 50% of the thymocytes. Expression of CD103 was particularly high in DN population. All the markers also co-expressed CD44. Co-expression with CD44v6 was high for CD49d, CD18, CD29 and CD62L and low for CD103. Co-expression with CD44v6 did not vary with other adhesion molecules at different stages of maturation except CD62L co-expression, which was less in DP (Fig.8D and E).

Since IL-3 and IL-7 are major growth factors for TC [174] we looked for their expression at different stages of TC maturation. Expression of CCL25/CCR9 an important chemokine/chemokine receptor pair for TC maturation [54] were checked by semi-quantitative PCR. IL-3, IL-7 and CCL25 expression were stronger in CD4 SP. IL-3R, IL-7R and CCR9 were highly expressed in DN. CCR9 expression was the highest in DP thymocytes (Fig.8F and G).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% stained cells
100
80
60
40
20
0
panCD44
CD44v6
CD44v7
BALB/c
SVEV
SVEVv7-/-
SVEVv6/7-/-
BMC
TC
B
% stained cells (BALB/c)
unsep.
lin+
lin-
CLP1
CLP2
DN
DP
CD4+
CD8+
C
TC
DN1
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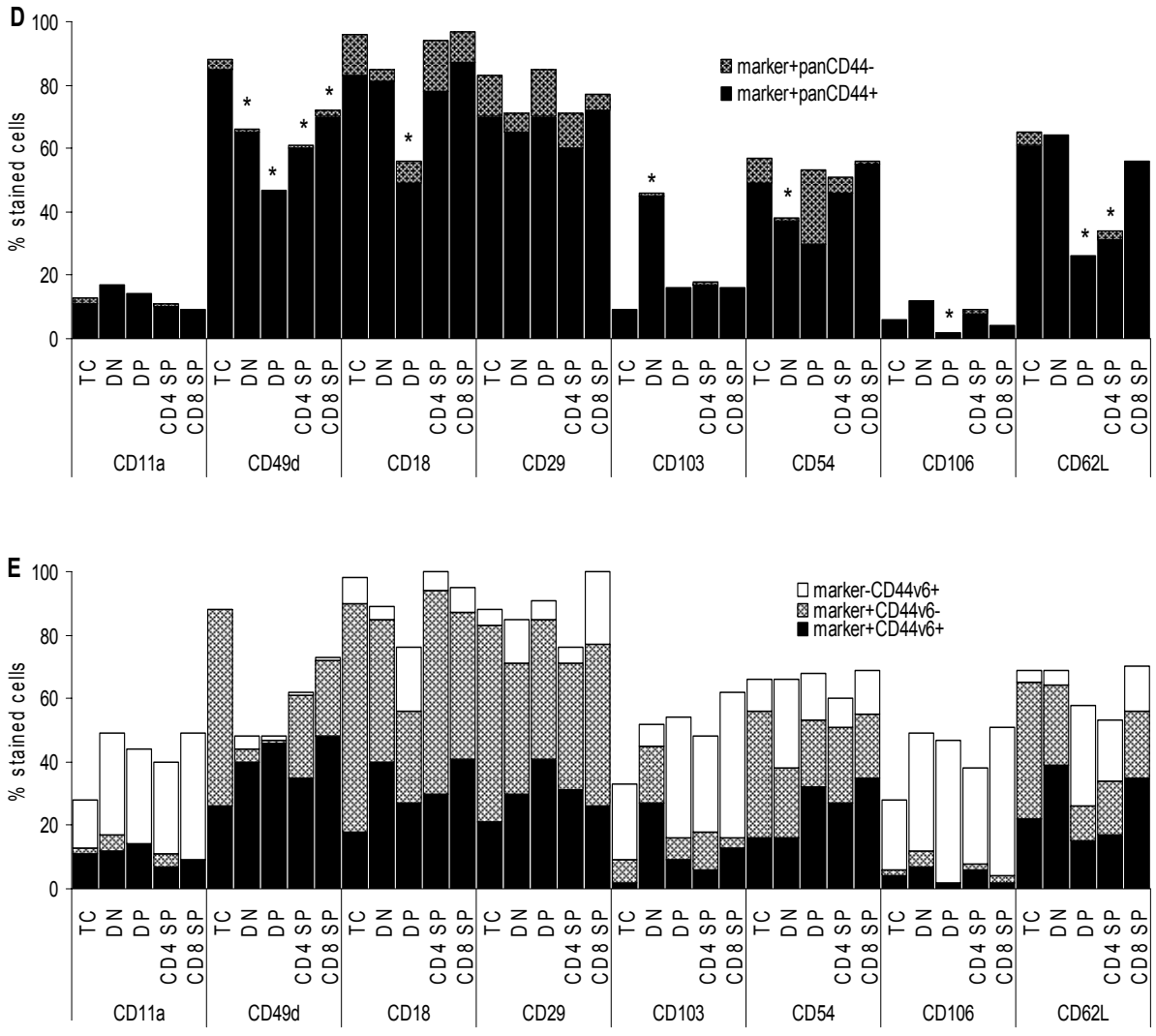
CD4 SP
CD8 SP
FL1-H
black line: neg. control
black area: panCD44
grey area: CD44v6


D
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CD103
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CD106
CD62L

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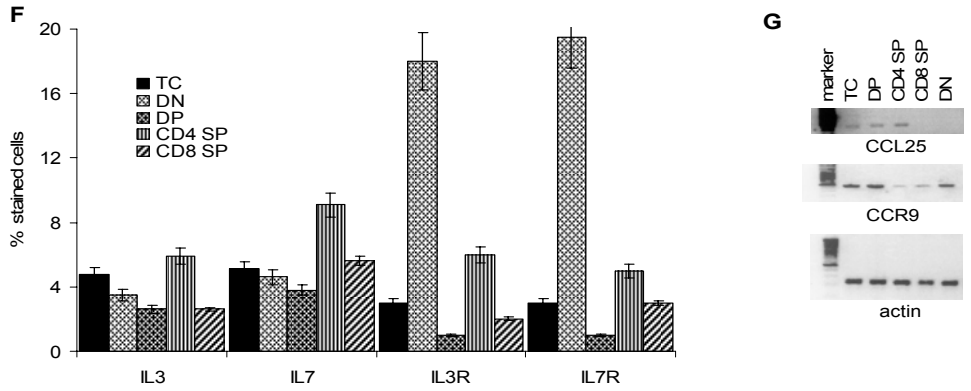


Fig. 8 Adhesion molecule, cytokine, chemokine and cytokine / chemokine receptor expression on subpopulations of BALB/c TC

(A) BALB/c, SVEV, SVEVv7$^{-/-}$ and SVEVv6/7$^{-/-}$ BMC and TC and (B) bone marrow- and thymus-derived progenitor subpopulations were stained with anti-panCD44, anti-CD44v6 and anti-CD44v7. The mean percentage ± SD of stained cells is shown. Differences between BALB/c and SVEV mice and between unseparated BMC / TC and subpopulations derived thereof are indicated by * (C) Examples on the intensity of CD44/CD44v6 expression on TC subpopulations. Single fluorescence overlays with the negative control are shown (D and E) Unseparated, DN, DP, CD4 SP and CD8 SP TC were double stained with (D) anti-CD44 or (E) anti-CD44v6 and antibodies against the indicated adhesion molecules or were stained (F) with anti-IL-3, -IL-7, -IL-3R or IL-7R. The percentage of single positive and double positive cells (mean values from 3-5 assays) is shown (G) CCL25 and CCR9 mRNA in TC subpopulations was evaluated by RT-PCR. Actin served as internal control. A representative example is shown.

Taken together, BALB/c TC express CD44 and CD44v6 at a relatively high frequency. CD44v6 expression varies with the maturation stage with a transient decline in DP. CD44v7 expression is low throughout. Irrespective of the maturation stage, integrins are mostly co-expressed with CD44v6, which does not account to the

same degree for ICAM. The selectin CD62L, highly expressed in TC, is also co-expressed with CD44v6, co-expression declines in DP.

3.8 Retarded thymus repopulation in anti-CD44 treated syngeneically reconstituted mice

In order to have a look whether CD44 standard or CD44v6/v7 isoforms are important for thymus homing and repopulation, lethally irradiated SVEV wild type mice with/without targeted deletion for CD44v6/7 or CD44v7 were reconstituted with BMC. Lethally irradiated and reconstituted Balb/c mice were used as an additional control. Alongwith TC we also checked bone marrow and spleen cells in parallel for better comparison. Balb/c and SVEV mice reconstituted with a good efficacy and reached normal numbers by week 4-6. CD44 v7$^{-/-}$ and v6/7$^{-/-}$ mice reconstituted with a lower efficiency in BMC and SC. The effect of CD44v6/7$^{-/-}$ was more pronounced in TC (Fig.9A). To assess if anti-CD44 treatment interferes with reconstitution mice were treated with anti-panCD44 (recognizes standard and variant CD44 isoforms) twice per week. The effect of inhibition was stronger in Balb/c than SVEV; probably due to higher CD44 expression in Balb/c. The effect became stronger in the bone marrow after 4-6wk of reconstitution (Fig.9B).

To evaluate the role of variant CD44 isoforms v6 and v7 in homing, SVEV BMC were transferred into wild type SVEV or SVEVv7/$^{-/-}$ and SVEVv6/7$^{-/-}$. Homing of SVEV BMC in the bone marrow of both knockout mice was reduced. In the spleen, slightly fewer cells were recovered in CD44v6/7$^{-/-}$ mice as compared to SVEV CD44v7$^{-/-}$ mice. In the thymus a weak effect was seen in the knockout mice receiving SVEV bone marrow, slightly lesser cells were recovered (Fig.9C). The other way round when bone marrow cells from knockout mice were reconstituted into SVEV mice, repopulation of BMC and spleen improved compared to the knockout mice. The recovery of TC in SVEV mice receiving BM from CD44v6/7$^{-/-}$ mice remained impaired (Fig.9D). This was an indication that CD44v6 on the thymic progenitors might be important in repopulating the thymus.

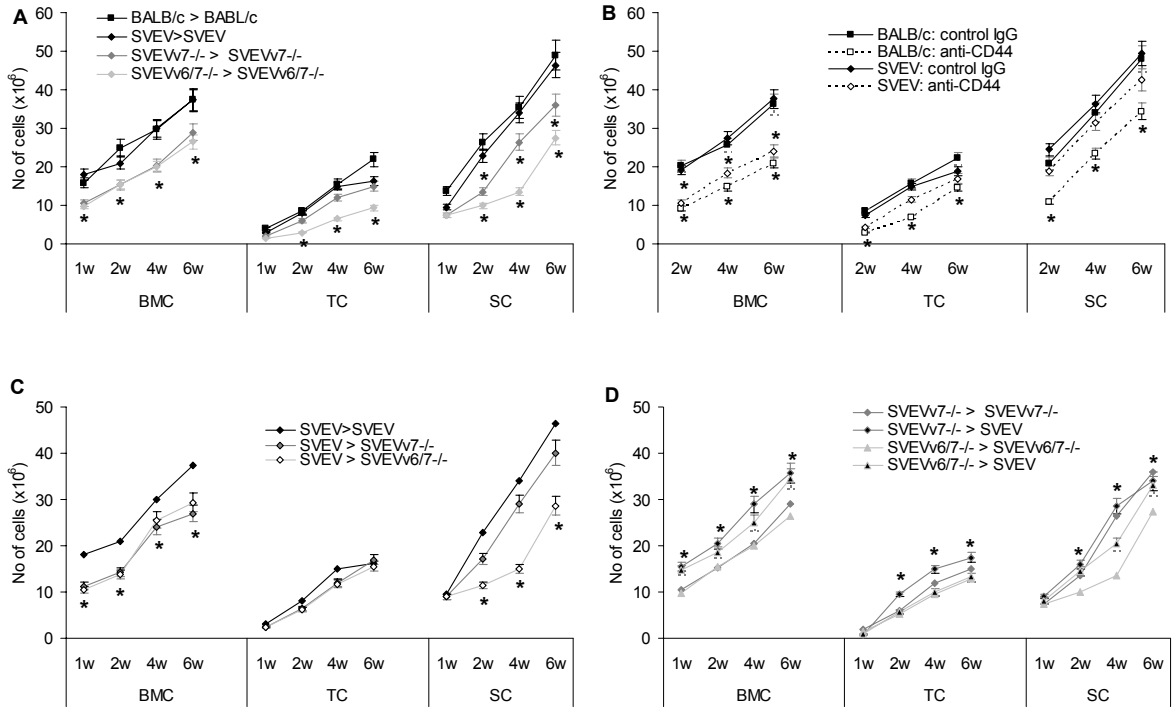


Fig. 9 The impact of CD44 on BMC reconstitution

(A) Lethally irradiated BALB/c, SVEV, SVEVv7$^{-/-}$ and SVEVv6/7$^{-/-}$ mice were reconstituted with 5x10^6 autologous BMC (B) BALB/c and SVEV mice, reconstituted as described, received 100µg control IgG or anti-CD44, i.v. twice / week (C) SVEVv7$^{-/-}$ and SVEVv6/7$^{-/-}$ mice were reconstituted with 5x10^6 SVEV BMC (D) Lethally irradiated SVEV mice were reconstituted with 5x10^6 SVEVv7$^{-/-}$ or SVEVv6/7$^{-/-}$ BMC (C and D) autologous reconstitution is included as control (A-D) Three mice / group were sacrificed after 1-6wk. The mean number ± SD of BMC, TC and SC is shown. Significant differences between SVEV and SVEVv7$^{-/-}$ / SVEVv6/7$^{-/-}$ mice (A, C, D) and between control IgG- and anti-CD44-treated mice are indicated by *.

From the finding that anti-panCD44 exerts a stronger effect on BMC recovery at 6wk than 2wk after reconstitution we assume that anti-panCD44 interferes not only with BMC homing/settlement, but also with BMC expansion/maturation. Settlement of BMC is likely facilitated by stromal cell CD44v7, because BMC homing, but not expansion is reduced in CD44v6/7$^{-/-}$ / CD44v7$^{-/-}$ mice. Thymus homing too is affected by an anti-panCD44 blockade. CD44v6/7$^{-/-}$ mice are burdened, in addition, by reduced TC expansion, that is not compensated in the CD44v6/7^{comp} (competent) host. This indicates a possible involvement of CD44v6 in TC expansion and/or maturation.

3.9 CD44 influences homing of hematopoietic progenitors into bone marrow and thymus

The importance of CD44 in BMC homing was confirmed by labeling BMC cells with CFSE and injecting into mice. Spleen, TC and BMC cells were isolated and checked for CFSE labeled cells by FACS. When CD44v6/7$^{-/-}$ and CD44v7$^{-/-}$ were reconstituted with bone marrow cells from these knockout mice fewer bone marrow cells were recovered (Fig.10A). However, when SVEV mice were reconstituted with bone marrow cells from CD44v6/7$^{-/-}$ mice, bone marrow reconstitution significantly improved. Reconstitution was still poor when SVEV BMC were injected into CD44v7$^{-/-}$ (Fig.10B). The migratory activity of BMC was checked using all mice strains including the knockouts. When CFSE BMC were reconstituted along with anti-CD44 treatment into mice, the recovery of BMC in Balb/c and SVEV was reduced drastically. Less BMC were recovered in knockout mice and anti-CD44 treatment did not have much of an impact. However, a higher number of cells were retained in the spleen of knockout mice after anti-CD44 treatment (Fig.10C).

Bone marrow cells are a mixture of stem cells, lymphoid and myeloid progenitors and several subpopulations. To see which subpopulation might be affected by a blockade of CD44 or by a deficiency in CD44v6 or v7 isoforms, bone marrow cells from Balb/c were separated into lin^{+} (lineage positive), CLP1 (lin-ckit^{+}IL-7Rα^{+}) and CLP2 (lin^{-}c-kit^{lo} B220^{+}) as indicated in materials and methods, CFSE labeled and injected into Balb/c mice. In order to explore the role of CD44 in the homing of individual populations, mice were giving injections of anti-panCD44. Lin^{+} and CLP1 population homed with high efficiency into BM and the homing was inhibited with anti-CD44 treatment. CLP2 homed with very less efficiency into BM (Fig.10D). To evaluate the role of isoforms in homing of these progenitors, the experiment was repeated with CFSE labeled SVEV BMC subpopulations injected into CD44v6/7$^{-/-}$ mice and vice-versa or injections of wild type bone marrow cells into wild type and knockout bone marrow cells into knockout mice. The SVEV lin^{+} and CLP1 cells homed efficiently into SVEV host bone marrow. Homing of CD44v6/7$^{-/-}$ lin^{+} and CLP1 into CD44v6/7$^{-/-}$ mice was severely impaired. The homing capacity improved especially in CLP1 population when BMC of CD44v6/7$^{-/-}$ were injected into SVEV CD44v6/7^{comp} (competent) mice.

However they did not reach the level of lin+ and CLP1 from SVEV BMC. In addition, the homing still remained impaired when SVEV lin+ and CLP1 cells were injected into CD44v6/7$^{-/-}$ mice (Fig.10E).

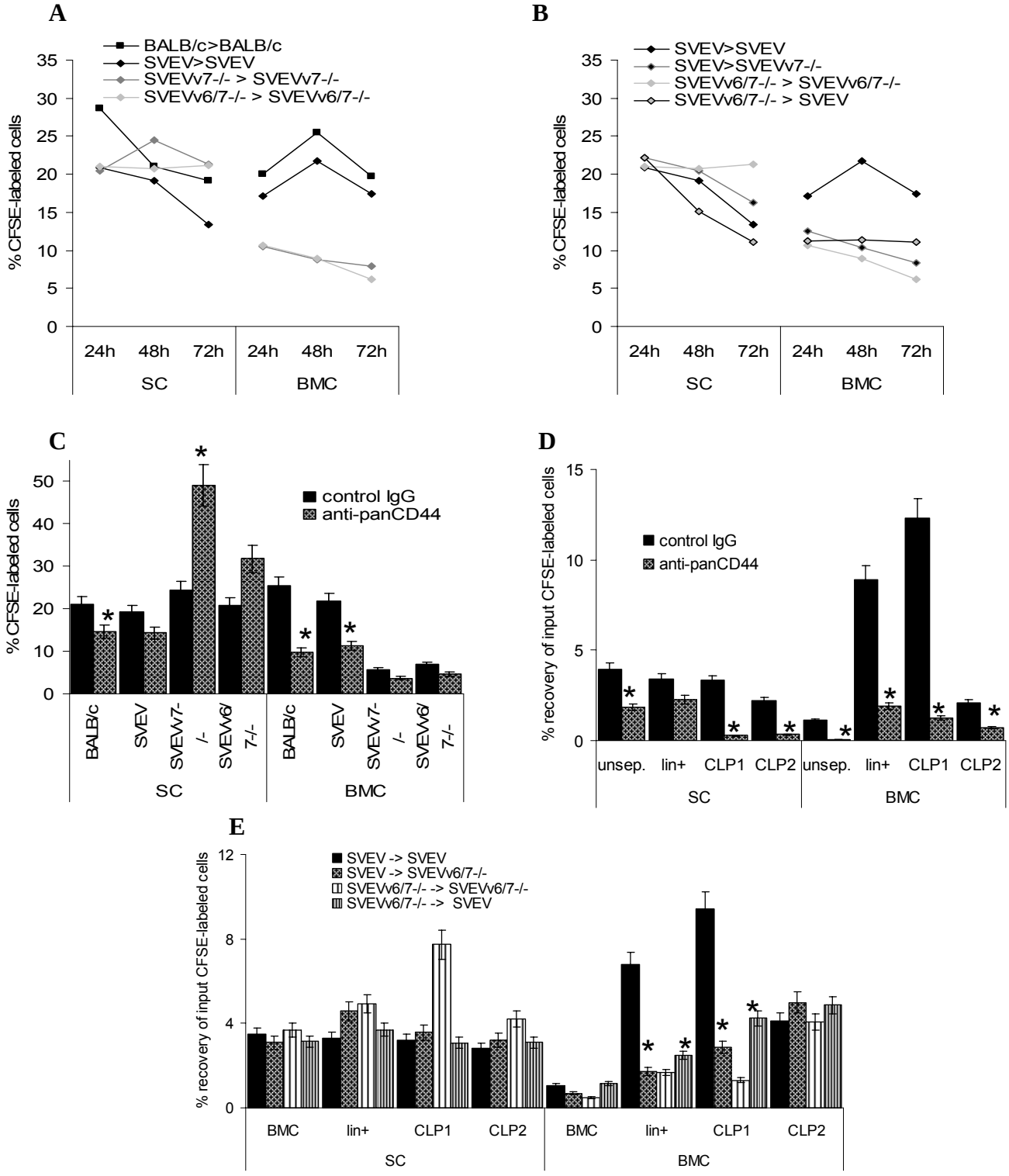


Fig. 10 The impact of CD44 on TC homing and reconstitution

(A) 1x10^{7} CFSE-labeled BALB/c, SVEV, SVEVv7$^{-/-}$, SVEVv6/7$^{-/-}$ BMC were i.v. injected into lethally irradiated syngeneic mice; (B) SVEV BMC were injected into SVEVv7$^{-/-}$ mice or SVEVv6/7$^{-/-}$ BMC were injected into SVEV mice; (C) CFSE-labeled BMC were loaded with anti-panCD44 before injection; (D)

CFSE-labeled subpopulations ($2x10^6$) of BALB/c BMC were injected concomitantly with 150µg anti-panCD44; (E) CFSE-labeled subpopulations ($2x10^6$) of SVEV BMC were injected into SVEVv6/7$^{-/-}$ mice and vice versa; (A and B) Mice were sacrificed after 24-72h or (C-E) 48h(A,B,C) The percentage of dye-labeled SC and BMC or (D,E) the % recovery of input CFSE-labeled cells in spleen and bone marrow was evaluated by flow cytometry. Mean values ± SD of 3 mice / group are shown. Significant differences between SVEV and SVEVv7$^{-/-}$ / SVEVv6/7$^{-/-}$ or between control IgG and anti-panCD44 are indicated by *.

Thus, CD44 considerably contributes to lin$^+$ and CLP1 marrow homing. Furthermore, stromal cell CD44v7 is important to retain “homed” lin$^+$ and CLP1. Retention of CLP2 appears to be independent of stroma cell CD44v7.

It is known that CD44 supports progenitor homing into thymus [179]. Since we also observed inhibition in homing of BMC into thymus using anti-CD44, we wanted to check which sub populations of BMC progenitors and TC subpopulations were susceptible to anti-CD44 treatment in homing into the thymus. We used Balb/c Lin$^+$, CLP1, CLP2, DN, DP, SP populations to check for their homing capacity into thymus as well as the role of CD44 using anti-panCD44. Lin$^+$ and CLP1 homed very poorly into thymus. CPL2 and DN1 homed with highest efficiency (2%) as compared to the rest, followed by DN2 and DN3. We could show in addition, that homing of these sub populations was inhibited with anti-panCD44 (Fig.11A).

We wanted to check next, if CD44 variant isoforms would be involved in the homing of these individual subpopulations into the thymus. The same experiment was further repeated using SVEV wild type and knockout for CD44v6/7. CLP2 homing was much higher as compared to unseparated BMC and TC in all mice. The lack of CD44v6 or v7 did not have any major impact on homing of these subpopulations into thymus. CD44v6 or v7 expression on the stroma of thymus doesn’t seem to have a major impact on homing of subpopulations into thymus, since transfer of SVEV bone marrow into CD44v6/7$^{-/-}$ mice had no effect on homing (Fig.11B).

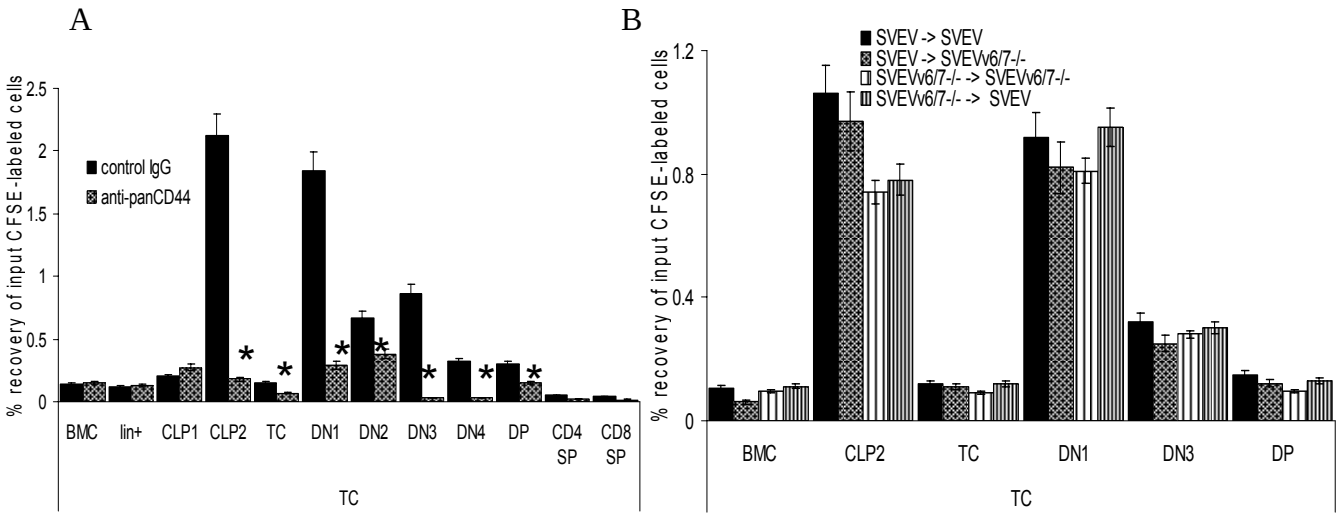


Fig. 11 The impact of CD44 on TC homing

(A) BALB/c-derived CFSE-labeled BMC ($1x10^7$) TC ($1x10^7$) and subpopulations ($1x10^6$ – $1x10^7$) thereof were i.v. injected into lethally irradiated BALB/c, concomitantly with 150µg anti-panCD44; (B) SVEV- or SVEVv6/7$^{-/-}$derived CFSE-labeled BMC ($1x10^7$) TC ($1x10^7$) and subpopulations ($1x10^6$ – $1x10^7$) thereof were i.v. injected into lethally irradiated SVEV or SVEVv6/7$^{-/-}$ mice. Mice were sacrificed after 48h. The % recovery of input CFSE-labeled cells in the thymus was evaluated by flow cytometry. Mean values ± SD of 3 mice / group are shown. Significant differences between SVEV and SVEVv7$^{-/-}$ / SVEVv6/7$^{-/-}$ or between control IgG and anti-panCD44 are indicated by *.

These findings confirm preferential CLP2 homing into the thymus. TC subpopulations also home into the thymus, the homing capacity inversely correlating with maturation. A blockade of panCD44 affects progenitor homing. Since thymus repopulation is delayed in CD44v6/7$^{-/-}$ mice, we speculated that CD44v6 may contribute to early stages of T progenitor maturation.

3.10 CD44v6 promotes proliferation of T progenitors in thymus

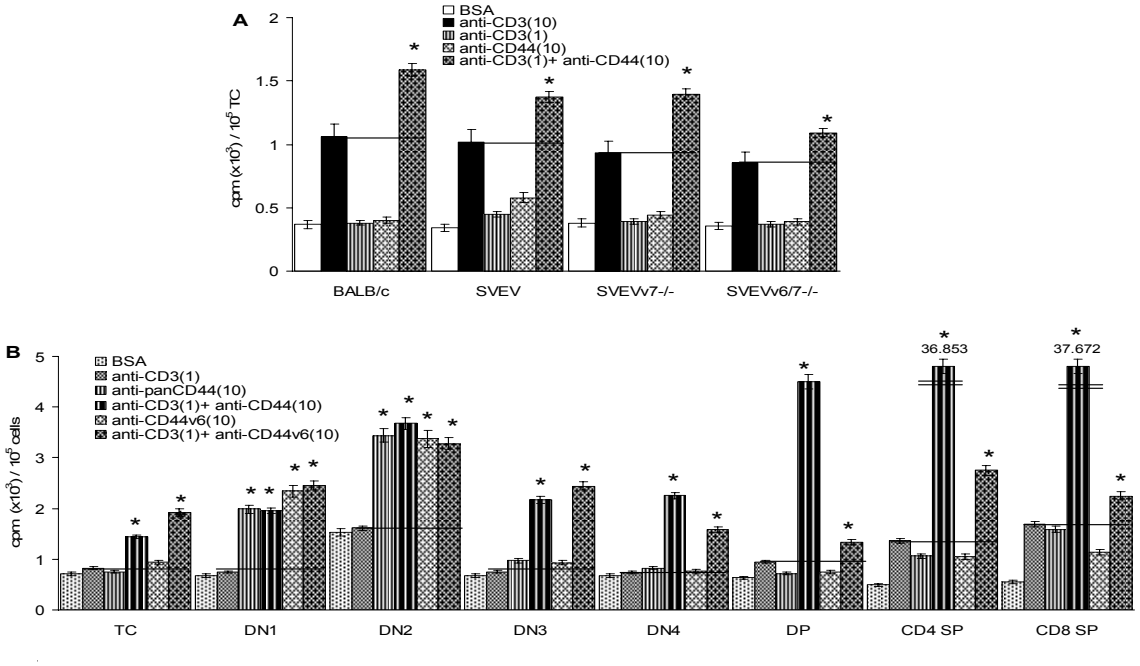
In peripheral T cells it was shown that, anti-panCD44 when cross-linked along with sub threshold levels of anti-CD3 increases proliferation of T cells as compared to sub threshold levels of anti-CD3 alone [150]. Since we observed delay in repopulation of the thymus in CD44v6/7$^{-/-}$ mice it was worthwhile to check for proliferation. To assess the role of CD44s or variant isoforms (particularly v6) with

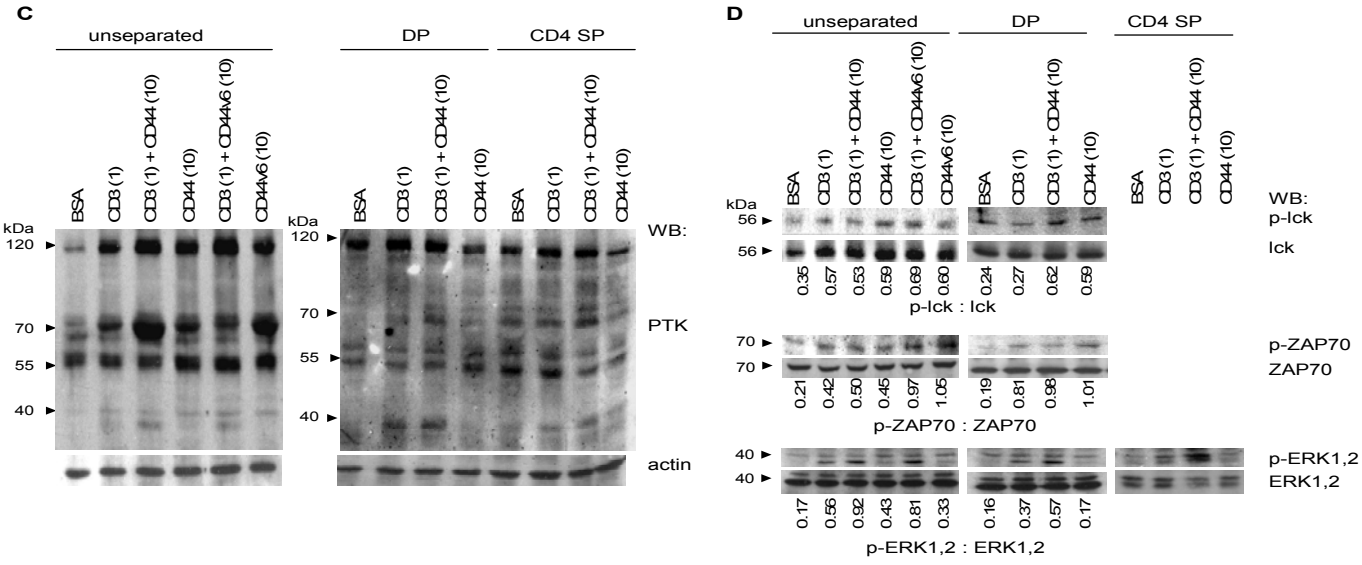
respect to pre-TCR, we repeated the same experiment in thymocytes as in peripheral T cells in two strains of mice Balb/c and SVEV including CD44v6/7$^{-/-}$ and v7$^{-/-}$ with SVEV background. Flat bottomed 96-well plates were coated with the specified antibodies o/n as described in materials and methods. We saw the same effect in unseparated thymocytes that CD44 alone could not increase proliferation. Instead, after concomitant cross-linking with anti-CD3, the proliferation was much higher than anti-CD3 cross-linking alone. The effect was comparatively less in the case of CD44v6/7$^{-/-}$ mice (Fig.12A).

In order to define the maturation stage of thymocytes at which CD44 has an effect, the experiment was repeated in different subpopulations of thymocytes. This time anti-CD44v6 cross-linking alone or with anti-CD3 concomitant cross-linking was also used since CD44v6/7$^{-/-}$ mice showed comparatively less proliferation in the previous experiment. Surprisingly, in the DN1 and DN2 subpopulations anti-CD44 cross-linking alone also showed high proliferation that was not increased by concomitant anti-CD3 cross-linking in addition. The same effect was also seen with a CD44v6 specific antibody cross-linking. In DN3 and DN4 subpopulations however, anti-panCD44/v6 cross-linking did not exert an effect by themselves. Only when they were concomitantly cross-linked with anti-CD3, thymocytes showed high proliferation rates which were higher than after anti-CD3 cross-linking alone, but not as high as in DP or SP. In the DP population anti-panCD44 cross-linking showed high proliferation after concomitant cross-linking with anti-CD3, and anti-CD44v6 could not mediate this effect as strong as pan CD44. In the SP populations proliferation was seen only when anti-panCD44 were concomitantly cross-linked with anti-CD3 and the effect was less pronounced with anti-CD44v6 cross-linking. SP cells weakly responded to CD44 or v6 cross-linking alone (Fig.12B). Since DP cells are the largest population of thymocytes (~85%) we suspect that the effect of CD44 alone on the proliferation of DN was hidden in total thymocytes.

Since we observed high proliferative stimulus in thymocytes with respect to anti-panCD44/v6 cross-linking, the next step was to gain insight into molecular mechanisms involved in mediating signals through panCD44/v6 or CD3 plus

panCD44/v6 leading to proliferation. When pre-TCR complex signals are triggered it leads to recruitment and phosphorylation of several tyrosine kinases to the vicinity of the TCR receptor which leads to further activation of downstream signaling events such as the mitogen-activated protein kinase (MAPK) family and PI3K family [74, 84]. So we looked for tyrosine phosphorylation events in reponse to anti-panCD44/v6 and or anti-CD3 cross-linking. Tyrosine phosphorylation became stronger when unseparated thymocytes, DP and SP were cross-linked with CD3 and panCD44/v6 in comparison to CD3 alone (Fig.12C). Next we looked for specific tyrosine kinases Lck and Zap-70 recruited at the pre-TCR complex. CD44/v6 cross-linking alone promoted phosphorylation of Lck and ZAP-70. But after this step panCD44/v6 could not mediate further events by themselves and required CD3 cross-linking. Since MAPK pathway is usually involved in mediating proliferation signals [180], thymocytes were activated with anti-CD3 and/or panCD44/v6 cross-linking and checked for ERK1/2 activation. Low level phosphorylation of ERK1/2 was mediated by CD3 cross-linking which was enhanced with panCD44/v6 and CD3 concomitant cross-linking in unseparated, DP and CD4SP TC (Fig.12D). To further confirm the pathway involved in proliferation we used MEK1/2 inhibitor and performed the proliferation experiment as before. DMSO was used as control since inhibitors were diluted in it. MEK1/2 inhibitor strongly interfered with the proliferation of DN1 and DN2 in response to panCD44/v6 cross-linking (Fig.12E). MEK1/2 also inhibited the proliferation of DP and SP cross-linked with CD3 alone. The effect of inhibition of proliferation in DP and SP was more strongly pronounced after CD3 plus panCD44 concomitant cross-linking (Fig.12F).


A
BSA
anti-CD3(10)
anti-CD3(1)
anti-CD44(10)
anti-CD3(1)+ anti-CD44(10)
cpm (x10³) / 10⁵ TC
BALB/c
SVEV
SVEVv7-/-
SVEVv6/7-/-
B
BSA
anti-CD3(1)
anti-panCD44(10)
anti-CD3(1)+ anti-CD44(10)
anti-CD44v6(10)
anti-CD3(1)+ anti-CD44v6(10)
cpm (x10³) / 10⁵ cells
36.853
37.672
TC
DN1
DN2
DN3
DN4
DP
CD4 SP
CD8 SP


C
unseparated
DP
CD4 SP
BSA
CD3 (1)
CD3 (1) + CD44 (10)
CD44 (10)
CD3 (1) + CD44v6 (10)
CD44v6 (10)
kDa
120
70
55
40
WB:
PTK
actin
D
unseparated
DP
CD4 SP
kDa
56
WB:
p-lck
lck
0.35 0.57 0.53 0.59 0.69 0.60 0.24 0.27 0.62 0.59
p-lck : lck
70
p-ZAP70
ZAP70
0.21 0.42 0.50 0.45 0.97 1.05 0.19 0.81 0.98 1.01
p-ZAP70 : ZAP70
40
p-ERK1,2
ERK1,2
0.17 0.56 0.92 0.43 0.81 0.33 0.16 0.37 0.57 0.17
p-ERK1,2 : ERK1,2

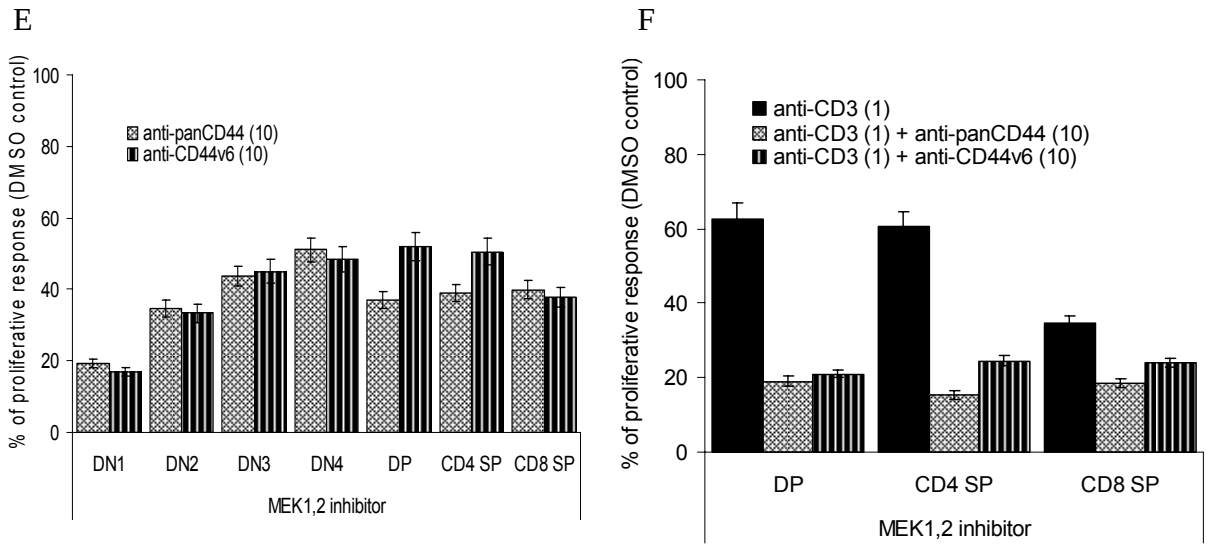


Fig. 12 CD44 promotes thymocyte proliferation:

(A) TC or (B) TC subpopulations were seeded in antibody-coated 96 well plates. Proliferation was evaluated by ^{3}H thymidine incorporation after 48h. Mean values of triplicates ± SD are shown. Significant differences between (A) anti-CD3 (10µg/ml) and anti-CD44 (10µg/ml) plus anti-CD3 (1µg/ml) or (B) between anti-CD3 (1µg/ml) and anti-panCD44/v6 (10µg/ml) or anti-panCD44/v6 (10µg/ml) plus anti-CD3 (1µg/ml) are indicated by *(C and D) TC, DP and CD4 SP were stimulated for 18min by seeding on antibody-coated plates. Cells were lysed and lysates were separated by SDS-PAGE. After protein transfer, membranes were incubated with (C) anti-phosphotyrosine or (D) anti-lck / anti-p-lck, anti-ZAP / anti-p-ZAP, anti-ERK1,2 / anti-p-ERK1,2. In (D) the ratio of p-lck: lck, p-ZAP70 : ZAP70, p-ERK1,2 : ERK1,2 are included (E and F) TC subpopulations were cultured for 48h on antibody-coated plates in the presence of 5µM MEK1,2 inhibitor. ^{3}H-thymidine incorporation (mean values of triplicates ± SD) is presented as % of the DMSO control.

Thus, CD44 ligation promotes lck and ZAP70 activation, at least partly, independent of TCR/CD3 complex ligation during TC maturation. At later maturation stages, CD44 co-operates with the CD3 complex supporting lck, ZAP70 and ERK1/2 phosphorylation.

3.11 CD44 protects thymocytes from apoptosis during maturation

Since CD44, particularly CD44v6 promoted proliferation of thymocytes, we were wondering whether it also plays a role in cell survival, since CD44v6/7$^{-/-}$ mice had a strong defect in reconstitution.

Thymocytes from SVEV and CD44v6/7$^{-/-}$ mice were cross-linked with anti-CD3 and/or anti-panCD44/v6 over night and checked for apoptosis by AnnexinV staining on FACS. SVEV and not CD44v6/7$^{-/-}$ mice were protected from apoptosis when cross-linked with anti-panCD44/v6 (Fig.13A). This was the first indication that CD44 protects thymocytes from apoptosis. We were inquisitive to know the signaling mechanism involved in panCD44/v6 mediated cell survival. Thymocytes were activated on antibody-coated plates. It was observed that increased apoptosis resistance mediated by panCD44/v6 was accompanied by Akt phosphorylation and Bcl-2 up-regulation. This effect was not seen in CD44v6/7$^{-/-}$ mice indicating the involvement of CD44v6 in cell survival (Fig.13B). We then looked for the maturation stage of thymocytes at which this effect was mediated. So the same experiment was repeated using different subpopulations of thymocytes. The protection of apoptosis in SVEV mice through panCD44/v6 cross-linking was the strongest in DN; DP were partly protected, on the contrary CD4 and CD8 SP were driven into apoptosis (Fig.13C). CD3 cross-linking at high level concentration promoted apoptosis in DP and SP TC. Cross-linking CD3 (sub threshold level) plus CD44 promoted apoptosis in DP and SP TC (Fig.13D). It should be mentioned that CD44 mediated apoptotic effects in DP and SP thymocytes were more pronounced for panCD44 as compared to CD44v6. To confirm the involvement of PI3K/Akt pathway the cross-linking experiment was repeated using PI3K inhibitor Ly49002. DN1 cells which were protected from apoptosis by panCD44/v6 cross-linking remained respiratory active with medium dose of Ly49002. DP and SP that were driven into apoptosis by CD3 and or panCD44/v6 cross-linking could survive in the presence of the inhibitor (Fig.13E). Thus expansion of DN population in response to panCD44/v6 cross-linking may partly also be due to increased cell survival.

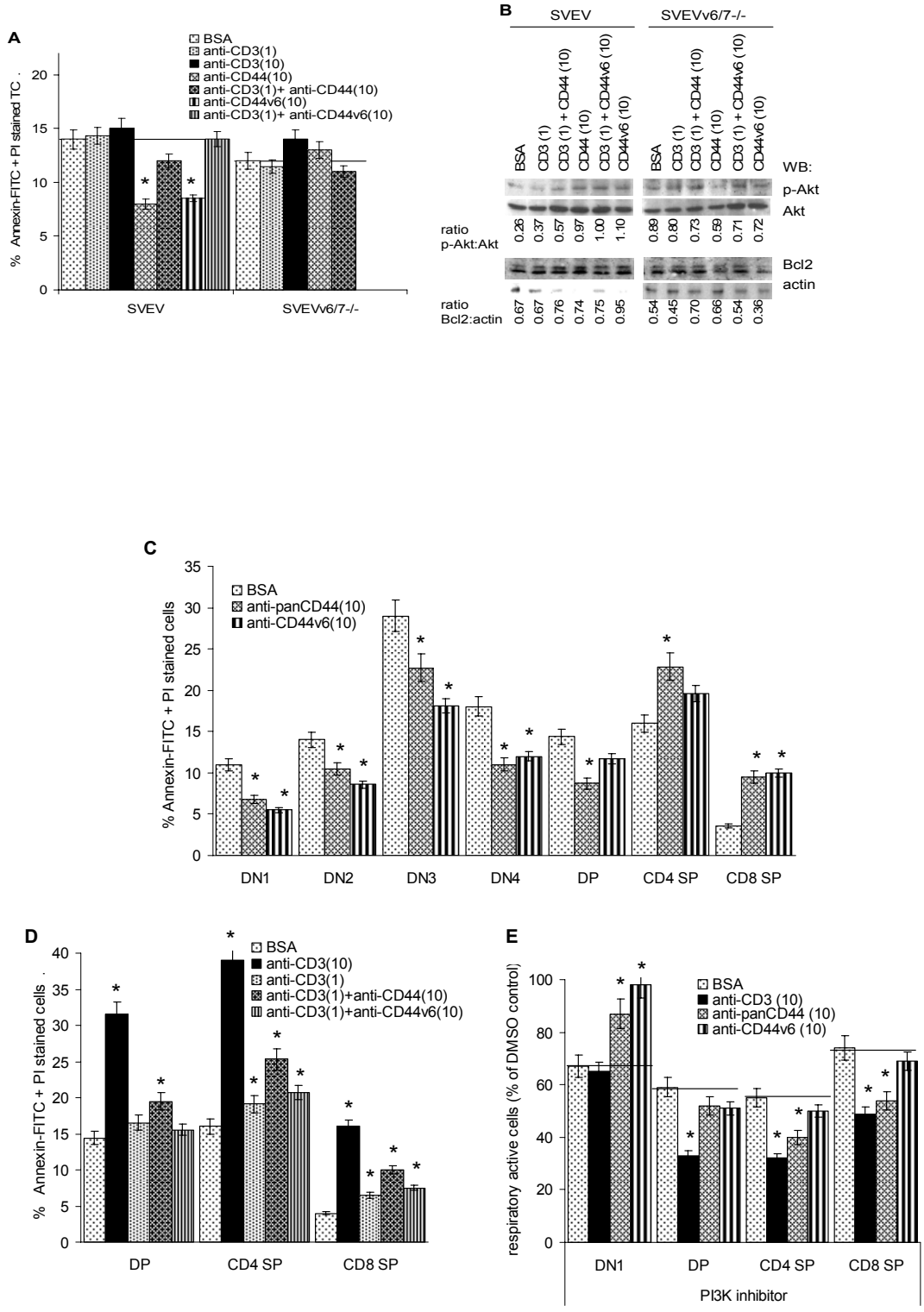


Fig 13 The involvment of CD44v6 in apoptosis resistance

(A) SVEV and SVEVv6/7$^{-/-}$ TC were seeded in antibody-coated 96-well plates. Annexin-FITC/PI staining was evaluated after 48h. Mean values ± SD of triplicates are shown. Significant differences to

BSA-coated plates are indicated by *(B) Unseparated TC were cultured for 24h in antibody-coated plates, lysed and, after separation by SDS-PAGE, blotted with anti-Akt / anti-pAkt, anti-Bcl2 and anti-actin (C and D) TC subpopulations were seeded in antibody-coated 96-well plates. Annexin-FITC/PI staining was evaluated after 48h. Mean values ± SD of triplicates are shown. Significant differences to BSA-coated plates are indicated by *(E) TC subpopulations were cultured in antibody-coated 96-well plates in the presence of 5µM LY294002 (PI3-kinase inhibitor). After 48h, respiratory active cells were evaluated by MTT staining. Mean values of triplicates ± SD are presented as % of the DMSO control. Significant differences are indicated by *.

4. Discussion

Allogeneic bone marrow transplantation could be an ultimate curative therapy for patients with hematological malignancies as well as solid tumors like renal cell carcinoma [14]. Since the suggestion of non-myeloablative or myeloreductive conditioning as compared to the conventional myeloablative regimen has come into picture, this approach could be extended to a vast majority of patients. Elderly patients suffering from hematological malignancies and solid tumors can now be given bone marrow transplantation with non-myeloablative conditioning regimen [11, 12]. However, even though tumor antigens exist and they have the ability to trigger an immune response it is known that patients suffering from tumors do not have sufficient immune responsiveness and this calls for a need of an external trigger like vaccination against specific tumor. In addition, vaccination protocol demands for a tolerance induction of donor T cells against the host [181]. This requires that donor T cells mature in the host thymus.

In contrast to other blood cells that are generated in bone marrow, T lymphocytes develop in the thymus. The thymus contains no self renewing cells and requires bone marrow progenitors to home into it and mature to form functional T lymphocytes. Thymus involution starts at young age resulting in decreased thymic cellularity as well as emigration capacity [31, 32]. Now the question is how efficient is the thymus to promote thymocyte maturation and produce functional T cells that can recognize and kill the tumor after allogeneic bone marrow transplantation? Strategies to overcome this problem could decrease infectious complications, malignant relapse, resulting in improved survival rate of recipients receiving allogeneic BMT [182].

In our first experimental setup we observed that thymus repopulation is severely impaired in the lymphoma bearing mice. In search for improving donor T cell tolerance and thymic reconstitution we discovered that even DP TC considered as late thymocytes are able to efficiently home into host thymus and mature, migrate to periphery and mediate GVT activity as functional T cells. Based on these findings we proceeded to further improve thymus homing with focus on CD44 that since many

years has been suspected to at least facilitate thymus homing, whereas its impact on thymocyte maturation has not yet been explored in detail.

4.1 Lymphoma bearing mice show poor thymic reconstitution after allogeneic BMT

Studies on mice receiving allogeneic bone marrow transplantation and tumor application (renal cell carcinoma) have revealed that there is poor repopulation of the thymus over weeks after reconstitution as compared to tumor free mice. In this case myeloreductive conditioning was applied and bone marrow cells were depleted of T cells. However, after weeks of reconstitution the number of donor derived T cells increased in the thymus consistently. Tolerance induction was achieved after 6 wk , probably due to maturation of donor derived cells in the host thymus developing into host tolerant T cells [173]. In our set of experiments with T and B lymphoma bearing mice given myeloreductive conditioning, donor cells in allogeneically reconstituted mice did not develop any tolerance towards the host. Thymus was very poorly populated even after weeks. Few thymic immigrants that were recovered were mostly host derived. It is known that strengthening the myeloreductive conditioning improves mixed donor host chimerism and results in better tolerance induction towards the host [173]. In our experiments the situation remained the same even when we used myeloreductive conditioning rather than non-myeloablative conditioning except that donor derived cell expansion could be improved slightly after strengthening the conditioning regimen.

The question arose as to why immune reconstitution is strongly impaired in lymphoma bearing mice? One possible explanation could be a competition between T lymphoma and thymic progenitors to reside in the thymus. The reason behind this could be that lymphoma bearing mice might be bearing the same molecules for homing into thymus as T cell progenitors. One likely target is the homing receptor CD44. It has been shown that CD44 is expressed on lymphoma bearing cells. Functional relevance of this has been described as CD44 being involved in adhesion and migration of these cells on HA vasculature or ECM which is indeed important for

tumor dissemination [183]. Although standard form of CD44 is basically dominant in many tumors the variant isoform CD44v6 has also been proposed to be involved in migration and metastasis of tumor cells [102, 107]. A study by Krause et al, has shown the involvement of CD44 in homing of leukemic stem cells in a chronic myeloid leukemia model [184]. At the same time CD44 is also present on thymic progenitors and early thymocytes and has been shown to support thymocyte homing [44, 59]. Till date the factors responsible for interference of T or B lymphomas with T progenitor homing have not been identified to our knowledge.

4.2 Cytokines promoted donor and host T cell expansion but failed to mediate tolerance induction in allogeneically reconstituted mice

In our first trials to improve thymic reconstitution we used the cytokines IL-3 and IL-7. Studies with IL-7 application after bone marrow transplantation have shown enhanced thymic output as well has peripheral T cell counts. IL-7 also leads to strong expansion of newly formed naive T cells [60, 174, 185]. Our results showed increase in both host and donor derived hematopoietic progenitor cells. The anti- donor and anti-host activity remained high making it an unsuitable approach for patients. One possible reason could be an over load of allogeneic BMCs that are accompanied by a high number of T cells under going extrathymic maturation [173]. In the next step we strengthened the myeloreductive conditioning and observed an increase in donor derived cells homing into thymus. This can be due to increased donor T cell chimerism (more donor T cells in comparison to host) due to the conditioning regimen received. On the other hand this regimen was disadvantageous since mice developed high GVHD. A possible explanation could be that since host T cells decreased considerably in the stronger myeloreductive regimen this led to a sooner domination of donor T cells recognizing the host as foreign [175]. Hence we wanted to establish a protocol whereby thymus reconstitution can be achieved soon after transplantation to increase donor tolerance towards the host, along with retention of tumor activity.

4.3 Injection of double positive thymocytes improves thymus reconstitution

In our approach we decided to inject DP TC along with BMC depleted of T cells. Our hypothesis was that DP TC might mature in the host thymus and hence become tolerant towards the host as well as kill tumor cells with high efficiency [4]. When we injected DP TC along with BMC depleted of T cells we observed efficient thymus repopulation. The DP TC selectively homed into thymus. A high percentage of cells recovered in the thymus was donor derived. In addition the number of T cells in the periphery also increased indicating that DP TC matured in the thymus and emigrated into the periphery. DP TC have the highest expression of CCR9. [54] We could confirmed this in our experiments as well. CCR9 is one of the chemokines essential for thymocyte homing as well as retention of thymocytes in the thymus [43, 45]. We speculate that DP TCs due to their high CCR9 expression can still receive signals form the thymic epithelial cells that express its chemokine partner CCL25 and thus preferentially home into the thymus. It would be of interest to investigate further molecules expressed on DP that facilitate their homing into thymus.

4.4 DP injection induces tolerance induction and mediates GVT activity

Reconstituting mice with DP TC resulted in decreased reactivity towards the host even after re-stimulation *in vitro.* However, there was sufficient reactivity against tumor without an increase in GVHD. Our approach also provided room for vaccination. Studies in animal models and humans indicated that donor T cells are able to kill tumor cells recognizing tumor-specific antigen or alloantigens expressed on normal and malignant cells [186]. We observed that 49 % (myeloreductively) and 82% (myeloablatively) conditioned mice reconstituted with T cell depleted BMC plus DP TC rejected the SP6 tumor. Whereas in the situation without DP TC injection only 21% (myeloreductive conditioning) and 46% (myeloablative conditioning) of vaccinated mice rejected the SP6 tumor. It has been a matter of great concern since long on how to separate GVHD from GVT due to their shared biology. Various

approaches have been proposed in this regard such as allograft T cell depletion, modulation of T cell response, donor lymphocyte infusion, use of T_{regs} and so on. But till now none have shown optimal results that could be used as a standard method in allogeneic BMT [187]. It is also believed that increasing the stem cell dose would be a promising approach to improve immune reconstitution after transplantation, however there is always a limitation in getting high dose of stem cells and there also lies the risk of GVHD with remaining T cells in the allograft [188]. Hence DP TC could be exploited in this regard. At least this knowledge could be broadened to use thymic progenitors that would preferentially home into thymus and mature in the host, thus becoming host tolerant as well as mediating GVT effects.

4.5 Injection of thymic precursors could be an ideal approach for better immune reconstitution and response in allogeneic BMT

Recent evidence suggests many progenitors that preferentially home into the thymus. Most likely targets are the CLP2 (lin-c-kit^{lo} B220^{+}), early thymic progenitors (ETP-DN1) and LSKs (Lin^{-}Sca-1^{+}c-kit^{+}) [50-52]. These progenitors can be isolated and injected into the host during bone marrow transplantation. They would home into the thymus of the host and develop inside the host thymic microenvironment and be tolerant towards the host and active against the tumor. Another approach would be to induce T lineage potential by culturing hematopoietic stem cells on a widely accepted *in vitro* system using OP9-DLI cells proposed by Zuniga Pflucker [189]. Since they are T lineage committed progenitors, they would preferentially home into thymus and mature. An elegant study by Zakrzewski et al. has used hematopoietic stem cell precursors that were artificially committed towards the T cell lineage using OP9-DL1 system and injected into mice along with bone marrow cells with T cell depletion. They were able to show that these precursors enhanced T cell reconstitution in thymus and peripheral reconstitution after hematopoietic stem cell transplantation and developed into any other functional T cells with high ratios of CD4 and CD8. Mice had enhanced resistance to infection and high graft vs tumor activity without induction of GVHD [182].

The approach of administration of T cell precursors to enhance T cell reconstitution after allogeneic BMT can have a number of advantages. In the first instance its effect on engraftment would reduce the failure of graft rejection and relapse of the tumor. It would lead to better T cell functioning and thus reducing the risk to infections and morbidity. They would also create a room for vaccination much earlier after allogeneic BMT [182].

Taken together these set of data suggest DP TC and clinically more relevant early thymic progenitors can reconstitute the thymus efficiently after allogeneic BMT. They can also create a room for vaccination and thus a better anti-tumor response, by overcoming to some extent the prevailing problems of GVHD and graft rejection. Allogeneic bone marrow transplantation would provide a better therapy to patients of all ages suffering from tumors since it relies on establishment of a competent immune system.

As mentioned before identifying thymic precursors that have high expression of molecules promoting their homing into thymus would add on to a better treatment in allogeneic BMT. In this regard, it is essential to identify molecules that support thymocyte homing and maturation. One of the homing molecules expressed in early thymocytes is CD44 [59]. So we explored the role of CD44 in homing, migration, proliferation and apoptosis which will be discussed further on.

4.6 CD44s and variant isoforms expression on thymocytes and thymic progenitors

PanCD44 (all isoforms including standard) expression was highest in Balb/c thymus as compared to SVEV, SVEV-CD44v6/7 knockout (KO) and SVEV-CD44v7 KO mice. Expression of CD44v6 and v7 remained the same in almost all strains. Expression of CD44 in individual thymocyte subsets revealed that its expression is high in almost all thymic subsets as well as CLP2 T cell progenitor. The intensity was lower in the DP population though. Expression of CD44v6 was highest in DN population and quite high in SP population, with decreased expression in DP TC. Data by Schwarzler et

al. in 2001 slightly contradicts to ours since they show that CD44 variant isoforms are not expressed after DN3 stage, but very low levels of CD44s remain, since we still see expression of v6 in DP stage as well as less amounts of v7.

4.7 CD44 promotes homing of progenitors into bone marrow and thymus

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 [190]. CD44 has also been shown to mediate adhesive interactions of stem cells with bone marrow vasculature [191]. 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 [192]. CD44v7 on bone marrow stromal cells contributes to progenitor settlement [167]. We could confirm the same *in vivo* using a syngeneic system. The decreased BMC settlement in $v7^{-/-}$ bone marrow led to increased settlement in spleen indicating that settlement in spleen doesn't require CD44v7. In addition we were also able to show the individual involvement of CLP1 and lin^{+} progenitors into bone marrow that was hampered using anti-CD44. As far as homing is concerned we and others were not able to show a role for CD44 variant isofoms in homing [167, 192, 193].

CD44 has also been described to be involved in thymus homing [179, 194]. We also observed that long term anti-CD44 treatment influences thymus homing. No involvement of variant isoforms was observed in thymus homing. We used thymic progenitors to check for their homing capacity. CLP2 cells home with high efficiency into thymus. Martin et al. have shown that CLP2 have a preference to home into thymus and develop into thymocytes and functional T cells [195]. We were able to show in addition that DN1 home with equal efficiency as CLP2. Anti-pan-CD44 treatment almost abrogates homing of these two populations. A possible explanation could be that IM7 that does not bind to the hyaluron binding site of CD44 interferes with CD44-integrin/ICAM/selectin since we saw a high co-expression of these

molecules with CD44. There was no evidence that CD44v6/v7 on thymus stroma influences homing. We only saw that recovery of TC in SVEV mice receiving BM from CD44v6/7 KO mice remained impaired. It is an indication for involvement of CD44v6 in repopulating the thymus and intrathymic maturation. This led us to check for the role of CD44s and isoform v6 in thymocyte maturation. Studies by Schwarzler et al., (2001) revealed that CD44v are necessary for thymocyte development till they acquire DP state and hence they might play a role in establishing initial interaction between thymocytes and stroma but not critical for immigration and transmigration step of progenitors into thymus [196]. So far we did not succeed in finding the ligands for CD44s and variants in the thymus. Studies are underway. But it has to be considered that such selective ligands do not exist. However, it may be possible that CD44 is associated with CD49d or other adhesion molecules as shown for activated T cells [154]. Another likely target is CD62L, since studies on BM adhesion to stromal CD44 have shown the involvement of CD62E in mediating these interactions [197].

4.8 CD44 promotes expansion of early thymocytes

Role of CD44 in promoting thymocyte expansion was supported by the finding that CD44 when cross-linked with sub-threshold level of CD3 provides a stronger proliferation stimulus as compared to CD3 alone, in the case of DP, CD4 and CD8 SP. So we propose that CD44 acts as a co-stimulatory molecule in these subsets of thymocytes just as described for peripheral T cells [150]. CD44 acting like CD28 as a co-stimulatory molecule for TCR mediated signaling is known already [150, 198].

Since proliferative signals of these thymocyte subsets were mediated through the pre-TCR we looked for the phosphorylation events of tyrosine kinases that get recruited towards the TCR upon activation [199]. We observed Lck and Zap-70 phosphorylation mediated by panCD44/v6 plus CD3 cross- linking as well as panCD44/v6 cross-linking alone. This phenomenon differs from that of peripheral T cells where association of CD44 with Lck and in turn Lck co-localisation with the TCR makes a way for recruitment and phosphorylation of several other kinases necessary for T cell activation [150, 151].

The studies of signaling mechanisms involved in the proliferative effect revealed the involvement of the MAPK pathway. The MEK/ERK pathway is central for T cell receptor signaling with respect to differentiation, proliferation and survival since it integrates cytoplasmic signals to mediate these events [99]. ERK1/2 activation has been reported in thymocytes to be involved in positive and negative selection. Reports show that weak ERK phosphorylation promotes positive selection whereas stronger activation of ERK promotes negative selection [200]. Some reports suggest that sustained ERK phosphorylation leads to positive selection [201]. With our set of experiments we cannot speculate the possibilities in this direction. However, we could show that DP and SP thymocyte subsets needed CD3 plus panCD44 cross liking and not panCD44 cross linking alone for MAPK signaling. This shows that early activation events downstream of CD44 might be uncoupled from Ras pathway in DP and SP cells. Uncoupling Ras activation form early activation events at the TCR complex such as Lck and Zap-70 phosphorylation has been described in the case of anergic CD4 T cells and several candidate molecules may play a likely role in mediating uncoupling such as early growth response genes (Egr), ubiquitin ligases CBL-B, GRAIL and ITCH [202]. CD44 is known to associate with Lck [151] and in turn Lck mediates activation of Ras/MAPK pathway in thymocytes [203]. This could be another possible explanation towards the results we observed.

Assessment of DN thymocytes provided differing results. Anti-panCD44 cross-linking alone was enough to provide a proliferative stimulus in the DN1 and DN2 subsets and this was mediated via MAPK pathway. Anti-panCD44 binds to all isoforms of CD44 including the standard form. We cross-linked anti-CD44v6 in addition to anti-panCD44 since we observed delayed repopulation in the CD44v6/7KO mice and also the proliferation in these mice was less as compared to wild type SVEV mice. We observed that proliferation of DN thymocytes was induced by the CD44v6 specific antibody. So we speculate that it is indeed CD44v6 that mediates these effects in early thymocytes. The proliferative response due to CD44 could be due to an interaction with an unknown ligand in the thymus. Our data are consistent with those of Graham et al., where they show CD44+ bone marrow chimeras as compared to CD44- have a higher capacity and preference to mature in thymus and develop into

functional T cells [194]. A role of CD44 in expansion of hematopoietic progenitor cells has been described before [192]. Transgenic mice bearing rat CD44v4-v7 have shown accelerated thymus repopulation [169]. In a CD44v6 over expressing thymoma line EL4 it was also shown that CD44v6 promotes proliferation via ERK1/2 [157]. This finding might extend to DN TC. Our findings clearly depict the role of CD44v6 in DN thymocytes expansion and accessory function of CD44 with respect to DP, CD4 and CD8 SP thymocytes.

4.9 CD44 contributes to apoptosis protection and cell survival in early thymocytes

Since we saw high proliferation of thymocytes in response to CD44s/v6 we were keen to look whether CD44 also plays a role in survival pathways of thymocytes. Indeed, panCD44/v6 protected thymocytes from apoptosis when cross-linked alone. This effect was absent in CD44v6/7 KO mice, indicating an involvement of CD44v6 in apoptosis protection. Our results are well in line with those by Marhaba et al. in which EL4 thymoma line transfected with CD44v6 show apoptosis resistance mediated through CD44v6 cross linking [157]. The same was observed in separated TC subpopulations of DN and DP TC when they were cross-linked with anti-panCD44/v6 alone. The SP TC on the contrary were driven into apoptosis when cross-linked with CD44.

Exploring the signaling pathway responsible for the increased cell survival in thymocytes revealed Akt phosphorylation and Bcl-2 upregulation after cross-linking with CD44v6 and this pathway was not activated in the CD44v6/7$^{-/-}$ KO mice. The involvement of PI3K/Akt pathway was confirmed using the PI3K inhibitor Ly49002. This could be a possible explanation for survival of DN TCs and expansion. Thymocyte development requires integration from several signaling events through pre-TCR, Notch, IL-7 for a programmed lineage commitment from DN and DP stage. The PI3K/Akt signaling acts at this point to translate these signals towards a functional outcome such as survival, proliferation, differentiation and allelic exclusion at the β-selection check point, where only thymocytes with a fully functional pre-TCR (TCR β) are allowed to cross the DN3 stage to develop into DN4 and further DP TCs

[70]. Thymocytes with deletions of components of the PI3K/Akt pathway as in PI3K (p110γ subunit), PDK1 and Akt isoforms have an arrest at the DN3 stage and very low double positive thymocytes in consequence [72, 204]. In SP thymocytes CD44 cross-linking alone promoted apoptosis of thymocytes. In addition, panCD44 cross-linked with CD3 led to apoptosis induction which is consistent with data on T helper cell line [150].

Taken together our data suggest that delayed thymus reconstitution with CD44v6/7 KO T progenitors is a consequence of early TC requirement of CD44v6 for expansion through MAPK pathway as well as survival of early thymocytes mediated via PI3K/Akt survival pathway and Bcl-2 upregulation that has an anti-apoptotic effect. In addition we were able to show for the first time that CD44 is involved in migration and homing of the T cell progenitors CLP2 and DN1. In later stages of thymocyte maturation the effect of CD44v6 vanishes and panCD44 acts as an accessory molecule similar to peripheral T cells in concert with CD3.

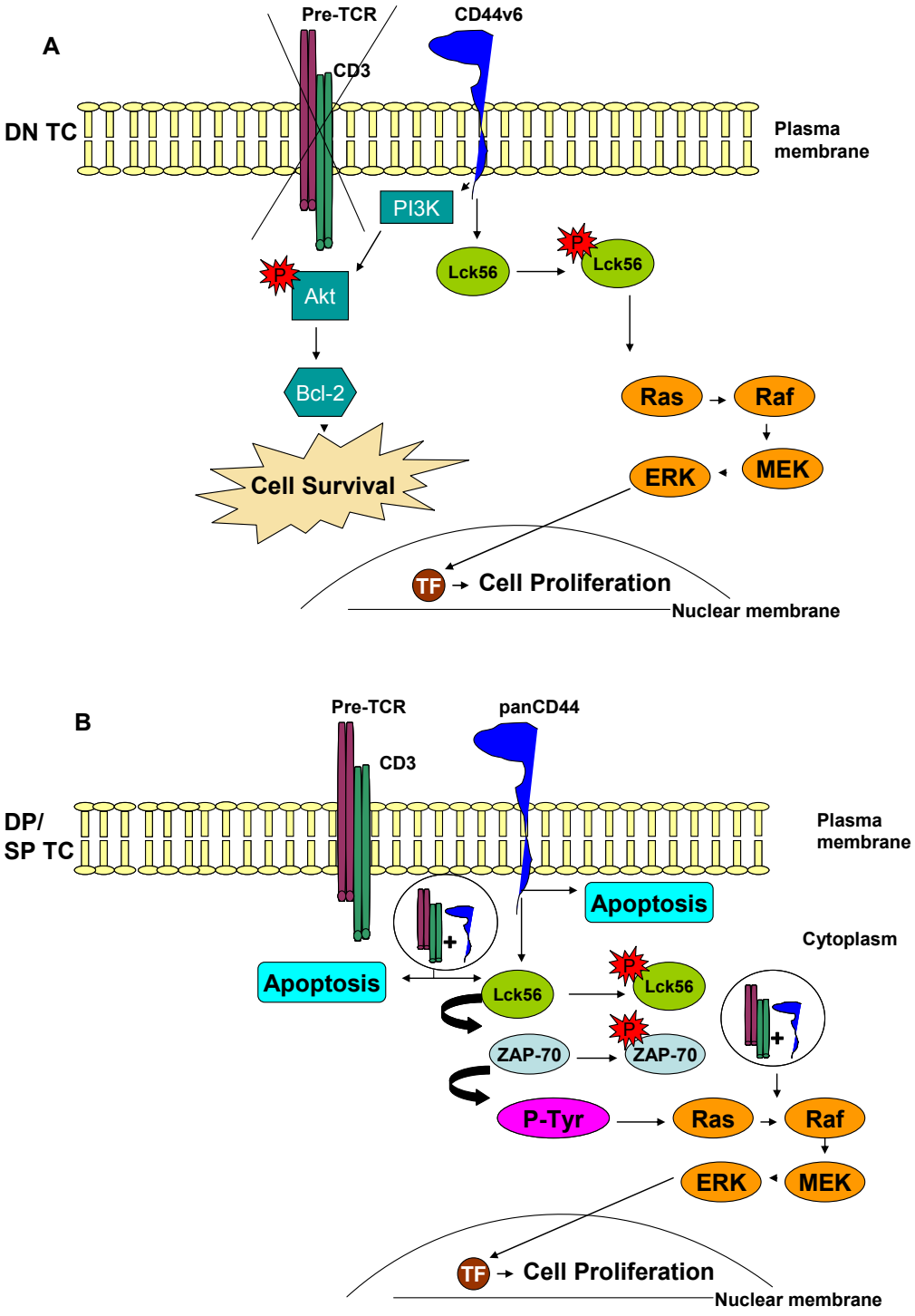
4.10 Future perspectives

Before exploring the therapeutic setup for improving thymus reconstitution in allogeneic bone marrow transplantation it would be worthwhile to investigate the mechanisms by which thymic progenitors home and mature in the thymus. Achieving host thymocyte reconstitution after bone marrow transplantation and retaining graft vs tumor activity without induction of GVHD would make this approach ideal for treatment of tumors and enhance survival rates of patients with hematological malignancies and solid tumors.

Based on the observation that CD44s is required in progenitor T cell homing, defining the ligand on thymic stroma that provides a microenvironment for the differentiation of thymocytes would be important for clinical translation. Application of immunohistochemistry on thymic slices with recombinant soluble molecules of CD44s and variants generated in our lab would be a good tool to identify potential ligands such as a special form of hyaluronic acid, chemokines (CCR9, CXCL12) or

cytrokines (IL-7). Of equal importance will be a detailed analysis on the mechanisms whereby CD44v6 facilitates thymocyte maturation. Ongoing studies involving co-immunoprecipitations of CD44v6 reveal the association of Lck (Src kinase). These studies have to be completed by checking for other potential partners interacting with CD44 such as CD49d, ICAM and CD62L. Should the hypothesis be correct that CD44 associates with CD49d or ICAM this would answer the question as to how CD44 mediates signaling in thymocytes by availing the associating molecules of one another such as Src kinases associated with CD44 and FAK associated with CD49d. In addition, it is also important to study the role of CD44 on the thymus stroma for thymocyte development and differentiation. The impact of CD44 on thymus homing, T cell maturation as well as on the thymic stroma can be therapeutically exploited to accelerate re-establishment of a competent immune system after allogeneic bone marrow transplantation.

Model for role of CD44 in thymocyte maturation



A. In DN TC CD44v6 alone mediates signaling events of Lck phosphorylation followed by activation of MAPK pathway leading to cell proliferation. On the other hand CD44v6 cross-linking mediates activation of PI3K/Akt survival pathway and Bcl-2 upregulation, leading to cell survival. B. In DP and SP TC panCD44 can phosphotylate Lck and Zap-70 partly independent of CD3, but acts as a co-stimulatory molecule in conert with CD3 to medate cell proliferation. CD44 alone leads to apoptosis in SP TC and concomitant crosslinking with CD3 can also lead to apoptosis in DP and SP TC.

5.1 Abstract

Active vaccination in the allogeneically reconstituted tumor bearing host essentially requires donor T cell tolerance. To create a basis for vaccination in the allogeneically reconstituted, lymphoma-bearing host, we elaborated a reconstitution protocol that supports thymus repopulation and tolerance induction. Myeloreductively conditioned, lymphoma-bearing mice were vaccinated after reconstitution with hematopoietic progenitor cells. In tumor-free mice, myeloreductive conditioning, together with NK cell depletion of the host and the transfer of T cell-depleted bone marrow cells, allows reconstitution without severe GVHD. However, in hematological malignancies, donor-derived T progenitor cells hardly immigrated into the thymus. As a consequence, the frequency of severe GVHD was significantly increased, which prohibited active vaccination. Thymus repopulation became improved by strengthening the myeloreductive conditioning, by supporting thymocyte expansion via IL-7, and most strongly, by a small dose of donor-derived $CD4^+CD8^+$ thymocytes, which preferentially homed into the thymus. Active vaccination in combination with this reconstitution protocol did not strengthen GVHD, but significantly improved survival time and survival rate of lymphoma-bearing mice.

The negative impact of hematological malignancies on thymus repopulation and central tolerance induction can, at least partly, be corrected by the application of a small number of donor-derived T progenitor cells.

Based on these findings we proceeded to further improve thymus homing with focus on CD44 that since many years has been suspected to at least facilitate thymus homing, whereas its impact on thymocyte maturation has not yet been explored in detail. CD44 is known as a T progenitor marker. Antibody blocking studies and cells/mice with a targeted deletion of CD44v6/7 or CD44v7 revealed that CD44 has a major impact on progenitor cell homing into marrow and thymus. Marrow homing additionally is supported by stromal cell CD44v7, but thymus homing is CD44v6-/CD44v7-independent. However, CD44v6 strengthens thymocyte expansion and apoptosis resistance. CD44v6-induced apoptosis resistance, most strongly in double negative (DN) cells, is accompanied by Akt activation. CD44v6-induced proliferation

of DN cells proceeds via the MAPK pathway. Instead, CD44-promoted proliferation of double-positive (DP) and single-positive (SP) thymocytes relies on CD44 supporting TCR/CD3 complex-initiated signal transduction.

Thus, CD44 plays a major role not only in progenitor cell homing into bone marrow, but also in thymus homing. In addition, CD44v6 promotes survival and expansion of early thymocytes. Thus, strengthening CD44v6 expression on thymic progenitors could well contribute to accelerated regain of immunocompetence.

5.2 Zusammenfassung

Allogene Knochenmarktransplantation nach nicht-myeloablativer Konditionierung wird in der Klinik in zunehmendem Maße als letzte Möglichkeit einer kurativen Therapie bei Tumorpatienten eingesetzt. Man weiß inzwischen, dass diese Therapieoption durch eine aktive Vakzinierung deutlich verbessert werden kann. Eine aktive Vakzinierung erfordert jedoch, dass die im Wirt gereiften Doner T-Zellen gegenüber dem Wirt tolerant sind. Hierzu müssen sie im Wirtsthymus reifen. Da bei älteren Menschen nur noch ein Thymusrudiment vorliegt, konnte dieses Problem in der klinik bisher nicht hinreichend gelöst werden. Daher habe ich im Rahmen meiner Dissertation versucht im Tiermodell Möglichkeiten aufzuzeigen, die die T-Zell-rekonstitution nach allogener Knochenmarktransplantation unterstützen.

Erste Untersuchungen zeigten auf, dass nach myeloreduktiver Konditionierung des allogenen Wirts eine verlässliche Rekonstitution mit T-Zell-depletierten und NK-Zell-depletierten Knochenmarkzellen ohne schwerwiegende *Graft versus Host* (GvH) Erkrankung erzielt werden kann. Dies war jedoch im tumortragenden Tier nicht der Fall. Progenitor T-Zellen wanderten nicht in den Thymus und ein erheblicher Prozentsatz der Tiere entwickelte schwere GvH Störungen. Die Repopulation des Thymus konnte durch stringentere Myeloreduktion, Applikation von IL-7 und insbesondere durch die Applikation von unreifen $CD4^+$ $CD8^+$ T-Zellen verbessert werden. Die $CD4^+$ $CD8^+$ Vorläufer-T-Zellen wanderten präferentiell in den Thymus. Nach der Reifung tolerierten die tumortragenden Tiere eine Vakzinierung, welche die Überlebenszeit und Überlebensrate der Tiere signifikant verbesserte.

Basierend auf diesen Ergebnissen und früheren Befunden, die eine Beteiligung von CD44 am Prozess der Immigration von Progenitor-T-zellen in den Thymus nahe-legten, untersuchte ich, inwieweit CD44 am *homing* der Progenitor-T-Zellen und an deren Reifung beteiligt ist. Untersuchungen mit blockierenden Antikorper, sowie mit Mäusen mit einer targetierten Deletion von CD44v6/v7 oder CD44v7 belegten, dass die CD44 Standardform maßgeblich am Einwandern hämatopoetischer Progenitorzellen in das Knochenmark und den Thymus beteiligt ist. Das Einwandern in das Knochenmark wird darüber hinaus durch CD44v7 auf Stromazellen begünstigt.

Hingegen ist die Wanderung in den Thymus CD44v6/CD44v7 unabhängig. Allerdings verstärkt CD44v6 die Expansion und Apoptoseresistenz von insbesondere frühen, sogenannten doppel-negativen Thmyozyten.

Die gesteigerte Apoptoseresistenz geht mit einer Aktivierung von Akt einher. Für die CD44v6-induzierte Proliferation doppel-negativer Thymozyten ist die Aktivierung der MAPK Signalkaskade verantwortlich. Dies gilt nicht für Thymozyten in einem späteren Reifestadium, in dem CD44 nur noch als akzessorisches Molekül die Aktivierung über den T-Zell-Rezeptor unterstützt.

Diese Befunde belegen, dass CD44 nicht nur das Einwandern von Progenitor-T-Zellen in den Thymus fördert, sondern darüber hinaus CD44v6 die frühen Stadien der Thymozytenreifung beschleunigt und somit eine wesentliche Rolle bei der Rekonstitution und Toleranzinduktion nach allogener Knochenmarkrekonstitution einnimmt.

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