Function of β1 and β7 integrins in the hematopoietic system

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List of abbreviations

bp, base pairs
BM, bone marrow
BSA, bovine serum albumin
cDNA, complementary DNA
DTT, dithiothreitol
ECM, extracellular matrix
EDTA, ethylenediaminetetraacetic acid
ERK, extracellular signal–regulated kinase
ES cell, embryonic stem cell
FA, focal adhesion site(s)
F-actin, filamentous actin
FAK, focal adhesion kinase
FBS, fetal bovine serum
FITC, fluorescein isothiocyanate,
g, gram
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
GM-CSF, granulocyte/macrophage colony-stimulating factor
GSK-3β, glycogen synthase kinase-3β
h, hour
HRP, horseradish peroxidase
ILK, integrin-linked kinase
kb, kilobase(s)
LN, lymph node
M, molar
M-CSF, macrophage colony-stimulating factor
min, minute
PAGE, polyacrylamide gel electrophoresis
PALS, periarteriolar lymphoid sheath
PAS/AGM, para-aortic splanchnopleura/aorta-gonad mesonephros region
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PI3K, phosphatidylinositol-3 kinase

PINCH, particularly interesting new cysteine-histidine rich protein

PIP_3, phosphatidylinositol 3,4,5-triphosphate

PP, Peyer's patches

RT-PCR, reverse transciption-polymerase chain reaction

s, seconds(s)

SDS, sodium dodecyl sulfate

Tris, tris(hydroxymethyl)aminomethane

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1. Introduction

1.1 Integrins

Cell-cell and cell-extracellular matrix (ECM) contacts are mediated by cell adhesion molecules (CAMs). These molecules are located on the surface of cells and belong to different families such as integrins, cadherins and transmembrane proteoglycans. Integrins are transmembrane proteins consisting of an α- and a β-subunit. To date, 18 α- and 8 β-subunits have been found which can heterodimerize in different combinations to form, as known so far, 24 different integrins with distinct ligand binding and signalling properties (Fig.1). The β1 integrin subunit can associate with 12 different α-subunits, thus forming the largest integrin subfamily (Hynes, 1992, 2002).

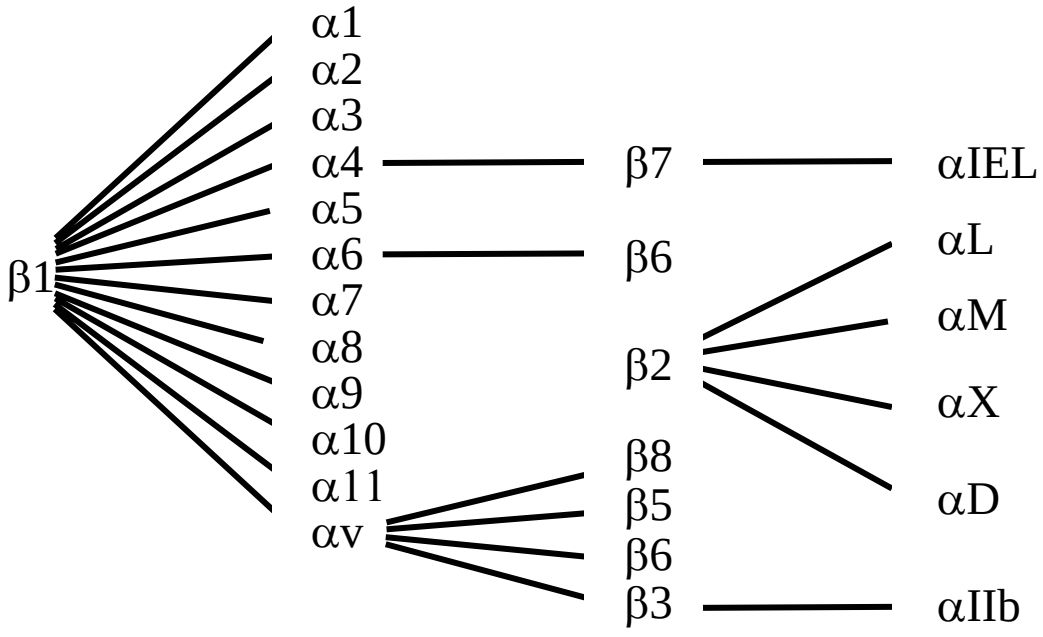


Figure 1: The integrin superfamily

Eight β integrin subunits associate with 18 α subunits in a noncovalent fashion giving rise to 24 heterodimeric cell surface receptors that can bind to ECM proteins and other cell surface molecules.

With the extracellular domain integrins of the β1 subfamily bind to different ECM proteins like collagens (α1β1, α2β1, α10β1), laminins (α3β1, α6β1, α7β1), vitronectin (αvβ1) and fibronectin (FN) (α3β1, α4β1, α5β1, α8β1, αvβ1) and to cell surface receptors like VCAM-1 (α4β1) (Fig. 2). Integrins α4β1 and α4β7 bind to FN, VCAM-1 and MAdCAM-1. The respective affinities, however, differ. Whereas α4β1 binds 30-45 fold stronger to VCAM-1 than to MAdCAM-1, α4β7 favours interaction with MAdCAM-1 by 3-15 folds over VCAM-1 (Day et al., 2002).

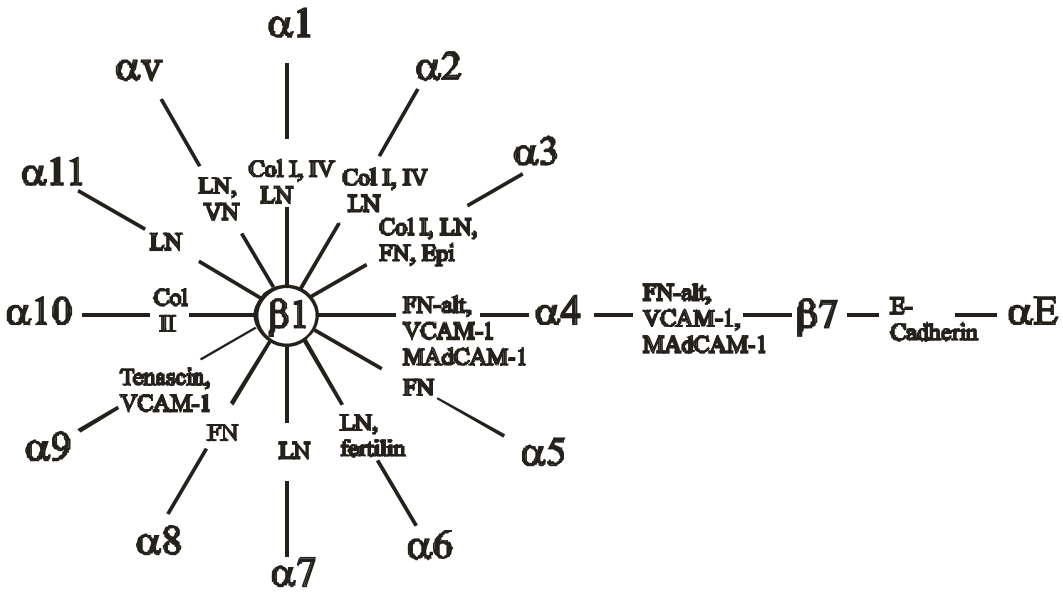


Figure 2: β1 and β7 integrins and their ligands

Integrin β1 and β7 subunits can heterodimerize as depicted by the lines recognizing the listed ligands, respectively. Col = collagen; FN = fibronectin; FN-alt = fibronectin alternatively spliced region; LN = laminin; VN = vitronectin.

While β1 integrin is ubiquitously expressed, other integrin subunits are predominantly found in certain tissues or cell populations. Integrin β7, for example, is exclusively found on white blood cells (Wagner et al., 1996) whereas β2 integrins are specifically expressed on leukocytes and associates with αL (lymphocyte function associated antigen-1 (LFA-1), αM (Mac-1), αX and αD subunits. Important ligands of β2 integrins include members of the ICAM-family. In addition, Mac-1 binds also to inactivated complement factor (iC3b), fibronectin and factor-X, αXβ2 to iC3b and type I collagen and αDβ2 to VCAM-1 (Plow et

al., 2000). The αv integrins which associate with β1, β3, β5, β6 and β8 bind to fibronectin and to vitronectin, except for αvβ8 which binds to fibronectin, vitronectin and fibrin (Chernuosov et al., 2003), and αvβ6 which additionally binds to tenascin-C and the latent form of TGF-β called the small latent complex. This procytokine is composed of the C-terminal TGF-β noncovalently linked to the latency-associated peptide (LAP) and inactive. Binding of αvβ6 to LAP leads to TGF-β activation which in turn modulates inflammatory cell function, growth inhibition and differentiation.

Integrin α subunits share a 25% amino acid identity. Some α-subunits, but not α4 integrin, contain an "Inserted" or "I-domain" or "von Willebrand factor A domain". I domains contain the major ligand binding sites and the metal ion-dependent adhesion site (MIDAS) which binds divalent cations that are important for integrin function.

There is an overall 37-45% amino acid identity between the β subunits with highest homology in the cytoplasmic and transmembrane domains. At the N-terminus, a cysteine-rich region shows sequence homology with membrane proteins including plexins and semaphorins. This region is therefore called the PSI domain for "plexins, semaphorins, and integrins". These cysteine-rich regions cooperate to restrain the integrin to the inactive conformation. Another evolutionarily conserved extracellular domain of β chains has about 240 residues, spanning from residue 100 to 340. Within this domain lays a putative metal-binding DXSXS sequence motif similar to that of the MIDAS in the I domain, which is therefore called the I-like domain. This region is involved in ligand binding and mediates association with the α subunit (Bajt et al., 1994, Wardlaw et al., 1990, Michishita et al., 1993, Ueda et al., 1994 and Rieu et al., 1994). The gene pactolus, probably derived from a duplication of β2 integrin (Garrison S. et al., 2001), demonstrates the importance of this motif. This gene contains a point mutation in its DXSXS region and, as a result, pactolus does not associate with an α subunit. Therefore pactolus is not considered as an integrin. While it is believed that integrins are expressed only as heterodimers on the cell surface (Whittaker and Hynes 2002), there are two reports that suggest that α4 in a B cell lymphoma cell line (Crowe et al., 1994) and α4 and α6 in a T cell lymphoma cell line

(Stroeken et al 1998) were present on the cell surface in the absence of any β subunit. No function could be addressed to these α chains on the cell surface.

The overall shape of the extracellular integrin dimer is a globular, ligand binding headpiece composed of both subunits. Two long stalk regions containing C-terminal cytoplasmic domains connect the headpiece to the transmembrane region of the α- and β-subunits, respectively (Fig. 3; reviewed by Shimaoka, Takagi, and Springer 2002)**.** Recent studies revealed that integrins can occur in an open and in a closed conformation and it is believed that these two conformations correspond to the ligand-bound and unbound form, respectively. In it unbound form, integrins adopt a bent conformation (Fig. 3, left panel). Ligand binding or conformational changes of the cytoplasmic domains lead to straightening and separation of the extracellular legs, allowing high affinity interaction with ligands (right). The open form allows ligand binding on the outside and the association of intracellular proteins inside the cell (reviewed by Hynes 2002). Integrins not containing an I domain, such as α4 and αv integrins, adopt similar conformations as shown in the crystal structure for αvβ3 (Xiong et al., 2001).

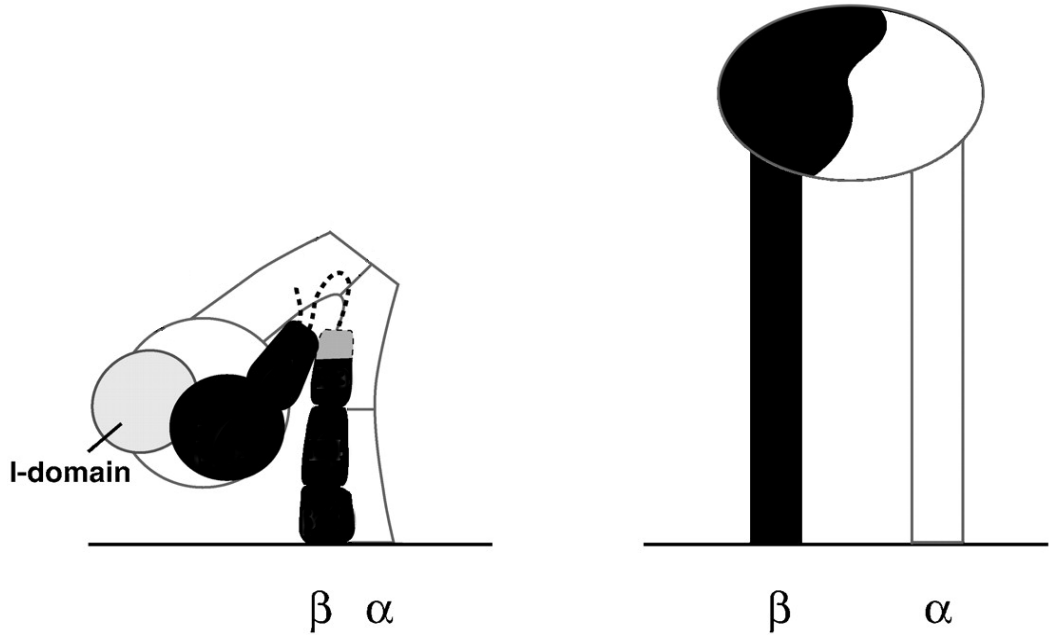


Figure 3: Arrangement of extracellular domains in the closed (left) and open (right) conformation according to αvβ3 crystal structure, with an I domain added (modified from Shimaoka et al., 2002)

Integrins in the inactive state occur in a bent (closed) conformation (left), while ligand binding or the association of intracellular molecules, mostly to the β chain, unfolds the receptor to the open conformation, which is upright with separated legs (right).

Different integrin heterodimers bind different extracellular ligands and induce different signalling pathways. However, since integrins also crosstalk with each other such that signalling by one integrin influences the affinity, avidity or signalling of other integrin receptors, integrin functions are dependent on the specific integrin composition of a cell.

Integrins are connected via linker molecules to the actin cytoskeleton inside the cell mainly via the β subunit. Thus by connecting the cytoskeleton to the ECM, integrins promote shape changes and migration of cells. They furthermore contribute to the mechanical stability of tissues. In addition to this structural role, integrins are also capable of transducing signals across the cell membrane in either direction. The binding of the extracellular ligands elicit signals inside the cell (outside-in signalling), which can promote migration, induce cell cycle progression, and prevent apoptosis. Since integrins, with the exception of β4, have only short intracellular domains without enzymatic activities, these signalling processes are initiated by the ligand induced association of the intracellular domain with signalling and adapter molecules. Ligand binding to integrins stimulates different intracellular signalling pathways, such as the activation of MAP kinases (Erk, JNK, p38), Rho GTPases, phosphoinositide 3-OH kinase (PI3-K) and calcium influx, which trigger changes in protein activities or gene expression (for review see Brakebusch and Fässler). Talin links the cytoplasmic tail of β integrins to the cytoskeleton and serves, in addition, as a platform for other scaffolding and signalling molecules as it binds phosphatidyinositol phosphate kinase type Iγ (PIPKIγ) (de Pereda et al., 2005), Phosphatidylinositol (4,5) bisphosphate (PIP2) (Ling et al., 2002), vinculin (Fillingham 2005) and focal adhesion kinase (FAK) (Chen et al., 1995) a non-receptor tyrosine kinase. Activation of FAK, via src or indirectly via integrin clustering plays a central role in integrin signalling as it promotes cell movement by activation of PI3-K and Rac1 and contributes to focal adhesion formation in a positive feedback mechanism (Fig.4).

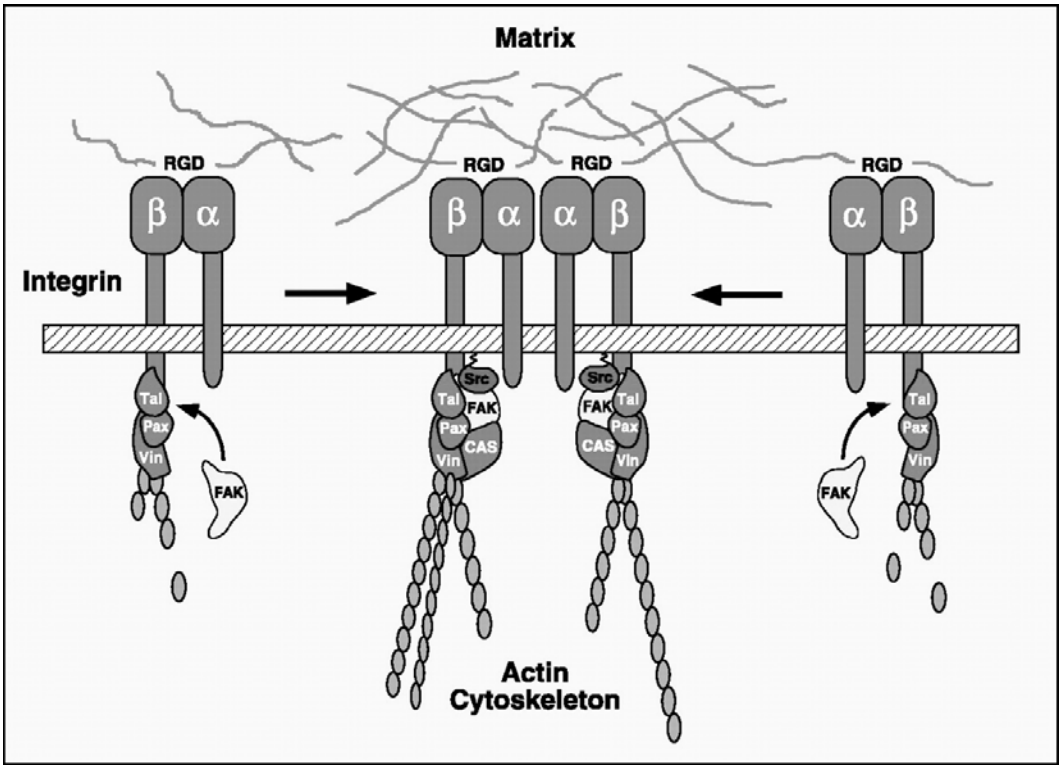


Figure 4: Formation of focal contacts involves extracellular and intracellular proteins
Matrix binding promotes integrin clustering, association with the cytoskeleton and activation of the focal adhesion kinase (FAK). This in turn promotes further integrin clustering and matrix organisation in a positive feedback mechanism. (RGD, Arg-Gly-Asp integrin binding motif; Tal, talin; Pax, paxillin; Vin, vinculin; CAS, $p130^{CAS}$; Giancotti and Ruoslahti 1999).

Integrin signalling can promote survival by activation of PI-3K. This activation inhibits apoptosis by activation of Akt which then blocks several cell death pathways (Faraldo et al., 2001). Activation of Erk was also described to prevent apoptosis, however, therefore the additional activation of Rac1 mediated via FAK might be necessary (Cho and Klemke 2000). In the absence of growth factors integrin mediated activation of JNK promoted cell survival of fibroblasts in a FAK-dependent, but Akt- and Erk-independent manner, while in the presence of growth factors this survival signal was mediated via PI3-K and Akt (Almeida et al., 2000).
Also the integrin-linked kinase (ILK) was reported to contribute to cell survival by phosphorylation of Akt (Persad et al., 2001) (Fig.5).

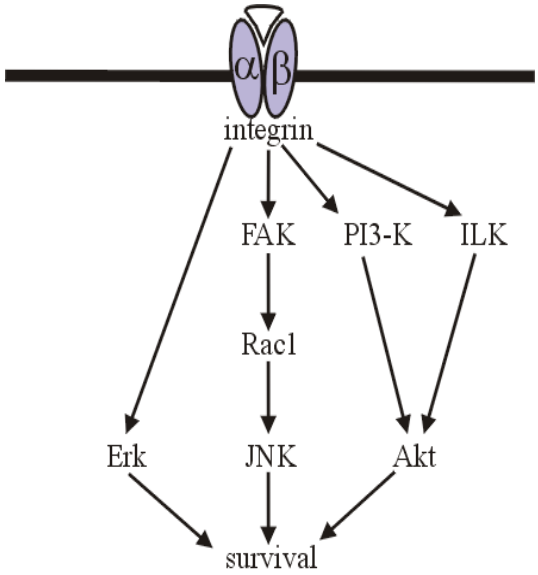


Figure 5: integrin signalling can promote survival

Integrins mediate activation of FAK, PI3-K and ILK and thereby contribute to cell survival and promote progression of the cell cycle.

ILK was described to directly bind to β1 and β3 integrin chains (Hannigan et al., 1996). Although ILK was shown in over-expression studies to induce phosphorylation of GSK-3β and Akt (Delcommenne et al., 1998) it is not clear whether this kinase activity is necessary for its biological function since ILK-deficient fibroblasts and chondrocytes did not show altered phosphorylation levels of GSK-3β and Akt (Sakai et al., 2003; Grashoff et al., 2003). It therefore seems likely that ILK exerts its function mainly by linking integrins to actin filaments and to signalling molecules such as paxillin, which was shown to bind α4 integrin directly (Liu et al., 1999), and PINCH (Fig. 6) (reviewed by Brakebusch and Fässler, 2003). It might also allow crosstalk between integrins and growth factor receptors by binding to receptor tyrosine kinases via PINCH and Nck-2.

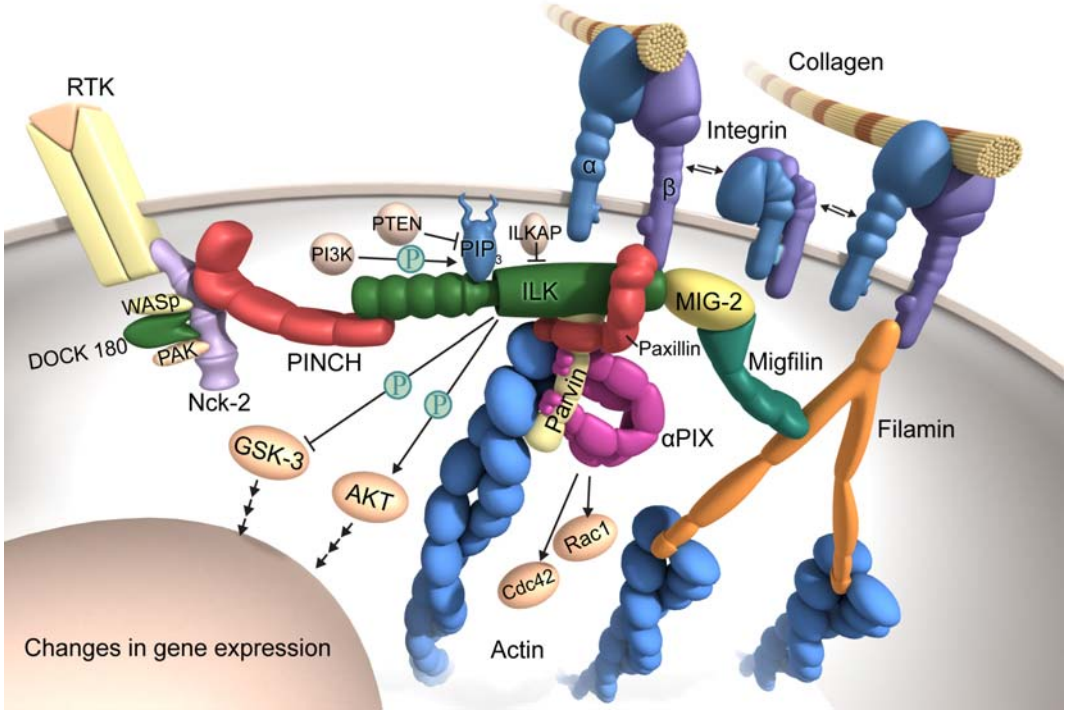


Figure 6: ILK provides a link between integrins and the actin cytoskeleton
Beta integrins bind ILK which, upon extracellular ligand binding, recruits several adaptor proteins that modulate actin dynamics and actin attachment to the integrin adhesion site.

FAK activation can promote cell cycle progression. Since FAK can be activated by integrins and by growth factor receptors, it is an important integration point of integrin and growth factor receptor signalling. Another integration point between these signalling pathways is that of the Rho-GTPases which can be activated by integrins and growths factor receptors by stimulation of guanine nucleotide exchange factors (GEFs) such as vav (Fig.7). Rho GTPases are crucial for the organization of the cytoskeleton, thereby influencing adhesion and migration (reviewed by Bishop et al., 2000). In addition, Rho GTPases are also regulating cell polarity, secretion, endocytosis, proliferation, survival and cell-cell contacts.

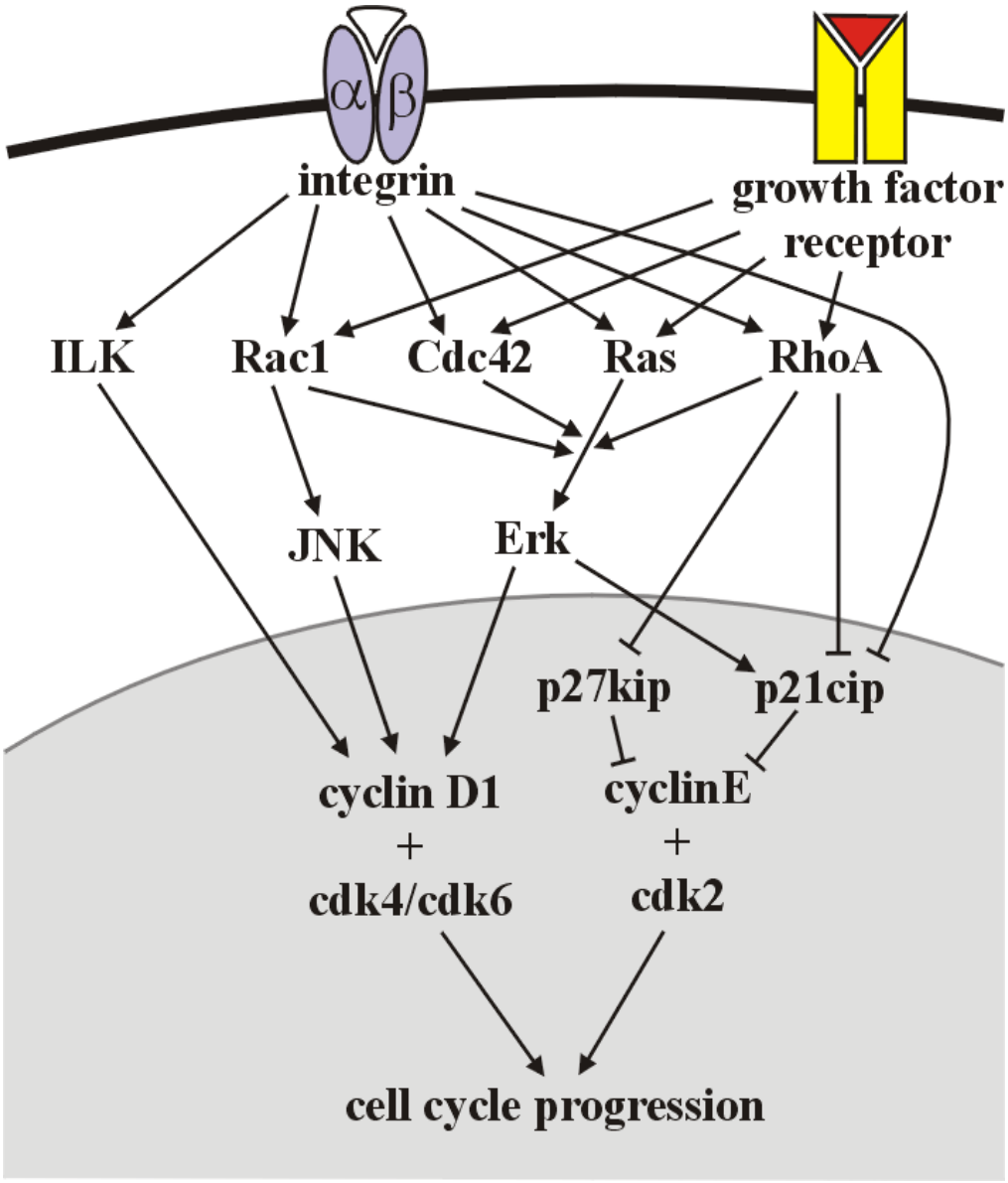


Figure 7: Crosstalk between integrin signalling and growth factor receptor signalling via Rho-GTPases.

Integrins and growth factor receptors can activate ERK and JNK via Rho-GTPases and thus modulate proliferation and survival of cells.

A remarkable feature of integrins in comparison to other adhesion molecules is that their ability to bind ligands is regulated by intracellular signalling. Intracellular processes can regulate the extracellular binding activity of integrins (inside-out signalling). The binding of talin to the cytoplasmic tail of inactive (closed conformation) β subunit, for example, leads to a separation of the cytoplasmic domains of the α- and β-chain and is accompanied by conformational

changes in them. This in turn straightens the extracellular stalk regions and opens the conformation of the integrin allowing ligand binding at the extracellular side.
Also chemokine signalling leads to a quick upregulation of integrin affinity, which is essential for the extravasation of blood cells into inflammed tissues. In addition, changes in the cytoskeleton, which lead to the clustering of the associated integrins, enhance the binding strength of integrin receptors by increasing the avidity. Finally, it was shown that also modulation of lateral mobility of integrins can be crucial for cell attachment.
Within a migrating cell new contacts need to be formed at the front, while the rear of the cell has to detach. Locking integrins into a high affinity state would prevent rear cell detachment and inhibit migration. Therefore disassembly of focal contacts and down regulation of integrin affinity and avidity via inside-out signalling at the rear of the cell are indispensable for the migration of cells. This implies that integrin activity is regulated differentially in different regions of a single cell. In migrating leukocytes, activation of RhoA mediates the detachment at the rear by decreased adhesion of $\alpha 4\beta 1$ and $\beta 2$ integrins (Worthylake et al., 2001).

1.2 Development of the hematopoietic system

All blood cells derive from hematopoietic stem cells (HSCs) which have the potential to self-renew and to differentiate into all hematopoietic lineages (Fig. 8). HSCs are generated outside the embryo proper, in the yolk sac (YS) and within the embryo in the para-aortic splanchnopleura (PAS)/aorta-gonad mesonephros (AGM) region. At around E8.5 when circulation starts in mouse, the HSCs are present in the fetal blood and at E10 start to colonize the fetal liver, which is the organ of hematopoiesis during embryonic life (reviewed by Morrison et al., 1995; Weissman 1994, Kondo et al., 2003). Later, hematopoietic progenitors seed the thymus, the spleen, other lymphoid organs and the bone marrow (BM) where blood cell development occurs in adulthood. In the adult mouse HSCs represent up to 0.05% of cells of the BM. HSC give rise to lymphatic, myeloid and erythroid precursor cells. Different cell types and developmental stages can be

distinguished by the expression of characteristic cell surface markers (Fig. 8). HSCs are described by a combination of marker proteins and defined by their ability to give rise to long-term multilineage reconstitution in lethally irradiated mice.

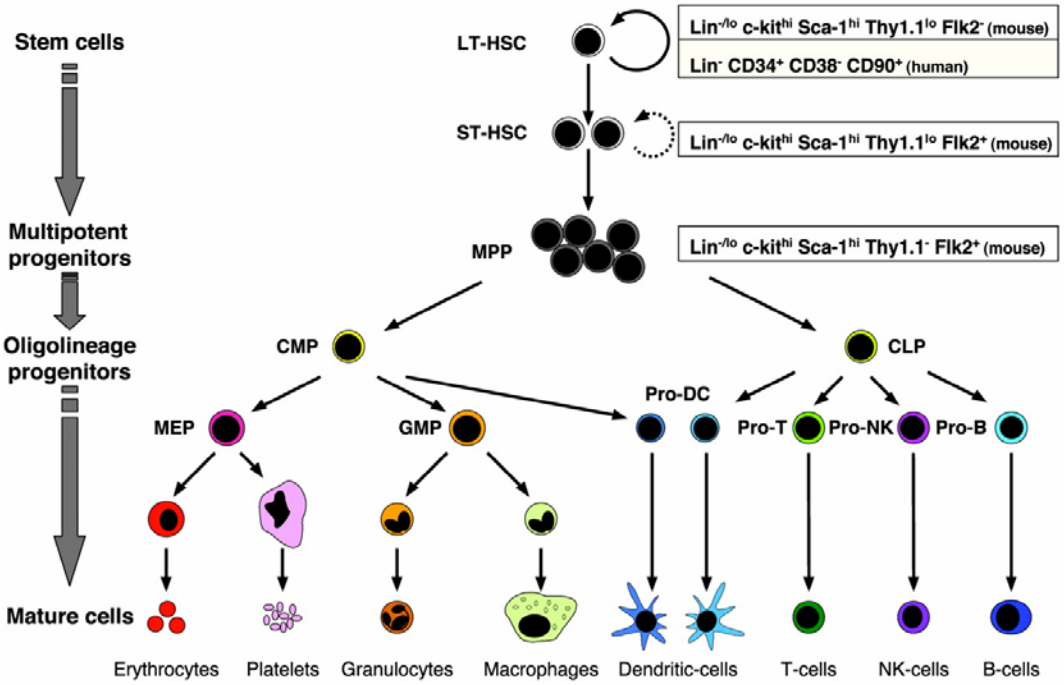


Figure 8: Cells of the hematopoietic system

All hematopoietic cells derive from hematopoietic stem cells (HSCs) that give rise to common lymphoid precursors (CMPs) and common myeloid precursors (CLPs), which further differentiate into different mature cell types. While lineage committed cells can be described upon their expression of certain markers on the cell surface, HSCs and precursor cells are usually identified by a combination of several surface markers (modified from Weissman et al., 2003).

Lymphoid development

Lymphocytes are responsible for the acquired immunity and the immunologic attributes of diversity, specifity, memory and self/nonself recognition. They continuously circulate in the blood and lymph, are capable of homing to secondary lymphoid organs and migrate into tissues. In mice, lymphocytes constitute 20%-40% of the blood leukocytes. During their development from CLPs, lymphoid cells become restricted either to the B cell, T cell or the NK cell

lineage. While B cells and NK cells develop within the BM, T cell precursors leave the BM, migrate to the thymus and mature there.

The course of B cell development is characterized by the expression of specific surface markers that characterize certain developmental stages (Fig.9). The first lineage specific marker B cells express is B220 (CD45R). This surface molecule is expressed at all stages from pre-pro B cells throughout mature stages and on activated B cells. After emigration from the BM, immature B cells increase B220 expression from medium (B220med) to high levels (B220hi) and become mature B cells. B220hi cells in the BM, therefore, are recirculating B cells from the periphery. CD19 is a member of the immunoglobulin superfamily and is expressed from the pro B cell stage throughout B cell development. IgM is present on immature B cells after the V, D, J rearrangements of the genes of the immunoglobulin heavy and light chains. In early mature cells a change in RNA processing of the heavy-chain primary transcript leads to production of two mRNAs encoding IgM and IgD. These cells leave the BM, enter the blood and subsequently home to peripheral lymphoid organs where the expression of membrane bound IgD increases.

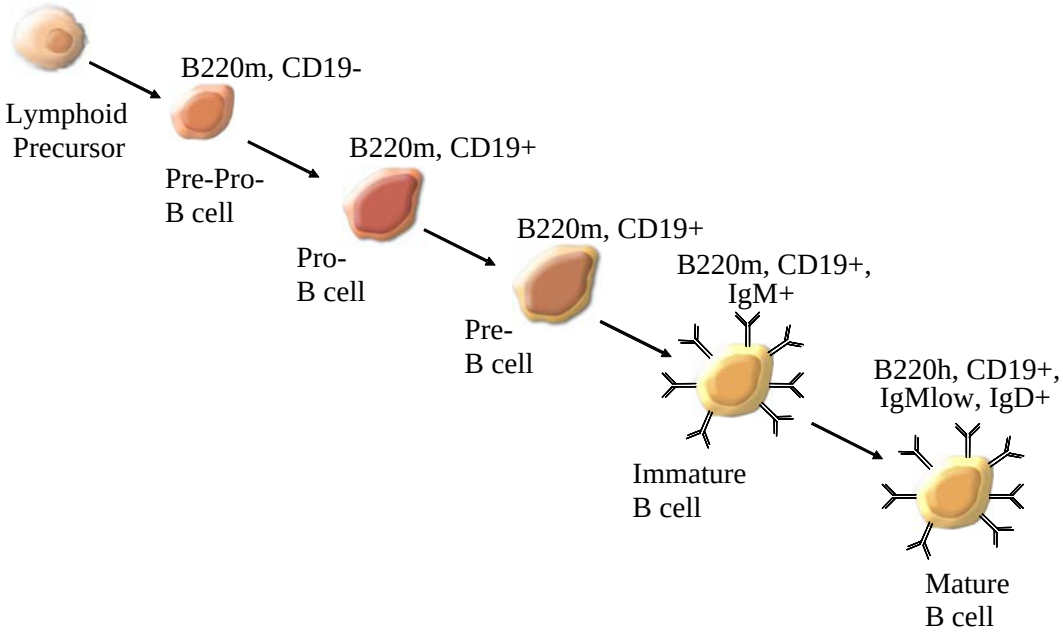


Figure 9: B cell development is accompanied by the expression of characteristic surface molecules.

B cell development occurs in the BM and originates from lymphoid progenitor cells. The expression of the surface markers B220, CD19, IgM and IgD are characteristic for a certain developmental stage on during B cell development. Mature B cells leave the BM and are found in the periphery.

T cell precursors leave the BM, enter the blood stream and then colonize the cortex (outer compartment) of the thymus. These precursors do not express molecules characteristic for mature T cells like CD4, CD8 or the T cell receptor complex. In the thymus, the T cell precursors are part of the so-called double negative (DN; CD4-CD8-) population. Other markers like c-kit, CD44 and CD25 allow subdividing the DN population into sequential developmental stages. Subsequent to switching off c-kit and reducing CD44 expression T cells start to rearrange their TCR genes. The majority of the thymocytes rearrange the TCR β-chain genes. Newly synthesized β-chains combine with the pre T α-chain and associate with the CD3 group to form a novel complex called the pre-T cell receptor (pre-TCR). The pre-TCR is thought to interact with an intrathymic ligand and to transmit a signal through the CD3 complex. This signal induces developmental progression to the CD4+CD8+ double positive (DP) state. At this stage T cells proliferate and undergo positive and negative selection restricting survival to MHC-restricted, self-tolerant T cells. During this process, DP T cells migrate from the cortex to the medulla (inner compartment of the thymus) and develop into either single positive (SP) CD4+ T cells or SP CD8+ T cells. SP T cells leave the thymus through postcapillary venules and home to secondary lymphoid organs and BM. The intrathymic development takes about 3 weeks, of which 2 weeks are at the DN, 3-4 days at the DP, and up to 2 days at the SP stage. Naive T cells are thought to survive about 5-7 weeks in the absence of antigen-stimulated activation.

Thymocytes with productive rearrangements of both the γ- and δ-chain genes develop into CD4, CD8 double negative CD3+ γδ T cells. These cells move to the periphery and account for less than 5% of thymocytes in adult mice.

Myeloid and erythroid cells

Common myeloid precursors (CMPs) develop either into megakaryocyte/erythrocyte progenitors (MEPs) or into granulocyte/monocyte precursor cells (GMPs). MEPs develop either into megakaryocytes to form platelets or into erythroblasts and later erythrocytes. GMPs give rise to both

granulocytes and monocytes, which mature into macrophages as soon as they leave the blood stream and enter tissues. Granulocytes can be subdivided on the basis of cellular morphology into neutrophils, eosinophils and basophils. Neutrophils and eosinophils are phagocytotic, whereas basophils are not. Neutrophils, besides macrophages are the major phagocytotic cells of the mouse. They are generated in the BM, released into the peripheral blood and circulate for 7-10h until they migrate into tissues, where they have a life span of a few days. Neutrophils constitute 50%-70% of the circulating white blood cells in mice and are much more numerous than eosinophils (1%-3%) or basophils (<1%). Because of their multilobed nucleus, neutrophils are often called polymorphonuclear leukocytes (PMN). Granulocytes can be detected by their expression of Gr-1 (Ly-6G). In the BM the Gr-1 expression level is directly correlated to the granulocyte differentiation and maturation. Also monocytes express this marker transiently in the BM, while in the periphery Gr-1 is expressed only on neutrophils.

Monocyte development starts in the BM. Promonocytes then leave the BM and enter the blood, where they enlarge and further differentiate into mature monocytes. After about 8h monocytes extravasate into tissues where they subsequently differentiate into tissue specific macrophages. Granulocytes, monocytes and macrophages express $\alpha_M\beta 2$ integrin (Mac-1).

Megakaryocyts mature within the BM and give rise to platelets, which are essential for blood clotting. Cells committed to the erythroid lineage develop to erythroid precursors and mature to erythroblasts, which give rise to erythrocytes. During their entire course of development, erythroid cells express the Ter119 (Ly-76) antigen, which is not detectable on HSC or any other lineages. Additionally, erythroblasts express CD71 (transferrin receptor), which allows, together with Ter119, a more detailed distinction of developmental stages (Fig. 10). The most immature erythroid cells express medium levels of both of these markers before CD71 expression becomes up regulated. These Ter119 medium CD71 high cells mature into CD71 high Ter119 high cells. During further development the Ter119 expression levels remain the same, while CD71 decreases until it is absent on mature erythrocytes. Ter119 medium, CD71 medium erythroid cells are erythroid progenitors that can be detected in a erythroid colony formation assay and have the potential for massive expansion, for example after acute erythrolysis (Socolovsky et al., 2001).

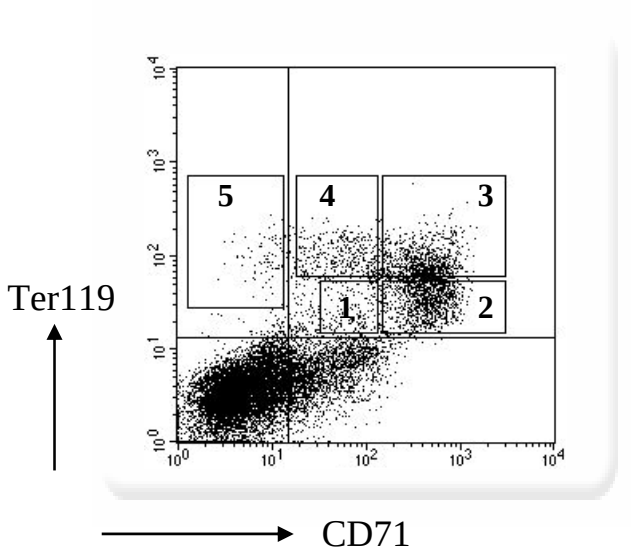


Figure 10: Different developmental stages of erythroid cells can be distinguished by their Ter119 – CD71 expression pattern.

BM cells stained for Ter119 and CD71 allow the distinction of different sequential developmental stages of erythroid cells (1-5) as indicated in the dot plot.

1.3 Role of β1 and β7 integrin in the hematopoietic system

Integrins play an important role in the migration of hematopoietic cells during development, in the adult animal and under pathological conditions (Gonzales-Amaro and Sanchez-Madrid, 1999). During the passage of leukocytes through the blood the integrins on the cell surface remain in a low affinity state in order not to make inappropriate interactions with ligands normally present in the blood or on endothelial cells. Leukocytes can loosely attach to the endothelium via selectins, which results in their rolling on the vessel wall. In the presence of chemokines, which are secreted constitutively in lymphoid organs or during inflammation, leukocytes with the corresponding chemokine receptors quickly upregulate integrin affinity leading to firm adhesion of leukocytes to the endothelium. Subsequently, the leukocytes cross the endothelial layer and the underlying basement membrane and migrate within the extravascular tissue. Although β1 integrins are crucial for the extravasation of HSC, their role in extravasation of

differentiated blood cells is less clear. Using β1-null chimeric mice it was shown that β1 integrin is not required for the generation of HSCs/HPCs in the PAS/AGM region and in the YS, but that it is crucial for the colonization of the fetal liver *in vivo* (Hirsch et al., 1996). Fetal β1-null HSC are not able to exit the blood stream, most probably due to a defective adhesion to the endothelial cells (Potocnik et al., 2000). However, they are able to survive and differentiate in liver and thymus organ cultures *in vitro* and in spleen *in vivo* indicating that the loss of β1 integrin does not affect the differentiation potential. Similarly, adult β1-null HSC can neither home to the BM nor migrate to other lymphoid organs. It was shown that adult β1-null HPC have a severely impaired adhesion to endothelial cells, suggesting that β1 integrin-mediated adhesion might be essential for all extravasation processes of HSC and HPC (Potocnik et al., 2000). Although antibody inhibition experiments suggested an important role of α4β1 integrin for the migration of adult HPC to the spleen (Williams et al., 1991), deletion of the α4 integrin gene did not interfere with the homing of HSC to the fetal liver or the BM (Arroyo et al., 1999). Also targeted disruptions of other β1 integrin associated subunits such as α3, α5 and α6, which are all expressed on HSC, did not impair BM homing, suggesting either the involvement of a yet unknown subunit binding to β1 or redundant function of various β1 integrins (Arroyo et al., 2000).

For the retention within the BM, HSC need to adhere to stroma cells and to the extracellular matrix. *In vitro* assays suggested that this adhesion also controls survival, proliferation and differentiation of HSC and HPC (Papayanopoulou and Nakamoto, 1993; Coulombel et al., 1997). Agents like granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) or α4 integrin antibodies (Papayanopoulou et al., 1995) can mobilize hematopoietic cells resulting in an increased number of colony-forming units in the peripheral blood. How these substances interfere with the attachment of HSC and HPC to the BM stroma, however, is not clear.

Within the BM, integrins are supposed to mediate the attachment of HSC to stroma cells and ECM, especially to fibronectin (Williams et al., 1991). This hypothesis, based on antibody inhibition experiments, was strengthened by the mobilization of HPC from the BM after injection of antibodies against α4 integrin, recognizing α4β1 and α4β7 integrin, and with antibodies against

VCAM-1 or fibronectin fragments, both ligands of α4β1 and α4β7 (Papayanopoulou et a., 1995; van der Loo et al., 1998). *In vitro* and *in vivo* α4β1-mediated attachment of HPCs to FN promotes proliferation (Yokota et al., 1998; Schoefield, 1998) and prevents apoptosis (Wang et al., 1998). However, the induced deletion of β1 integrin in adult mice did not lead to increased release of HPCs into the periphery indicating that β1 integrin is not essential for the retention of HSCs/HPCs in the BM (Brakebusch et al., 2002).

Colonisation of Peyer's patches (PP), a lymphoid tissue of the gut, is dependent on the presence of α4β7 on leukocytes and MAdCAM on the endothelial cells in the intestine (Wagner et al., 1996; Pabst et al., 2000). Neither α4-null nor β7-null lymphocytes could home to PP *in vivo* (Arroyo et al., 1996; Wagner et al., 1996). Since αEβ7 is expressed only on few lymphocytes others than IELs it was concluded that α4β7 is essential for the seeding of PP. In chronic inflammation, α4β1 integrin mediates extravasation of lymphocytes into the affected tissues (Yang et al., 2003). For the extravasation of neutrophils, β2 integrin is of major importance. Patients lacking a functional β2 integrin show severe immunodeficiencies and virtual absence of granulocytes in extra vascular tissues (Roos et al., 2001). On the other hand, β2 integrin-deficient mice do show some extravasation of neutrophils, suggesting that other molecules can also mediate the firm adhesion of these cells to the endothelium of inflamed tissues (Scharffetter-Kochanek et al., 1998). Potential candidates for this function are β1 integrins. In line with this hypothesis is that α9β1 is highly expressed on granulocytes and contributes to the transendothelial migration of neutrophils *in vitro* (Taooka et al., 1999).

Antibody inhibitions experiments suggested that lymphocyte development in adult BM depends upon α4β1 integrin mediated adhesive interactions between hematopoietic cells and stroma cells (Miyake et al., 1991). The analysis of α4-null somatic chimeric mice, which in all tissues have different contributions of α4 deficient cells, confirmed this observation. In the absence of α4 integrin, B cell development stopped before the pro-B cell stage. Furthermore, adult T cell progenitors could not emigrate from the bone marrow resulting in severe thymus atrophy after birth (Arroyo et al., 1996). Injection of α4-deficient BM cells into the blood, however, resulted in thymus colonization and generation of CD4+ and

CD8+ T cells, indicating that α4 integrin is not crucial for thymus colonization and T cell differentiation per se. Mice with a complete or restricted deletion of the VCAM-1 gene showed normal hematopoiesis, indicating that interaction of α4β1 or α4β7 with VCAM-1 is not essential for lymphopoiesis (Leuker et al., 2001, Koni et al., 2001, Friedrich et al., 1996).

The loss of α4 integrin also affects erythro- and myelopoiesis. In adult α4-null chimeric mice no erythrocytes derived from α4-null HSC could be detected. Furthermore, the number of monocytes and granulocytes was severely reduced due to a decreased expansion on the level of the progenitor cells. As an explanation for the impaired differentiation of α4-null HPC it was proposed that α4 integrin is crucial for the migration of progenitor cells through the BM stroma, which is considered to be necessary for the development *in vivo* (Arroyo et al., 1996). However, no similar defects were observed in mice lacking VCAM-1, a major ligand of α4β1 integrin (Leuker et al., 2001).

α4 integrin can dimerize with β1 and with β7 integrin. Since mice lacking β7 integrin demonstrated normal hematopoiesis (Wagner et al., 1996), it was assumed that the loss of α4β1 is responsible for the impaired hematopoiesis. This predominant role of α4β1 integrin for adult hematopoiesis was tested recently using β1 mutant BM chimeric mice (Brakebusch et al., 2002). Surprisingly hematopoiesis was normal in these mice questioning the requirement of α4β1 integrin in adult hematopoiesis. Two explanations could account for this unexpected result. First, the role of α4 integrin was studied in somatic chimeric mice and the deletion of the α4 gene was therefore not restricted to cells of the hematopoietic system. A deletion of the α4 integrin gene on other cells types, in particular BM stroma cells might have an impact on hematopoietic development. Second, α4β1 and α4β7 integrins might have redundant functions in early hematopoiesis so that only the absence of both molecules impairs hematopoiesis. This hypothesis was tested in this work by generating β1β7 mutant BM chimeric mice. These animals lack β1 and β7 integrin expression restricted to the hematopoietic system. Since the α4 subunit can only dimerize with β1 and β7 integrin, we expected an indirect loss of α4 integrin from the cell surface equivalent to a knockout of the α4 integrin gene.

1.4 Aim of the project

Based on published evidences of α4 somatic chimeric mice, α4β1 and α4β7 integrin are indispensable for normal hematopoiesis. Since β7 knockout mice and β1 mutant BM chimeras had normal hematopoiesis, it was concluded that only simultaneous loss of α4β1 and α4β7 integrin as in α4-null chimeric mice, results in hematopoietic defects. This PhD project aimed to test this hypothesis.

β1β7 mutant BM chimeras, in which both α4β1 and α4β7 integrin are absent were generated and analysed. The analysis focused on hematopoietic stem and precursor cell maintenance and function as well as on the development of different blood cell lineages. During the analysis a conditional knockout of α4 integrin in the hematopoietic system focusing on HPC distribution was published giving this project the further aim of investigating the reason for conflicting results.

2. Materials and Methods

2.1 Generation of mice with a deletion of the β1 and the β7 integrin genes in the hematopoietic system

Mice carrying a β1 integrin gene flanked by loxP sites (fl/fl) (Brakebusch et al., 2002) were mated with mice with a neomycin-disrupted β1 integrin gene (+/-) (Fässler and Meyer, 1995), mice lacking a functional β7 integrin gene (β7-/-) (Wagner et al., 1996) and with mice expressing the cre recombinase under the control of the (polyinosinic-polycytidylic acid) polyIC-inducible Mx-promotor (+Mx) (Kühn et al., 1995). The Mx-promotor is silent in healthy mice but can be transiently activated upon application of Interferon α (IFN-α) or IFN-β or of synthetic double stranded RNA (polyIC) which induces IFNs.

As a result mice were obtained carrying one conditional and one null allele for the β1 integrin gene, a constitutive deletion of both β7 integrin alleles and a transgene for the cre recombinase controlled by the Mx-promotor (β1β7 mutant: β1 (fl/-) β7 (-/-) +Mx), β1 (fl/fl) β7 (-/-) +Mx). β7 null mice with a conditional and a wt allele for β1 and transgenic for the cre transgene (β1(fl/+) β7(-/-) +Mx) or with two conditional alleles for β1 without the cre recombinase (β1 (fl/fl) β7(-/-)) served as control animals (β7 mutant). Since the polyIC stimulated Mx-promoter is not only active in the hematopoietic system but also in various other tissues, we transplanted bone marrow (BM) from β7 mutant and β1β7 double mutant mice before knockout induction into lethally irradiated normal recipient mice, to restrict the gene deletion to the hematopoietic system. BM cells were isolated from femurs of (β1 (fl/-) β7 (-/-) +Mx or β1 (fl/fl) β7 (-/-) +Mx) and (β1 (fl/+) β7 (-/-) +Mx or β1 fl/fl β7 (-/-)) donor mice. BM chimeras were generated by tail vein injection of 10^6 bone marrow cells into lethally irradiated (6 MV X-rays, 10 Gy, 6.2/min) B6.SJL recipient mice. Donor cells expressed the surface marker Ly5.2 while host cells expressed Ly5.1, allowing a simple distinction by FACS analysis. After reconstitution of the hematopoietic system, 4-8 weeks after BM transfer, the

deletion of the β1 integrin gene was induced by three intraperitoneal injections of 250µg polyIC at two day intervals obtaining β1β7 mutant BM chimeras and β7 mutant BM chimeras.

Mice lacking β1 and β2 integrins in the hematopoietic system were obtained by intercrossing β1 (fl/fl) +Mx mice with mice carrying a constitutive gene deletion for β2 integrin (Scharffetter-Kochanek, K. et al., 1998) and subsequent polyIC treatment.

2.2 Genotyping of β1, β2 and β7 mutant mice

Mice were earmarked and genomic DNA was isolated at 2-3 weeks of age by digesting tail pieces in buffer containing 0.2% SDS, 100 mM Tris-HCL, pH 8.0, 200 mM NaCl, 5 mM EDTA, pH 8.0 and 100 µg/ml proteinase K at 55°C over night. DNA was isolated the next day using a phenol-chlorophorm extraction and isopropanol precipitation according to Sambrook and Russell 2001. β1 conditional and wild type allele were distinguished using the primers 5´- AGG TGC CCT TCC CTC TAG A – 3´ and 5´- GTG AAG TAG GTG AAA GGT AAC – 3´. A touchdown program was run with 10 cycles starting from 63°C to 53°C annealing temperature, decreasing 1°C each cycle, and subsequent 35 cycles at 53°C annealing for 30s, 30s denaturing at 94°C and 30s elongation at 72°C each cycle. This reaction gave rise to a 345 bp product for the wild type allele and a 450bp product for the conditional allele. The β1 integrin null allele was amplified with the primers 5´- AGG TGC CCT TCC CTC TAG A – 3´ and 5´- TAA AAA GAC AGA ATA AAA CGC AC – 3´ amplifying a 210 bp fragment. The β7 integrin wild type allele was detected applying a similar touchdown program starting with 10 cycles from 65°C to 55°C decreasing 1°C each cycle and subsequent 35 cycles at 55°C for 30s each cycle using the primers 5´- GAC CAG TGC CTA GGC TGC – 3´ and 5´- CCT CTA CTC CCG TCG GTC - 3´. If the DNA contained a functional β7 integrin gene a 600 bp band was obtained. The presence or absence of the cre-transgene was assessed by using the primers 5´- TTC GGA TCA TCA GCT ACA CC -3´ and 5´- AAC ATG CTT CAT CGT CGG – 3´ applying the

same program described for the detection of the β1 integrin wild type or conditional allele above. DNA from mice carrying the cre transgene gave rise to a 419 bp fragment. The primers 5´- GCC CAC ACT CAC TGC TGC TTG – 3´ and 5´- CCC GGC AAC TGC TGA CTT TGT – 3´ were used to amplify a 467 bp fragment for β2 integrin wild type mice and the latter one combined with the primer 5´- AGG ACA GCA AGG GGG AGG ATT -3´ giving rise to a 140 bp fragment. The same touchdown program used for the detection of the cre-transgene was used for the genotyping of β2 integrin knockout allele. PCRs for all of these reactions was conducted in 20 µl reaction volume containing 2 µl purified genomic DNA and a mastermix of the following components listed for one reaction: 0.2 µl 100 pmol/µl of primer I and II, 0.4 µl 10 mM dNTPs, 2 µl 10xPCR buffer, 0.8 µl 50 mM MgCl, 0.08 µl 5 U/µl Taq-polymerase and 14.32 µl H_2O using a Biometra® Thermocycler. Recombinant Taq-polymerase, MgCl and 10xPCR buffer was purchased from Invitrogen™. All reactions were electrophoretically separated on a 2% agarose gel containing 0.5 µg/ml ethidium bromide and photographed on a UV-table.

2.3 Animal treatment

Mice were bred and housed in compliance with the German Law for Welfare of Laboratory Animals. All animal experiments were approved by the local ethic committee. Blood samples were obtained from the retro-orbital plexus under anaesthesia using the inhalation anaesthetic IsoFlovet ® form Schering-Plough Animal Health. Acute haemolysis was assessed after two daily intraperitoneal injections of 60 mg phenylhydrazine (PHZ; Sigma) per kg mouse weight at day 1 and 2. Mice were scarified for analysis at day 4. Animals were scarified by cervical dislocation.

2.4 Türk staining

Whole blood of β7 mutant and β1β7 mutant BM chimeras was isolated, diluted 1:10 with Türk stain (0.01% of methilrosaniline chloride and 1.0% acetic acid) and differentially counted for polymorphonuclear and mononuclear cells in a haemacytometer.

2.5 Flow cytometry

Single cell suspensions were prepared by gently pushing the dissected organs through 70 µm cell strainers (Becton Dickinson) in a Petri dish. Cells were then transferred into a 50 ml Falcon tube and washed in 20 ml FACS-buffer (1% bovine saline albumin (BSA) in phosphate buffered saline (PBS 8.4 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 137 mM KCl, pH 7.4). After subsequent centrifugation at 1300 rpm at 4°C the cells were resuspended in 10ml FACS-buffer and counted in a haemacytometer. For phenotype analysis, $1x10^6$ cells from bone marrow (BM), spleen and lymph nodes (LN) were incubated with fluorescently labelled antibodies in 50µl antibody solution for 30 min at 4°C in the dark. Erythrocytes in blood samples were lysed using ACK-buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA) before staining (Coligan et al., 1995).

The following primary antibodies were used in flow cytometry: hamster anti-β1 integrin (Ha2/5), rat anti- β7 integrin (M293), rat anti-α4 integrin (R1-2, 9C10, 5/3 and PS/2), rat anti-B220 (RA3-6B2), rat anti-CD19 (1D3), rat anti-IgM (R6-60.2), rat anti-IgD (11-26c.2a), rat anti-CD4 (H129.19), rat anti-CD8 (53-6.2), rat anti-CD3 (17A2), rat anti-Gr-1 (RB6-8C5), rat anti-Mac-1 (M1-70), rat anti CD71 (C2), rat anti Ter-119 (Ter119), mouse anti-Nk1.1 (PK136), rat anti sca-1 (D7), rat anti c-kit (2B8) rat anti-Ly-5.1 (A20), rat anti-Ly-5.2 (104) (all Pharmingen, USA). Primary antibodies were conjugated with FITC (Fluorescein), PE (Phycoerythrine) or biotin and used at 1:200 dilutions in FACS-buffer to block unspecific binding. After washing the cells with 200 µl FACS-buffer they were incubated in 50 µl secondary antibody solution for 30 min or fluorescently

labelled streptavidin for 10 min at 4°C in the dark, washed again and analysed by FACS. Biotinylatyed antibodies were detected by streptavidin PE (Southern Biotechnology, 1:2000 dilution) or streptavidin Cy-5 (Jackson Immunoresearch, 1:500 dilution). Rat anti-α4 integrin 5/3 and PS/2 were detected with donkey anti rat Cy5 as a secondary antibody (Jackson Immunoresearch, 1:500 dilution). Dead cells were excluded from the analysis by propidium iodide (1 µg/ml) counterstaining. Erythrocytes and debris were excluded by FSC/SSC gating.
In all dot plot analyses shown the percentage of cells in the respective quadrant is indicated. The cells in the lower left quadrant show no staining significantly distinct from the autofluorescence of these cells. For the analysis of platelets 5µl antibody solution containing β1 integrin antibody (Ha2/5) FITC-labelled and GPIb-IX (p0p1) PE-labelled (a gift from B. Nieswandt) both diluted 1:10 in PBS was added to 1µl whole blood. After 15 min incubation at room temperature in the dark 100 µl PBS was added and samples were analysed by FACS.

2.6 Colony formation assay

For colony formation assays leukocytes were suspended in 300 µl Iscove's modified Dulbecco’s medium (IMDM) with 2% FCS in a 15 ml tube and mixed with 3 ml methylcellulose supplemented with growth factors and cytokines and plated into three wells of a 6 well plate after dissipation of air bubbles.
For colony assays detecting granulocyte/monocyte precursors 180.000 BM cells, 3.600.000 splenocytes or 250 µl whole blood were added to 3 ml MethoCult GF M3534 (StemCell Technologies, Vancouver, Canada), containing erythropoethin (Epo), interleukin-3 (IL-3), IL-6, and stem cell factor (SCF). In blood samples erythrocytes were lysed by incubation in ACK-lysis buffer for 5 min at room temperature prior to culture. For pre-B cell colony assays 800.000 BM cells were mixed with MethoCult M3630 (StemCell Technologies) containing IL-7. To measure the amount of erythroid colony forming units (CFUe) 600.000 BM cells or 1.200.000 splenocytes were mixed with 3 ml Methocult M3334 (StemCell Technologies) containing 10 µg/ml Insulin, 200 µg/ml iron saturated human transferrin and 3 units/ml Epo were plated into three wells of a 6 well plate. To

prevent the cultures from drying out the interspaces of the 6 well plates were filled with PBS. GM and pre-B colonies were counted after 7 days of incubation at 37°C, 5% CO_2 under a dissection microscope. For FACS analysis colonies were picked into a 96 well plate with 100 µl PBS in each well, centrifuged and stained with β1 integrin and Gr-1 antibody for GM colonies and β1 integrin and B220 antibody for pre-B colonies, washed, resuspended in 100µl PBS and analysed by FACS. CFUe colonies were stained with benzidine (Sigma, B3503) after 3 days of incubation at 37°C, 5% CO_2. For staining, benzidine was dissolved in 12% acetic acid and immediately before usage complemented with H_2O_2 to a final concentration of 0.3%. 1 ml staining solution was added to each well of a 6 well plate. Haemoglobinized colonies turned dark blue and were counted under a dissection microscope.

CFUe colonies were then picked into PCR tubes containing 100 µl PBS and centrifuged for 5 min at 1400 rpm at 4°C. The supernatant was discarded and the cells were subsequently incubated in 5 µl alkaline lysis solution (0.2 M KOH, 0.05 M Dithiothreitol (DTT)) for 10 min. After addition of 5 µl of neutralisation solution (0.9 M Tris pH 8.3, 0.3 M KCl, 0.2 M HCl) genomic DNA was subjected to a random preamplification as described by Lin Zhang et al., (1992). In brief, each PCR reaction was performed in a total volume of 50 µl containing 20 µl random 15-mere 100 pmol/µl primers, 0.6 µl 10 mM dNTPs, 6 µl of a solution containing 0.025 M MgCl, 0.1% gelatine, 0.1 M TrisHCl pH 8.3, 1 µl Taq polymerase purchased from Invitrogen™ and 20.4 µl H_2O. The random preamplification PCR program includes 50 primer extension cycles. Each cycle consists of a denaturation step at 92°C of 1 min, an annealing step at 37°C of 2 min and an extension step of 4 min at 55°C. After random preamplification of the genomic DNA 6 µl of each sample of this reaction was genotyped by genomic PCR in a 20 µl PCR reaction as described above.

2.7 Separation of splenocytes by MACS

Single cell suspension from the spleen was prepared by gently pushing the dissected organ through a 70 µm cell strainer (Becton Dickinson) in a Petri dish.

The cells were transferred into a 50 ml Falcon tube and washed in MACS-buffer (0.5% BSA, 2 mM EDTA in PBS). Splenocytes from a whole spleen were divided into three fractions and stained with B220, CD4 and CD8 antibodies (all FITC labelled), respectively as described for FACS analysis. Magnetic sorting was carried out with MACS beads according to the manufacturer's instructions (Miltenyi biotec). Briefly, for usage of anti-FITC microbeads FITC labelled cells were resuspended in 90 µl MACS-buffer per $1x10^7$ cells and 10 µl MACS anti FITC-microbeads were added. After 15 min incubation at 4°C the cells were washed with 2 ml MACS-buffer and centrifuged at 300 g for 10 min. After resuspending cells in 500 µl MACS-buffer cells were applied to a MiniMACS separation column attached to the MiniMACS separation unit (magnet) equilibrated with 500 µl MACS-buffer and placed at the MACS Multistand. The column was washed three times with 500 µl buffer to remove all unlabelled cells. Then the MACS column was removed from the magnet and placed on a collection tube. To elute all cells 1 ml buffer was applied to the column and firmly flushed out using the plunger supplied with the column. Since the eluted cells were FITC labelled the purity of the sort was checked subsequently by FACS.

2.8 Southern blot analysis

Genomic DNA was isolated from BM, spleen and thymus single cell suspensions using proteinase K digestion, phenol-chloroform extraction and isopropanol precipitation as described above (3.2). Southern blotting was performed according to standard procedures (Sambrook and Russell, 2001). DNA was digested with EcoRI and probed with a fragment of the lacZ gene, which detects only the targeted allele. Membranes were exposed to X-ray films and the resulting bands quantified using Bio-PROFIL Bio-1D V97.03 software (Vilber-Lourmat©).

2.9 Iodination of cell surface proteins and subsequent immunoprecipitation and gel electrophoresis

BM cells were washed with PBS in a polystyrene tube and cell surface proteins labelled with ^{125}I using the lactoperoxidase method as described previously (Lang et al., 1987). In brief, $5x10^7$ BM cells were resuspended in 0.5 ml PBS pH 6. Under continuous stirring 5.7 µl lactoperoxidase 88 U/ml, 1 mCi ^{125}I (Amersham) and 25 µl H_2O_2 (diluted immediate before use 1:1000) were added. After 5 min additional 2.8 µl LPO and 12 µl H_2O_2 were added and incubated under stirring for 5 more minutes. The iodination reaction was terminated by adding 0.5 ml solution containing 1mM DTT and 1 mg/ml tyrosine. Cells were washed twice and then lysed for 30 min in 2 ml lysis buffer (50 mM Tris-HCL, pH 7.4, containing 0.15 M NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 1%Triton X-100) containing proteinase inhibitors (Fässler et al., 1995). After centrifugation at 10.000 g for 15 min the supernatant was transferred to a new tube. Then the lysate was precleared with 30 µl Protein-G beads for 1 hour at 4°C and an immunoprecipitation (IP) was performed using anti α4 integrin monoclonal antibody (clone 5/3) and anti α_M integrin antibody (clone M1/70) according to Zeller et al., 1998. The supernatant and the precipitates were resuspended and heated in 20 µl – 50 µl Laemmli buffer containing β-mercaptoethanol at 95°C for 5 min and separated on a 6% SDS polyacrylamide gel. To estimate protein sizes the protein molecular weight markers were run in a separate lane (Broad range BioRad®). The protein was transferred onto a Nitrocellulose membrane (Protran® from Schleicher & Schuell) by Western blotting in transblot-buffer containing 48 mM Tris-HCl, 39 mM glycin, 0.03% w/v SDS and 20% methanol in water. To visualise the protein weight marker the membrane was first washed 3 times in water for 5 min each and then stained in 1% (v/v) Ponceau S (Sigma) in H_2O for 30 s to 1 min and subsequent destained in water until bands appear on the membrane. After the bands of the protein weight standard were recorded by photocopy the residual Ponceau S stain was extracted from the protein bands with several washes with water. Without drying out the membrane at any point it was then enwrapped in foil and exposed to X-ray sensitive film.

For paxillin immunodetection blots were blocked in 5% non-fat dry milk/TBS pH 7.5/0.1%Tween20 (blocking solution) for 1 h at RT and probed with monoclonal

anti paxillin antibody in blocking solution for 1 h at RT or 4°C overnight. Blots were then washed with blocking solution 6 times for 5 min each and incubated with the secondary horseradish peroxidase-labelled antibody diluted in blocking solution for 1 h at RT. The blots were washed with TBS 5 min 5 times and signal was detected with ECL+Plus™ kit (Amersham Pharmacia).

2.10 Soluble VCAM-1 binding assay

The binding to VCAM-1 was tested by incubating 400,000 BM cells in 50 µl with soluble VCAM-1 (Makarem et al., 1994) labelled with oregon green (kindly provided by M. Humphries University of Manchester, UK) in Tris buffered saline (TBS) (150 mM NaCl, 10 µM Tris-HCL, pH 7.4) at room temperature. To activate integrin heterodimers, incubation and washing steps were carried out in the presence of 1 mM $MnCl_2$. Integrin independent binding was assessed in the presence of 2 mM EDTA. After 15 min incubation 50 µl β1 and α4 integrin antibody solution diluted 1:100 was added and incubated 15 more minutes. Cells were washed in the presence of 1 mM $MnCl_2$ and 2 mM EDTA respectively and analysed by FACS.

2.11 Generation of a cell line deficient for β1, β2 and β7 integrins

To generate a fibroblastoid cell line deficient for β1, β2 and β7 integrin, mice conditional for β1 integrin, positive for the cre-transgene and deficient for β7 integrin were crossed with animals lacking a functional β2 integrin gene. The offspring of this mating were intercrossed and the pregnant mice sacrificed at day 13.5 counting the day of the plug as day 0.5. The embryos were prepared aseptically, the head and inner organs removed and the remaining material cut into small pieces. After incubation in 1xtrypsin/EDTA for 10 min at 37°C and breaking tissue pieces by pipetting up and down the material was taken up in

DMEM medium containing 10% fetal bovine serum (FBS) and seeded into a tissue culture flask. For immortalisation culture medium of 865-SV40 largeT cells encoding retrovirus that can transduce the SV40 large T antigen into murine cells was centrifuged and filtered with a celluloseacetate membrane pore size 0.2µm (Renner GmbH Darmstadt/Germany). Together with polybrene at final concentration of 8 µg/ml, the virus containing supernatant was added to the cells to be immortalized. 12h later the medium was exchanged for EF medium and the cells were expanded and cloned by limiting dilution. Two isolated (β2(-/-)β7(-/-) β1(fl/fl) clones were infected with an adenovirus carrying the cre recombinase. The cre-mediated deletion of the β1 integrin gene was confirmed on isolated clones by FACS analysis.

2.12 Reverse transcription polymerase chain reaction (RT-PCR)

For transcription analysis total RNA was prepared using TRIZOL® Reagent according to the manufacturer's instructions (Invitrogen). RNA was reverse transcribed with Superscript^{TM} III reverse transcriptase (Invitrogen) using gene specific primers. For β7 integrin the primer 5´- GCT TGA AGA GTG ACC CAG AAA TCC - 3´ was used. The primer anneals to two different exons in order not to obtain any PCR products from contaminations of genomic DNA. For first strand synthesis 1 µg total RNA, 2 pmol of the gene specific primer, 1µl 10mM dNTPs and 10 µl H_2O were mixed and heated to 65°C for 5 min and then incubated on ice. Each sample was then complemented with 4 µl 5x first strand buffer (Invitrogen), 1 µl 0.1 M DTT and 1 µl Superscript reverse transcriptase and incubated at 55°C for 1h for synthesis. The reaction was inactivated by heating at 70°C for 15 min. In PCR detecting β7 integrin the primers 5´- GCT TGA AGA GTG ACC CAG AAA TCC - 3´ and 5´- AGC AAT GGT GTC TAC ACG AAC AGC – 3´ resulted in a 392bp product.

As an internal standard, expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a cellular housekeeping gene, was analyzed using the primers 5'- GGT GAA GGT CGG AGT CAA CGG ATT TGG TCG – 3´ and 5'-GGA TCT CGC TCC TGG AAG ATG GTG ATG GG-3' resulting in a 520bp product. The PCR

reaction was carried out in a 50 µl volume using 2 µl cDNA from the first strand reaction according to the manufacturer's instructions. The resulting DNA products were separated by 2% agarose gel electrophoresis containing 0.5 µg/ml ethidium bromide and photographed.

2.13 Transformation of fibroblastoid cells with α4 integrin cDNA

To express α4 integrin in fibroblastoid cells, target cells were infected with a retrovirus encoding α4 integrin cDNA (Gosslar et al., 1996). The cell line (GP+E-86) continuously producing this virus was kindly provided by B. Holzmann (Technical University Munich). In brief, subconfluent GP+E-86 cells were grown over night in fresh EF medium. The next morning the medium was collected, centrifuged and filtered with a cellulose acetate membrane pore size 0.2 µm (Renner GmbH Germany). This medium was complemented with polybrene to a final concentration of 8µg/ml and added to the target cells that were 50% - 80% confluent. After approximately 12h the medium was exchanged for fresh EF medium. The expression of α4 integrin was analysed by FACS analysis.

3. Results

3.1 Generation of mice with a deletion of the β1 and β7 integrin genes in the hematopoietic system

To test whether α4β1 and α4β7 have redundant functions mice were generated lacking both receptors in the hematopoietic system.

To achieve this goal, mice carrying a conditional knockout for β1 integrin, a β1-null allele and a cre recombinase transgene under the control of the polyIC inducible Mx-promotor were intercrossed with mice lacking a functional β7 integrin gene (Wagner et al.,, 1996). Thus, mice were obtained, which are deficient for β7 integrin and carry an inducible β1-null gene (β1 (fl/-) β7 (-/-) Mx-cre+ or β1 (fl/fl) β7 (-/-) Mx-cre+) and, as controls, mice, which are deficient of β7, but constitutively express β1 (β1 (fl/+) β7 (-/-) Mx-cre+ or β1 (fl/fl) β7 (-/-)). Since the Mx-promotor is strongly active in HSC, but also in many non-hematopoietic cells such as hepatocytes and endothelial cells, injection of polyIC into the conditional knockout mice would result in a loss of the β1 integrin gene in many tissues. To restrict the deletion to the hematopoietic system, BM from these mice was transplanted into lethally irradiated recipient mice after the complete repopulation of the hematopoietic system. The ablation of the conditional β1 gene was induced by three intraperitoneal injections of 250µg poly IC at two day intervals. The constitutive knockout of the β7 integrin gene was checked by genomic PCR of the BM donor mice and by FACS staining of cells derived from β7 integrin deficient BM chimeras in each experiment. In all analyses, no wild type β7 gene was detected (Fig. 11A).

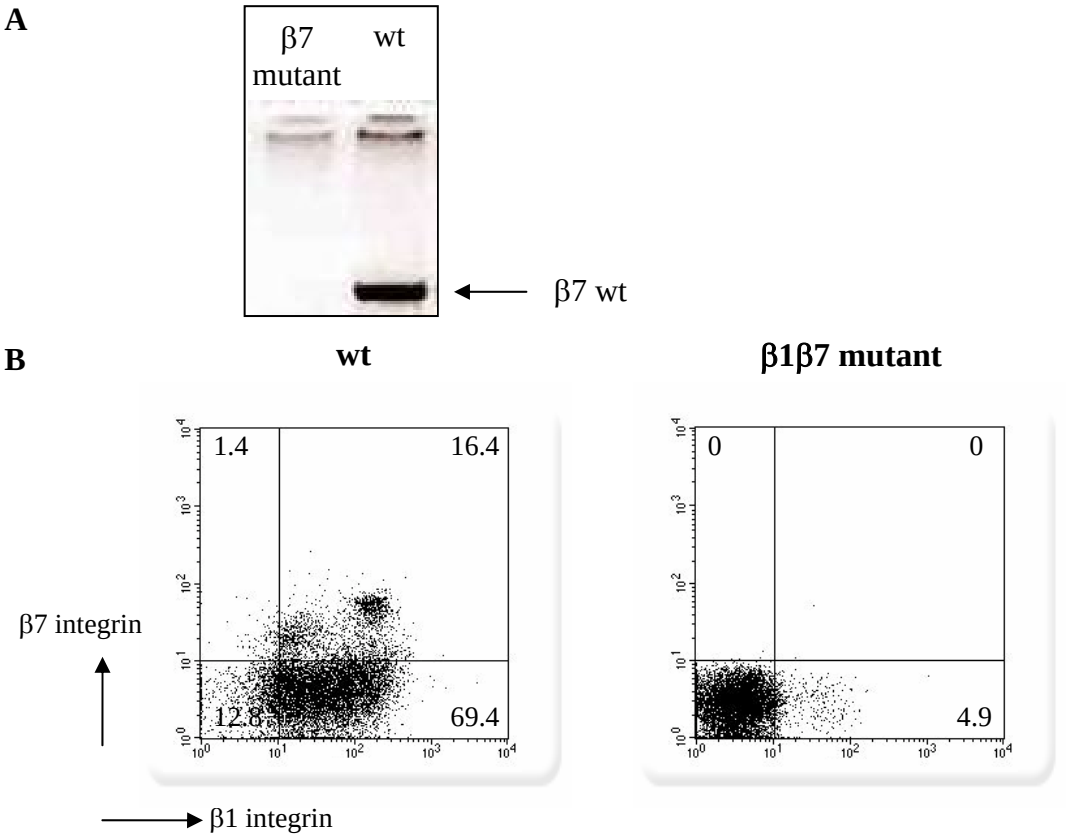


Figure 11: Loss of β1 and β7 integrin expression in BM cells of β1β7 mutant mice

A: PCR analysis of genomic DNA isolated from mouse tail showed no band for the β7 integrin wild type allele in a β7-deficient mouse.

B: Single cell suspensions from BM of wild type and β1β7 mutant mice were prepared 2 months after polyIC injection, stained with antibodies against β1 and β7 integrin and analysed by FACS. No β7+ and only little residual β1+ cells were detected.

FACS analysis confirmed the complete loss of β7 integrin expression, while in wild type mice approximately 18% of the cells express β7 integrin (Fig. 1B). Before knockout induction more than >85% of wild type BM cells expressed β1 integrin. 2 months after polyIC injection less than 5% of BM cells of β1β7 mutant mice expressed β1 integrin as determined by FACS (Fig. 1B).

To analyse the time course of the ablation of the β1 integrin gene we monitored the loss of β1 integrin expression on platelets, since platelets express high amounts of β1 integrin allowing a clear distinction of β1+ and β1- platelets. In addition, platelets and their precursors have a short life span of less than 2 weeks. A deletion of the β1 integrin gene on HSC is, therefore, quickly detected on

platelets. At day 0 β7 mutant and β1β7 mutant BM chimeras received the first of three injections of polyIC and blood was taken from the retro-orbital plexus after 2, 4, 7, 10, 14, 21 and 30 days. Platelets of these blood samples were checked for their β1 integrin expression. Two days after the first polyIC injection there were already β1 negative platelets detectable in β1β7 mutant BM chimeras (Fig. 12). In these mice the relative amount of β1 deficient platelets increased continuously to reach 93% after 14 days and 97% after 21 days and later. In β7 mutant mice, on the other hand, close to 0% of the β7 mutant platelets were β1 integrin negative at all time points.

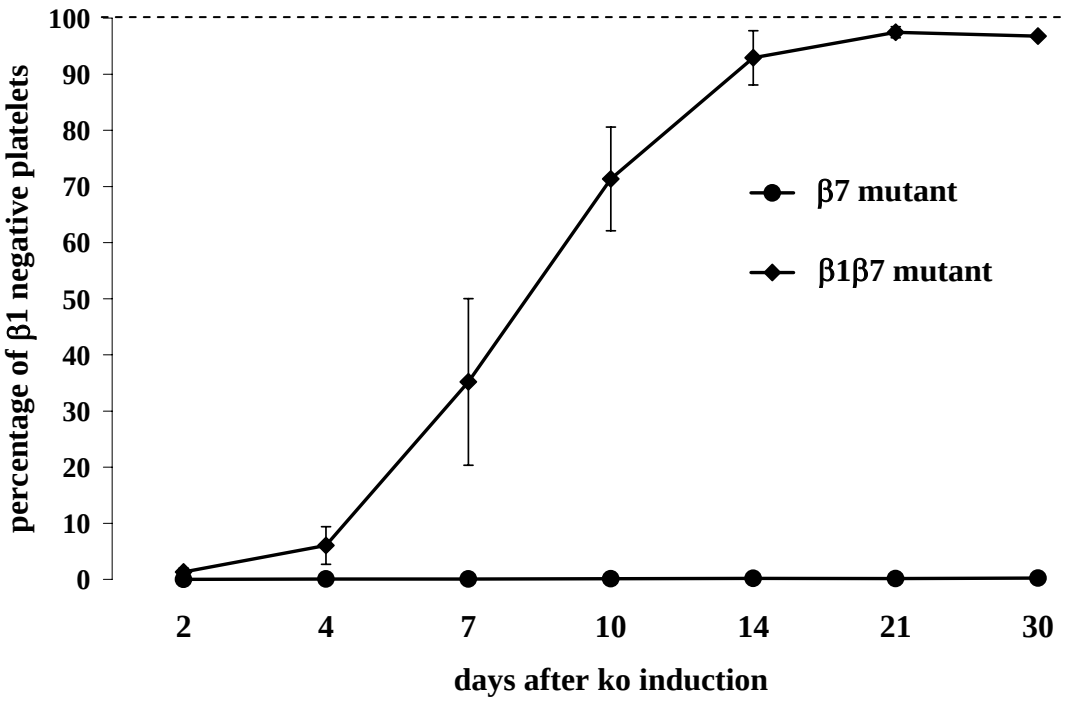


Figure 12: β1 integrin expression on platelets is quickly reduced after knockout induction
Percentages of β1 integrin negative platelets isolated from β7 mutant and β1β7 mutant BM chimeric mice are shown at indicated time points after the first of three polyIC injections (day 0). Error bars show the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 9/6).

These data show that the deletion of the β1 integrin gene can be induced within a few days in a β7 mutant background. Furthermore, they confirm that the development of megakaryocytes and platelets is not crucially dependent on β1 and β7 integrin.

To test the deletion of the β1 integrin gene in different lymphoid organs, Southern blot analysis of BM, thymus and spleen of β1β7 mutant and β7 mutant BM chimeras was carried out 10 months after the transient induction of the β1 gene deletion (Fig. 13).

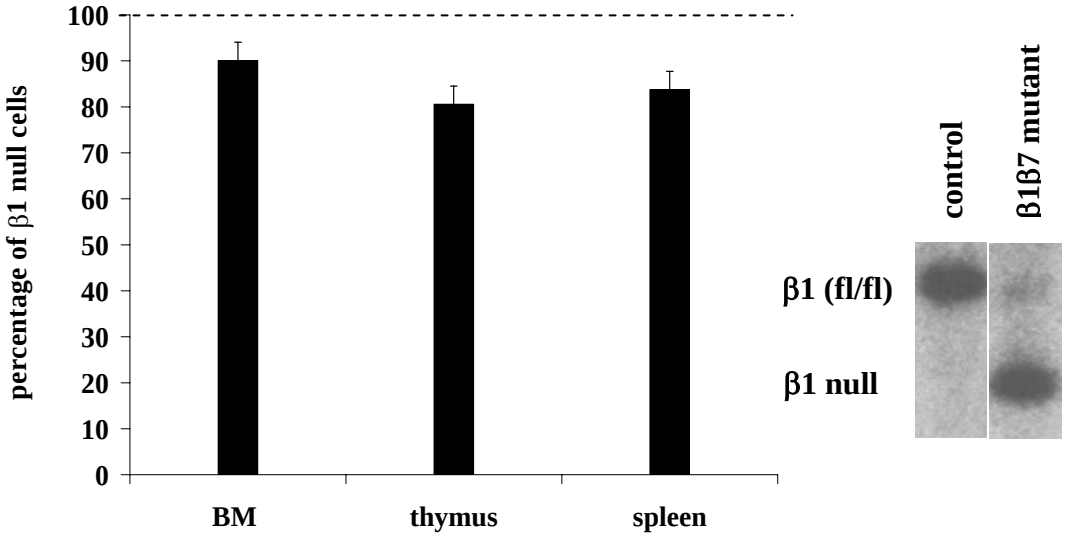


Figure 13: High efficiency of β1 integrin gene deletion in BM, thymus and spleen even 10 months after induction of the β1 gene deletion

DNA was isolated from single cell suspensions from BM, thymus and spleen from β1β7 mutant BM chimeras 10 months after polyIC treatment. Southern blot analysis detecting the conditional and the null allele was performed (a representative result is shown on the right panel). Band intensities were quantified and visualized in a bar graph. Error bars show the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 3/3).

An efficient knockout of the β1 integrin gene was detected in BM, thymus and spleen 10 months after the knockout induction (Fig. 13). Since only HSCs can sustain hematopoiesis for more than 3 months, these data indicate that β1β7 deficient HSCs are maintained *in vivo*.

3.2 Cellularity of lymphatic organs in the absence of β1 and β7 integrin

In order to investigate the development of different hematopoietic cells that derive from HSCs, we first checked the cellularity of different lymphoid organs. Lymphocyte migration to PP was reported to depend on β7 integrin (Wagner et al., 1996). As expected only small PP with severely decreased cellularity were found in β1β7 mutant BM chimeras.

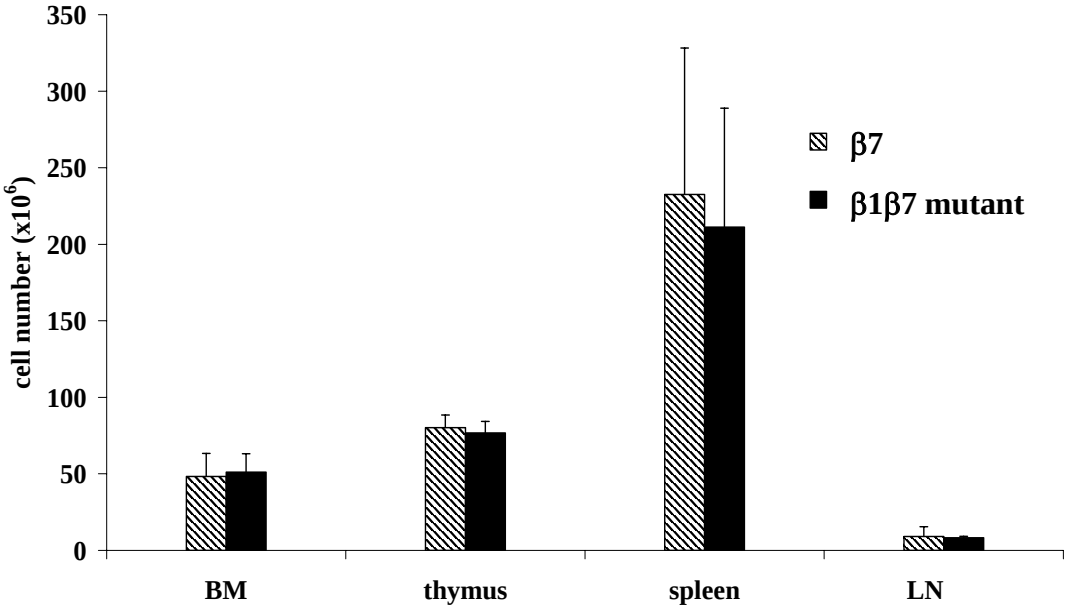


Figure 14: Normal cellularity of lymphoid organs of β1β7 mutant BM

Single cell suspensions from BM, thymus, spleen and lymph nodes (2 inguinal, 2 axial, 1 para-aortic) from β7 mutant and β1β7 mutant BM chimeric mice 2 months after the gene deletion were made and cells counted using a haemacytometer. The bar graph shows the absolute cell number in the respective tissues. Error bars show the standard deviation. n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): (4/4).

Neither 2 months (Fig. 14), nor 10 to 12 months (data not shown) after induction of the gene deletion any differences were observed in the cellularity of BM, thymus or spleen of β7 mutant and β1β7 mutant BM chimeras, giving no evidence for defective hematopoiesis in the absence of β1 and β7 integrins.

3.3 Analysis of polymorphonuclear and mononuclear cells in the peripheral blood

To determine the concentration of mononuclear (monocytes, lymphocytes) and polymorphonuclear (neutrophils) that are characterized by a lobed, fragmented nucleus, Türk staining of whole blood was conducted and cells counted using a haemacytometer. No significant differences were found comparing β7 mutant with β1β7 mutant mice (Fig. 15).

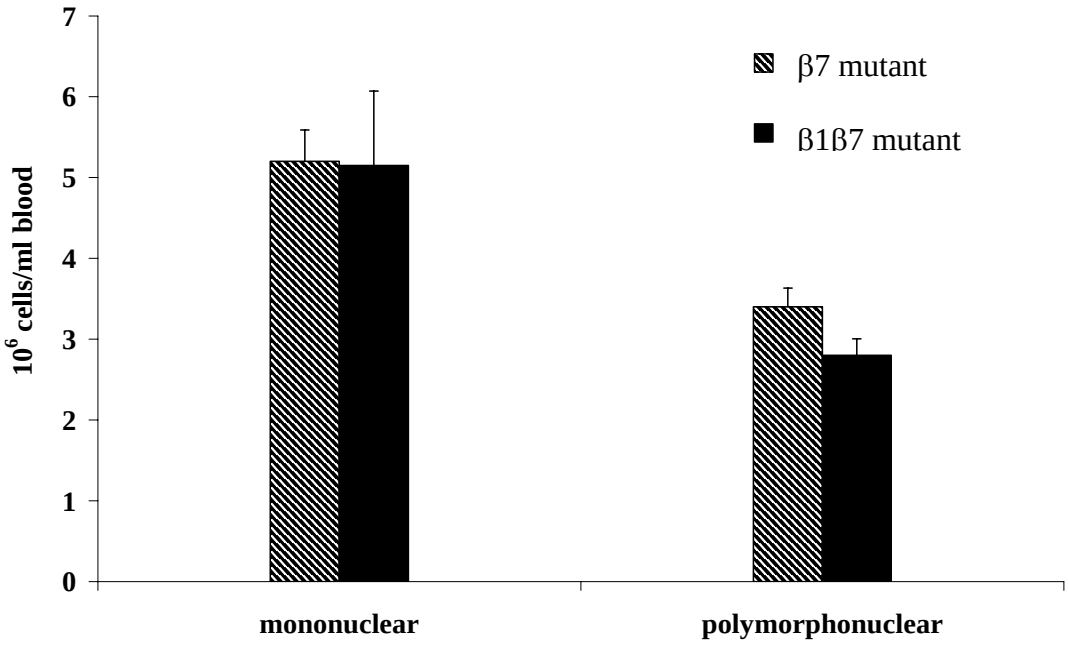


Figure 15: Normal concentrations of mononuclear and polymorphonuclear cells in the peripheral blood of β7 mutant and β1β7 mutant BM chimeras

Whole blood was collected retro-orbitally from β7 mutant and β1β7 mutant BM chimeric mice 6 months after the gene deletion. The blood was diluted 1:10 with Türk stain and differentially counted in a haemacytometer. The bar graph shows the concentration of the respective cells in the blood. Error bars show the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 4/4).

3.4 Retention of HSCs and HPCs in the absence of β1 and β7 integrin

α4 integrins were shown to be crucial to retain HPCs within the BM as antibodies blocking α4 led to a release of precursors into the blood (Papayannopoulou et al., 1995). In β1 mutant BM chimeric mice, however, HSCs were maintained in the BM and no increased release of HPCs was observed. To assess whether the simultaneous loss of β1 and β7 integrin reduces the amount of HSCs and HPCs in the BM, we determined the amount of lin- c-kit+ cells in the BM of β1β7 and β7 mutant mice 10 months after knockout induction. No significant reduction of HSCs/HPCs was detected in the absence of β1 and β7 integrins, suggesting that α4 integrins are not crucial for the retention of HSCs/HPCs in the BM (Fig. 16).

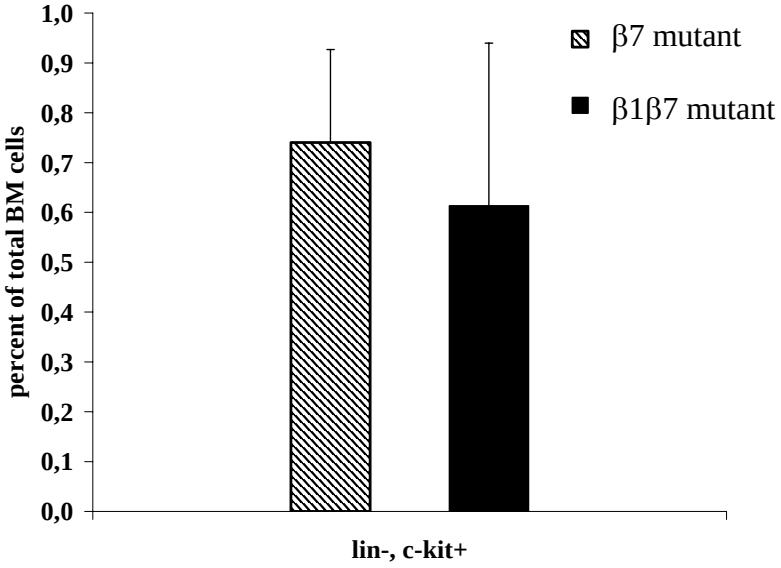


Figure 16: No differences in the number of HSCs/HPCs in the BM between β7 mutant and β1β7 mutant BM chimeras 10 months after the induction of the β1 integrin gene deletion.

Single cell suspensions were made from BM from β7 mutant and β1β7 mutant BM chimeras 10 months after the gene deletion and stained with a lineage cocktail containing the lineage markers B220, CD4, CD8, Mac-1, Gr-1, Nk1.1 and Ter119 and with c-kit. Cells derived from the host mice were excluded by a Ly5.1 counterstaining. The bar graph shows the relative amount of lineage negative, c-kit positive cells which should include all HSCs/HPCs. Error bars show the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 3/3).

3.5 Lymphopoiesis in the absence of β1 and β7 integrin

3.5.1 B cell development *in vitro*

Since previous studies suggested that B cell development was dependent on α4 integrin (Arroyo et al., 1999), but neither on β1 integrins (Brakebusch et al., 2002), nor on β7 integrins (Wagner et al., 1996) alone, we investigated whether β1 and β7 integrins have a redundant function in B cell development. To test for the development of pre B cells in the absence of β1 and β7 integrins *in vitro*, pre-B-colony formation assays with BM cells were performed. Both β7 mutant BM as well as β1β7 mutant BM gave rise to pre-B colonies of similar morphology (Fig. 17).

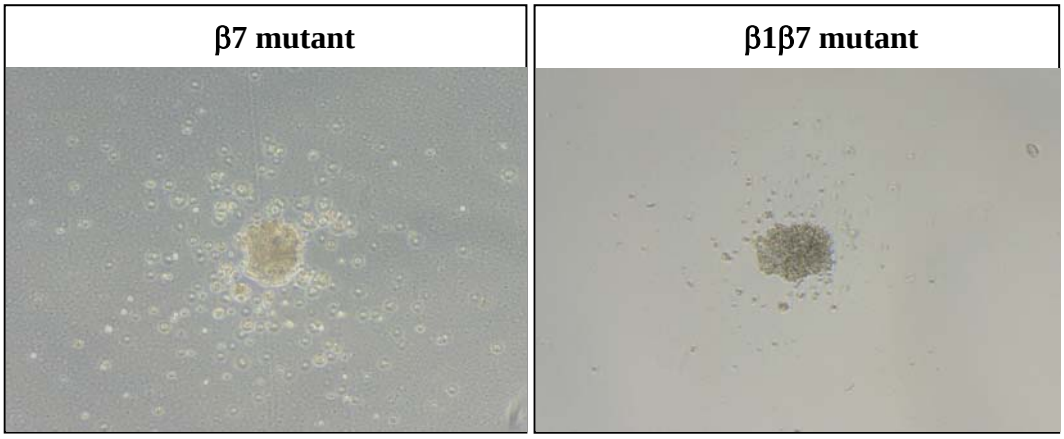


Figure 17: Pre-B cells from β7 mutant and from β1β7 mutant BM form colonies that are morphologically similar.
Single cell suspensions were made from BM from β7 mutant and β1β7 mutant BM chimeras 2 months after the gene deletion. 800000 BM cells were seeded into MethoCult M3630 medium and colonies were photographed (magnification 50x) 7 days later.

FACS analysis of the colonies confirmed that β7 mutant colonies were β1 integrin positive, while most of the β1β7 mutant colonies did not express β1 integrin (Fig. 18).

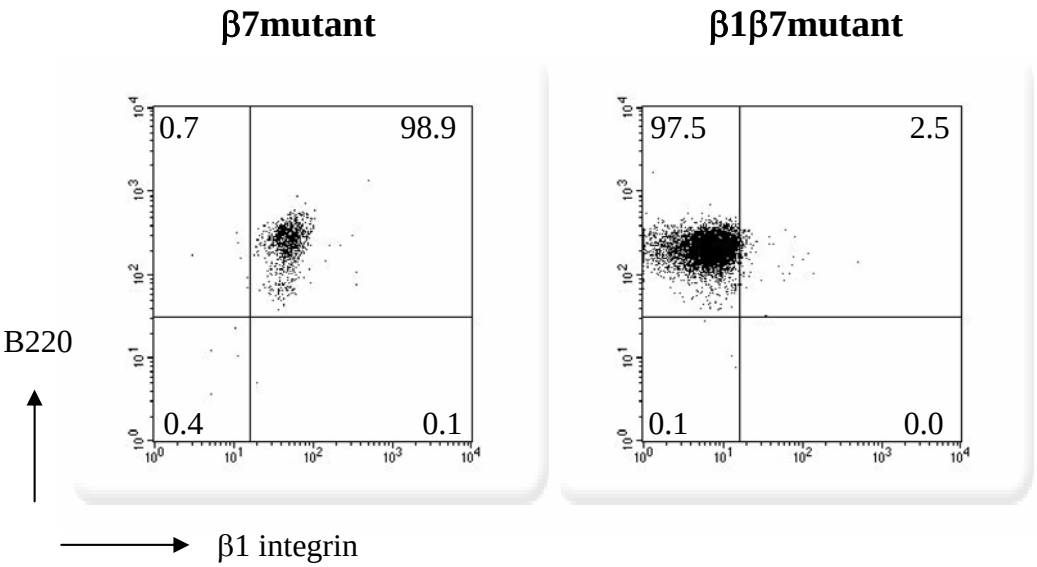


Figure 18: β1 integrin is absent on pre-B cell colonies forming from BM cells of β1β7 mutant BM chimeras.

Randomly picked pre-B cell colonies derived from BM of mice 2 months after knockout induction were stained with the B cell marker B220 in combination with β1 integrin and analysed by FACS. Colonies derived from β7 mutant BM chimeric mice were positive for β1 integrin and could clearly be distinguished from β1 negative clones derived from β1β7 mutant BM chimeras as the example shows.

36 of 39 colonies (92.3%) of β1β7 mutant BM did not express β1 integrin, while all colonies tested derived from β7 mutant BM expressed β1 integrin. No host derived colonies expressing Ly5.1 were detected. These results suggest already that there is no strong impairment of pre-B cell development, since the ratio of β1 integrin positive and negative colonies resembles the overall efficiency of β1 deletion of 90% in the BM as determined by Southern blot.

3.5.2 B cell development *in vivo* in the absence of β1 and β7 integrins

B cell development and integrin expression of β1β7 mutant BM chimeras were monitored in BM, spleen and LN by FACS analysis using various stage-specific surface markers:

A

BM	popul. size	
(n=4/4)	**β7mutant**	**β1 β7mutant**
B220med	**5.2**±1.6	**5.5**±1.2
B220 hi	**5.7**±1.8	**4.7**±1.1
CD19	**12.9**±6.2	**10.5**±2.5
IgM	**6.0**±1.1	**5.1**±1.4
IgD	**8.5**±6.5	**4.7**±1.1

Spleen	popul. size	
(n=4/4)	**β7mutant**	**β1 β7mutant**
B220hi	**40.5**±12.0	**37.7**±12.8
CD19	**38.9**±13.9	**36.9**±10.5
IgM	**34.6**±3.5	**32.8**±9.3
IgD	**39.7**±1.6	**45.5**±15.7

LN	popul. size	
(n=4/4)	**β7mutant**	**β1 β7mutant**
B220hi	**19.8**±4.0	**18.3**±4.5
CD19	**17.4**±4.3	**16.1**±5.5
IgM	**8.4**±3.7	**9.0**±3.5
IgD	**15.0**±1.7	**16.7**±7.0

B

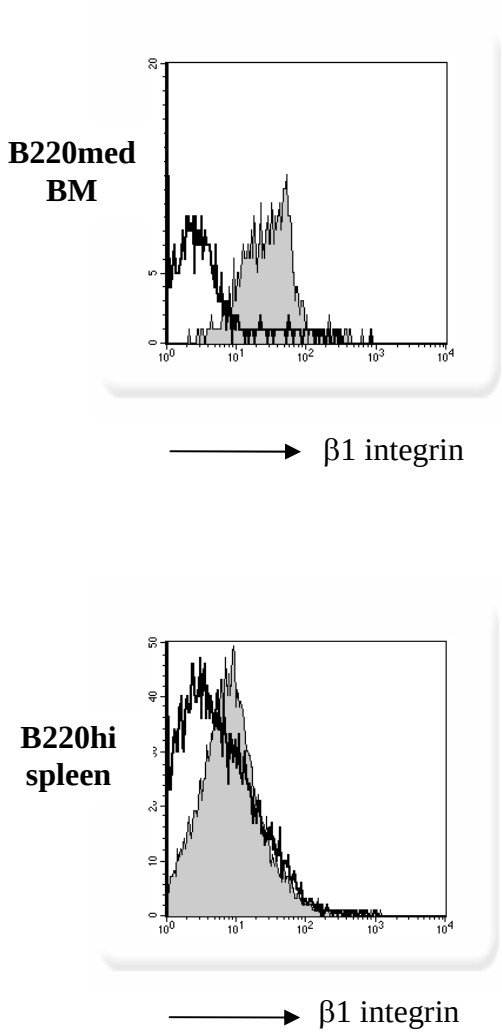


Figure 19: Normal B cell population sizes in β1β7 mutant mice

A: Single cell suspensions from BM, spleen and LN of β7 mutant and β1β7 mutant BM chimeras 2 months after polyIC treatment were prepared, stained with antibodies against B220, CD19, IgM, IgD and against β1 integrin and analysed by FACS. The averages of the population sizes in the respective tissues are shown with standard deviations. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 4/4).

B: Representative histogram overlays show the β1 integrin expression of β7 mutant controls (filled) and of β1β7 mutant BM chimeras (line). The upper diagram visualizes the loss of β1 expression in β1β7 mutant on immature B cells in the BM (B220 medium cells), the lower one shows the difference in β1 expression for mature B cells in the spleen (B220 high).

B220 (pre-pro-B and later), CD19 (pro-B and later), IgM (immature B) and IgD (mature B). 2 months after knockout induction no significant differences were obtained between β7 and β1β7 mutant BM chimeras for the sizes of the populations characterised by these markers in any of these tissues investigated (Fig 19). All B cell subpopulations in β1β7 mutant BM chimeras showed a loss of β1 integrin expression as determined by FACS. This reduction was more obvious on B220medium cells, which express higher levels of β1 integrin than B220high cells.

10 to 12 months after the knockout induction of the β1 integrin gene again B cell development was analysed in BM and spleen using the B cell lineage affiliated markers B220, CD19, IgM and IgD (Fig. 20).
Also 10 to 12 months after the induction of the β1 integrin gene deletion, B cell development was rather normal, although a slight reduction of B220med cells was observed. In LN a small reduction in the number of B cells was detected. However, only 3 β7 mutant and 3 β1β7 mutant tissues were analysed. More mice have to be analysed to test the significance of these alteration.

A

BM	**popul. size**	
(n=5/5)	**β7mutant**	**β1 β7mutant**
B220med	**3.7**±1.6	**1.6**±0.8 *
B220 hi	**7.8**±3.9	**9.7**±7.6
CD19	**9.1**±3.7	**8.6**±6.7
IgM	**4.3**±1.7	**6.6**±3.6
IgD	**4.7**±2.4	**5.5**±4.1

spleen	**popul. size**	
(n=5/5)	**β7mutant**	**β1 β7mutant**
B220hi	**50.7**±9.7	**39.7**±11.3
CD19	**48.6**±11.6	**40.7**±8.4
IgM	**28.4**±7.0	**22.3**±6.5
IgD	**36.7**±9.6	**28.9**±4.8

LN	**popul. size**	
(n=3/3)	**β7mutant**	**β1 β7mutant**
B220hi	**57.9**±2.8	**38.9**±11.0 *
CD19	**58.4**±2.5	**39.9**±11.1 *
IgM	**20.3**±6.1	**11.3**±4.0 *
IgD	**47.0**±0.3	**31.4**±11.3 *

B

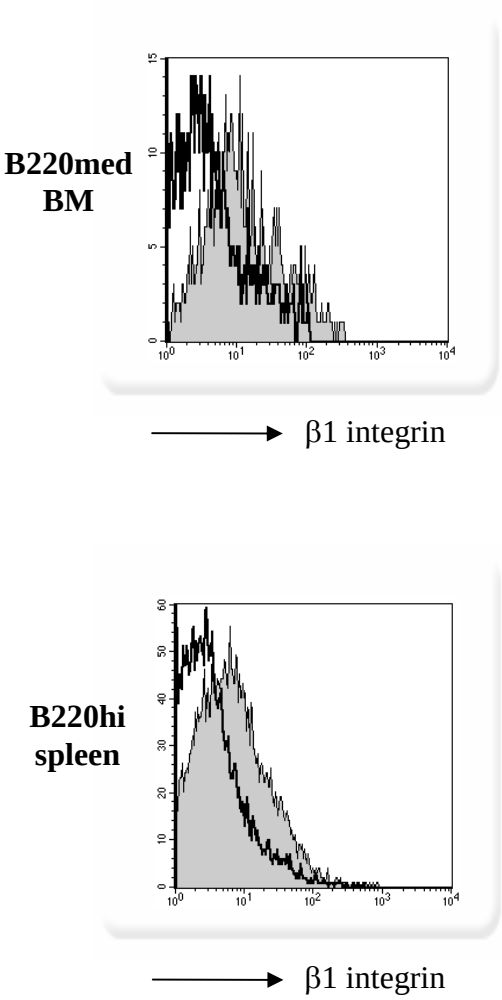


Figure 20: Normal B cell population sizes in β1β7 mutant mice

A: Single cell suspensions from BM, spleen and LN of β7 mutant and β1β7 mutant BM chimeras 10 to 12 months after polyIC treatment were prepared, stained with antibodies against B220, CD19, IgM, IgD and against β1 integrin and analysed by FACS. The averages and standard deviation of the population sizes are shown in the tables for the respective tissues, asterisks indicate a significant difference according to student's *t* test. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): BM 5/5, spleen 5/5, LN 3/3).

B: Representative histogram overlays show the β1 integrin expression of β7 mutant controls (filled) and the β1 expression of β1β7 mutant β1β7 BM chimeras (line). The upper diagram visualizes the loss of β1 expression in β1β7 mutant on immature B cells in the BM (B220 medium cells), the lower one shows the difference in β1 expression for mature B cells in the spleen (B220 high).

The low expression of β1 integrin on B220hi cells even in the β7 mutant control mice made it difficult to quantify the loss of β1 integrin in this subpopulation in β1β7 mutant mice using FACS. Therefore, we determined the knockout efficiency in B220+ B cells from spleen, purified by MACS beads, on genetic level using Southern blot (Fig.21).

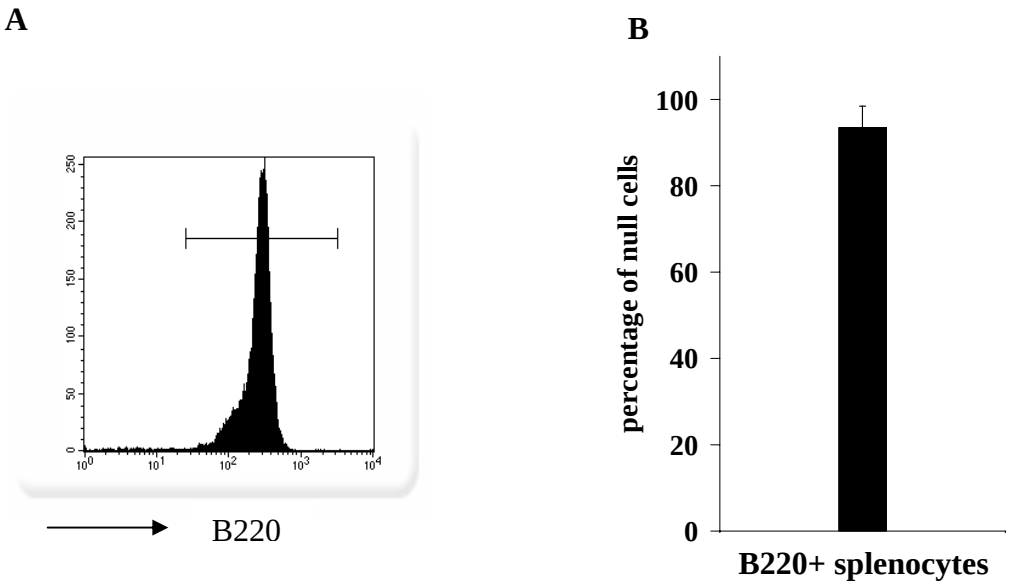


Figure 21: B220+ B cells have an efficient deletion of the β1 integrin gene

Single cell suspensions from spleen of β7 mutant and β1β7 mutant BM chimeras 6 months after polyIC treatment were prepared, stained with B220-FITC antibody and subsequently sorted using anti FITC MACS beads.

A: FACS analysis of the B220+ enriched fraction indicated higher than 95% purity (representative histogram is shown)

B: DNA was prepared from MACS-enriched B220+ splenocytes, Southern blot performed and densitrometically evaluated. The bar graph shows the relative amount of B220+ cells deficient for a functional β1 integrin gene. Error bar shows the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 5/5).

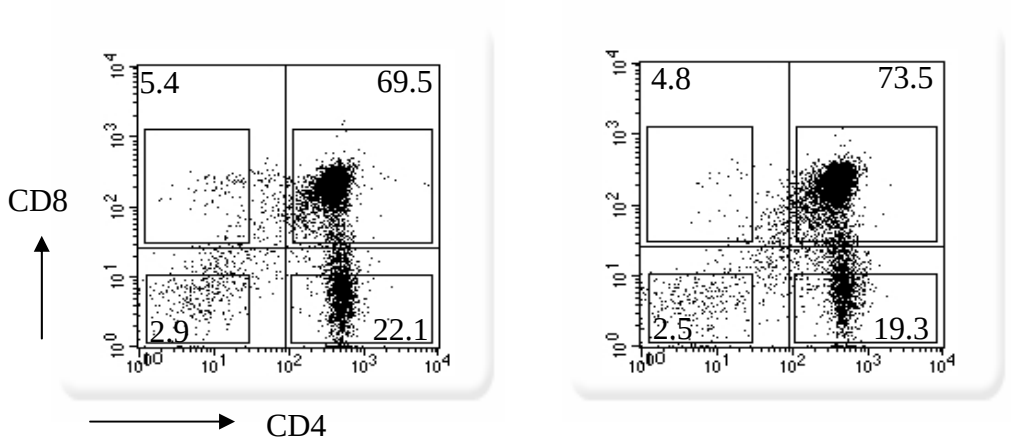
These data indicate that in the absence of β1 and β7 integrins B cells can fully mature and migrate to spleen, LN and, as B220hi cells, from the periphery back to the BM.

3.5.3 T cell development in the absence of β1 and β7 integrins

T cell precursors originate from the BM and migrate to the thymus. In the thymus they differentiate from thymocytes that lack CD4 and CD8 (double negative (DN)) via thymocytes that express both CD4 and CD8 (double positive (DP)) into mature T cells expressing either CD4 (CD4 single positive (CD4SP)) or CD8 (CD8 single positve (CD8SP)) (Fig. 22).
Using α4 null somatic chimeras α4 integrin was reported to be essential for the emigration of T cell precursors from the BM to the thymus (Arroyo et al.,, 1996) while individual loss of α4β1 or α4β7 did not effect thymus colonisation (Brakebusch et al., 2002, Wagner et al., 1996). To test whether α4β1 and α4β7 have overlapping function in this respect T cell development was studied in β1β7 mutant BM chimeras by FACS analysis of thymocytes using the T cell specific markers CD4 and CD8. In contrast to the expectations the relative amount of DN, DP, CD4SP and CD8SP cells in the thymus was unaltered in β1β7 mutant BM chimeric mice as compared to β7 mutant BM chimeras (Fig. 22).

No significant difference was found in the population sizes of DN thymocytes, which contain the early thymic immigrants indicating that thymus colonisation was not altered in β1β7 mutant BM chimeric mice which lack both α4β1 and α4β7 integrin. Also further thymocytes maturation to DP, CD4SP and CD8SP cells was normal in β1β7 mutant BM chimeric mice as indicated by the similar population sizes in β7 mutant BM chimeras (Fig. 22). Normal numbers of CD4 and CD8 T cells in spleen, LN and BM of β1β7 mutant BM chimeras 2 and 10 to12 months after induction of the β1 gene deletion suggested unimpaired migration of these cells to secondary lymphoid organs and to the BM (Fig. 23 and 24). Due to severe age dependent thymus involution in β7 mutant and β1β7 mutant BM chimeras thymocytes were not analysed.

A



B

thy	popul. size	
(n=4/4)	**β7mutant**	**β1β7mutant**
DN	**3.0**±1.2	**2.0**±0.4
DP	**70.2**±2.9	**76.5**±4.3
CD4SP	**20.0**±3.4	**17.3**±3.0
CD8SP	**6.7**±1.0	**4.2**±2.0

C

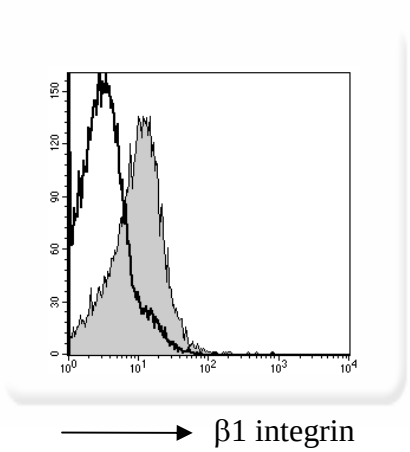


Figure 22: Normal T cell development in the absence of β1 and β7 integrins

Single cell suspensions from thymus of β7 mutant and β1β7 mutant BM chimeras 2 months after polyIC injection were prepared, stained with antibodies against CD4, CD8 and β1integrin and analysed by FACS.

A: The dot blots show a representative staining of DN, DP, CD4SP and CD8SP thymocytes for both β1 mutant and β1β7 mutant BM chimeras.

B: The averages and standard deviation of the population sizes are shown in the table for the respective subpopulations. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 4/4).

C: A representative histogram displays β1 integrin expression on DP T cells of β7 mutant (filled) and β1β7 mutant (line) mice, indicating efficient loss of β1 integrin in β1β7 BM chimeras.

A

BM	popul. size	
(n=4/4)	**β7mutant**	**β1β7mutant**
CD4+	**3.7**±2.3	**1.8**±1.5
CD8+	**2.2**±1.4	**1**±0.4

spleen	popul. size	
(n=4/4)	**β7mutant**	**β1β7mutant**
CD4+	**38.3**±5.9	**33.1**±10.9
CD8+	**11.3**±4.8	**8.4**±3.2

LN	popul. size	
(n=4/4)	**β7mutant**	**β1β7mutant**
CD4+	**57.4**±7.4	**56.7**±1.9
CD8+	**24.0**±5.8	**22.7**±2.4

B

BM	popul. size	
(n=5/5)	**β7mutant**	**β1β7mutant**
CD4SP	**2.8**±1.0	**3.3**±2.4
CD8SP	**3.5**±1.0	**4.4**±2.2

spleen	popul. size	
(n=5/5)	**β7mutant**	**β1β7mutant**
CD4SP	**32.0**±10.2	**27.6**±9.1
CD8SP	**11.6**±6.1	**14.1**±7.5

LN	popul. size	
(n=3/3)	**β7mutant**	**β1β7mutant**
CD4SP	**24.6**±1.5	**32.8**±4.2 *
CD8SP	**13.8**±2.5	**22.4**±8.4

Fig. 23: β1β7 deficient T cells home normally to spleen, LN and BM

Single cell suspensions from spleen and LN of β7 mutant and β1β7 mutant BM chimeras 2 months (A) and 10 to 12 months (B) after polyIC treatment were prepared, stained with antibodies against CD4 and CD8 in combination with antibodies against β1 integrin and analysed in FACS. The averages and standard deviation of the population sizes are shown in the tables for the respective tissues, the asterisk indicates a significant difference according to student's *t* test ($p<0.05$). (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 5/5).

In the LN of β1β7 mutant BM chimeric mice 10-12 months after the induction of the β1 integrin gene deletion more CD4+ and CD8+ T cells were observed. No conclusion can be made whether this difference is within the range of mouse to mouse variations or due to alterations in the migration of β1 and β7 deficient T cells to LN as only 3 mice were analysed. More mice need to be analysed to clarify this point. FACS analysis of DP thymocytes indicated an efficient loss of β1 integrin expression in β1β7 mutant BM chimeric mice Fig. 22C. Since CD4SP, CD8SP and naïve peripheral T cells express only little amounts of β1 integrin, the deletion efficiency in these populations was tested on genomic level. Southern blot analysis of thymocytes 10-12 months after the knockout induction, including around 20-25% CD4SP and CD8SP cells showed a gene deletion efficiency of more than 80% suggesting that most of the SP cells have to be β1 integrin deficient (Fig. 23). Southern blot of MACS beads enriched CD4 and CD8 cell from the spleen proved the high knockout efficiency on mature T cells (Fig. 14).

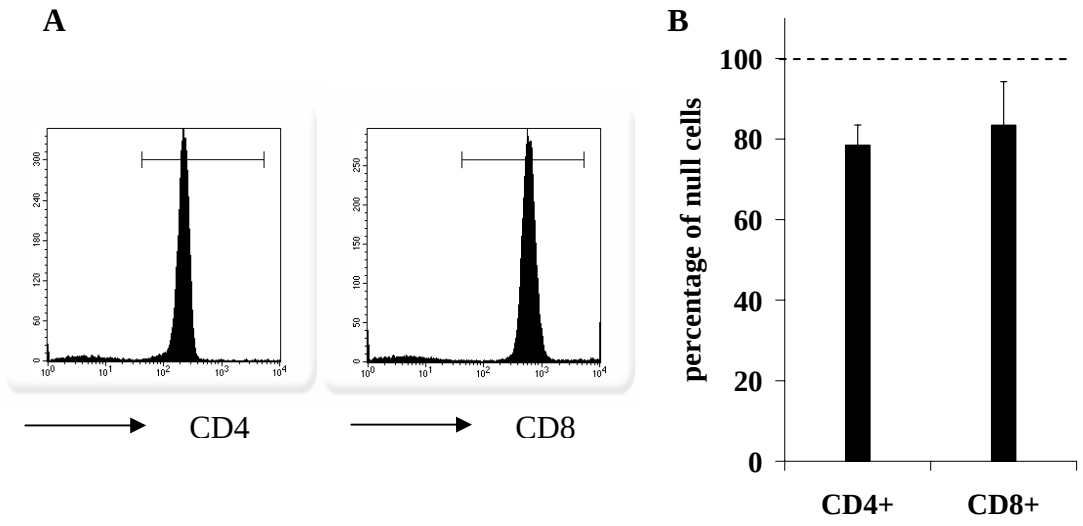


Figure 24: CD4+ and CD8+ T cells have an efficient deletion of the β1 integrin gene

Single cell suspensions from spleen of β7 mutant and β1β7 mutant BM chimeras 6 months after polyIC treatment were prepared, stained with CD4-FITC and CD8-FITC antibody and subsequently sorted using anti FITC MACS beads.

A: FACS analysis of the CD4+ and CD8+ enriched fraction indicated higher than 95% purity (representative histogram is shown)

B: DNA was prepared from MACS-enriched CD4+ and CD8+ splenocytes, Southern blot performed and densitrometically evaluated. The bar graph shows the relative amount of

CD4+ and CD8+ cells deficient for a functional β1 integrin gene. Error bar shows the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 5/5).

These data indicate, that β1 and β7 integrins are neither essential for the migration of T cell precursors to the thymus, nor for T cell development and trafficking.

3.6 Myeloid and erythroid development in the absence of β1 and β7 integrins

3.6.1 Myeloid and erythroid development *in vitro*

α4 integrins are suggested to be required for normal myeloid differentiation in fetal liver (Arroyo et al.,, 1999). In adult somatic α4 null chimeric mice, the number of myeloid cells was low and no erythrocytes were detected originating from α4 null HSCs. β7-null mice and β1 mutant BM chimeras, on the other hand, displayed normal myeloid and erythroid development (Wagner et al.,, 1996; Brakebusch et al., 2002). To test whether the defect described for the α4-null somatic chimeras can be observed in the absence of both α4β1 and α4β7 integrins, first the capacity of myeloid and erythroid progenitors was tested to form granulocyte/monocyte (GM) and colony forming units (erythroid) (CFUe) colonies, respectively. GM colony assays were conducted with BM cells, splenocytes and peripheral blood after erythrocyte lysis from β7 mutant and β1β7 mutant BM chimeric mice 2 months after the β1 integrin gene deletion. CFUe assays were performed with BM cells and splenocytes (Fig. 25).

β1β7 mutant and β7 mutant control cells from all tissues formed GM and CFUe colonies that were morphologically similar (Fig. 25). To assess whether colonies derived from β1β7 mutant BM chimeric mice are deficient for β1 integrin, GM colonies were analysed by FACS for β1 expression (Fig. 26A) and the small CFUe colonies were genotyped by genomic PCR (Fig.26B).

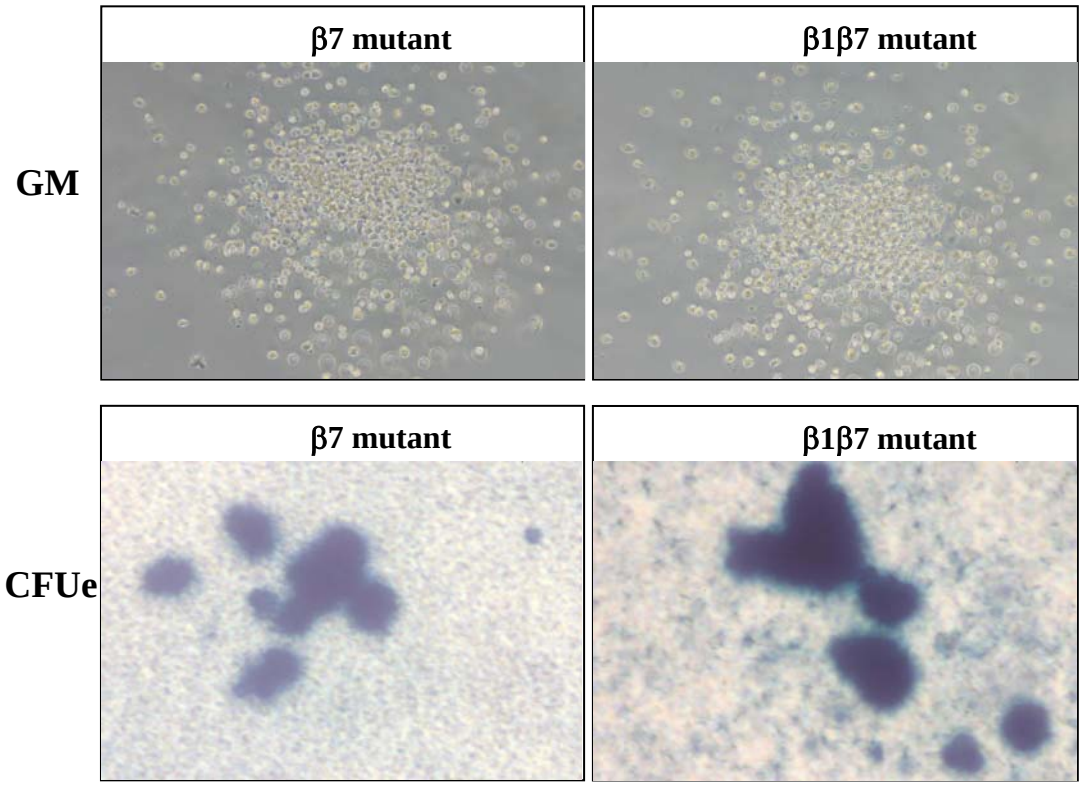


Figure 25: Granulocyte/monocyte progenitor cells (upper panel) and erythroid progenitor cells (lower panel) from β1β7 mutant mice form colonies that are indistinguishable from β7 mutants

Single cell suspensions were made from BM from β7 mutant and β1β7 mutant BM chimeras 2 months after the gene deletion. 180.000 BM cells were seeded into MethoCult GF M3534 medium and photographed (magnification 50x) 7 days later. For CFUe 600.000 BM cells were seeded in MethoCult M3334, stained with benzidine 3 days later and photographed (magnification 100x). Examples of colonies derived from BM are shown.

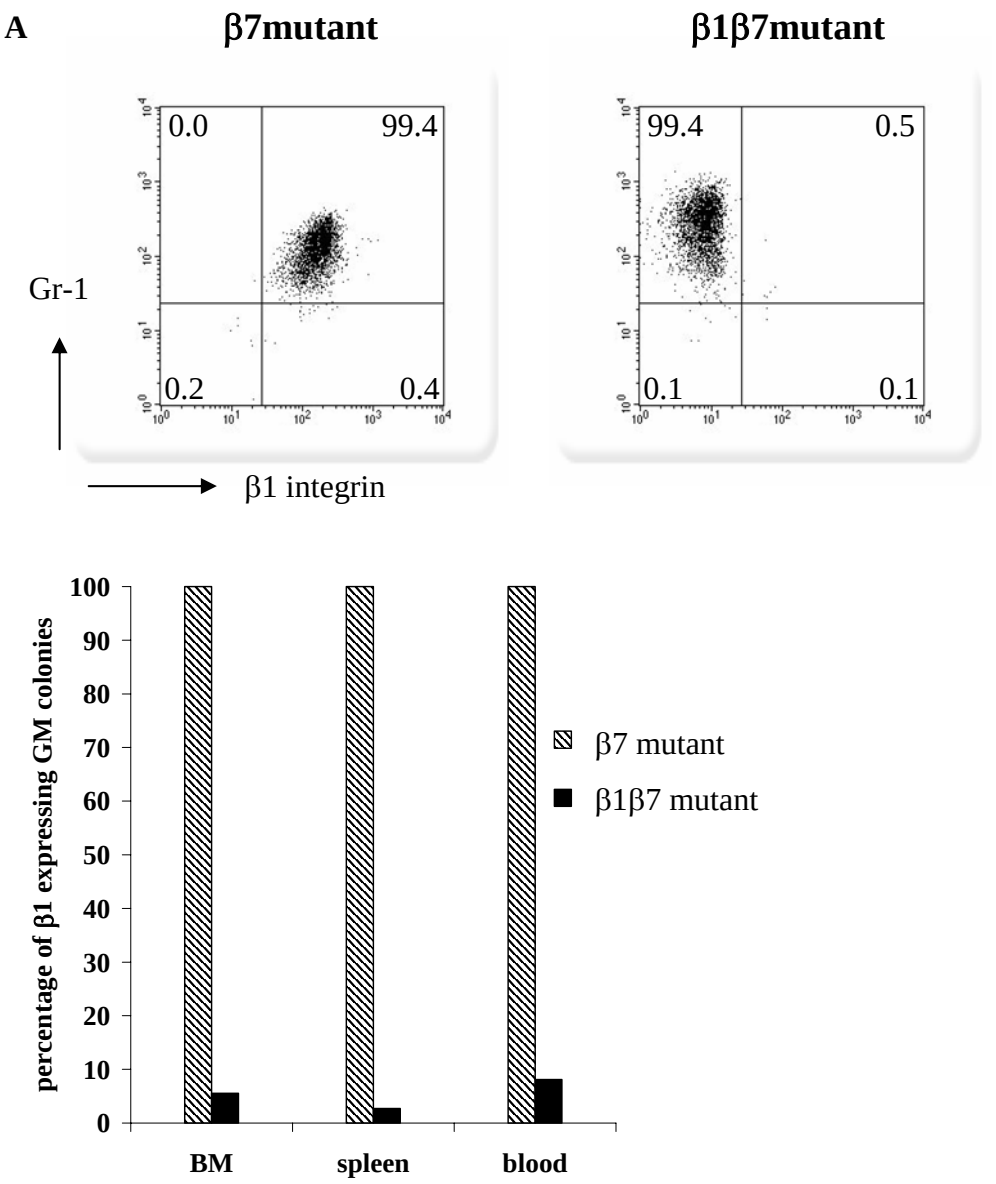


Figure 26 B: Efficient loss of β1 integrin on GM precursors of β1β7 BM chimeras

A: Randomly picked GM colonies derived from BM, spleen or PB of mice 2 months after knockout induction were stained with the granulocyte marker Gr-1 in combination with β1 integrin and analysed by FACS. Colonies derived from β7 mutant BM chimeric mice are positive for β1 integrin and can clearly be distinguished from β1 negative clones derived from β1β7 mutant BM chimeras as the example shows. Nearly all β1β7 mutant colonies were β1 integrin deficient. Colonies of three mice were combined and β1+ and β1- colonies of β7 mutant and β1β7 mutant BM chimeras are shown as percentages, respectively.

B

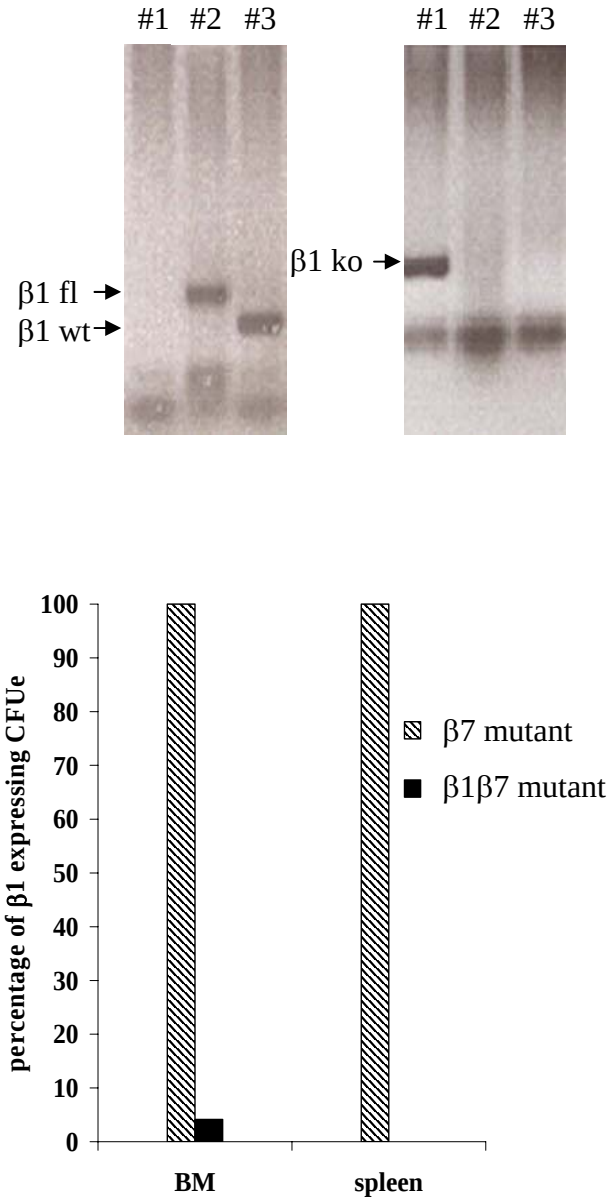


Figure 26 B: Efficient loss of β1 integrin on CFUe of β1β7 BM chimeras

Genotyping of randomly picked CFUe of BM or spleen of β7 mutant and β1β7 mutant BM chimeras enables distinction between the β1 integrin null allele (#1), conditional allele (#2) and wild type allele (#3). The results of three exemplary colonies, one for each genotype, are shown. Genomic DNA was randomly amplified and then analysed by specific PCRs detecting β1 (fl), β1 (null) and β1 (wt). CFUe from β1β7 mutant BM chimeras were β1 deficient. Colonies of four mice were combined and β1+ and β1- colonies of β7 mutant and β1β7 mutant BM chimeras are shown as percentages, respectively.

All GM colonies analysed from BM (37), spleen (38) and peripheral blood (PB) (36) from β7 mutant mice were positive for β1 integrin as expected. From β1β7 mutant BM chimeras only 2 of 36 colonies from the BM, 1 of 37 of the spleen and 3 of 37 colonies derived from PB were positive for β1 integrin. All 19 CFUe colonies (13 in BM, 6 in spleen) derived from the β7 mutant control were positive for β1 integrin, while in β1β7 mutant BM only 1 of 24 colonies and none of three in the spleen was β1 integrin positive. This result shows first, that in the absence of β1 and β7 integrin granulocyte/monocyte and erythroid precursors have the potential to form a colony *in vitro* and second, that the efficiency of the β1 integrin gene deletion is also very high on the myeloid and erythroid lineage.

3.6.2 Myeloid and erythroid development *in vivo*

To investigate the role of β1 and β7 integrins in myeloid and erythroid differentiation *in vivo*, the development of monocytes, granulocytes, and erythroblasts was monitored in β1β7 mutant BM chimeric mice 2 months after the β1 integrin gene deletion by FACS analysis using the markers Mac-1 (monocytes, granulocytes), Gr-1 (granulocytes), and Ter119 (erythroblasts), respectively (Fig. 27).

In BM of β7 mutant and β1β7 mutant BM chimeras similar amounts of granulocytes, monocytes and erythroblasts were observed indicating no developmental defects in the absence of both α4β1 and α4β7 integrins.

To prove that HSCs and HPCs continuously provide myeloid and erythroid cells we analysed β1β7mutant BM chimeras 10 to 12 months after the β1 integrin gene deletion (Fig. 28).

Even after 10-12 months no significant differences in the population sizes of Gr-1+, Mac-1+ and Ter119+ cells were found between β7 mutant and β1β7mutant BM chimeric mice. The percentage of β1 integrin expressing Gr-1 medium cells increased slightly from 5.6%±4.7% at 2 months to 9.3%±2.7% at 10-12 months after knockout induction. Whether this points to a subtle competitive advantage of

β1+ granulocytes precursors versus β1 null progenitors has to be checked by analysis of more mice.

BM	popul. size	
(n=4/4)	β7mutant	β1β7mutant
Gr-1+	50,7±4.9	63.5±11.0
Mac-1+	64.4±10.6	73.5±14.2
Ter119+	19.3±3.5	14.1±8.6

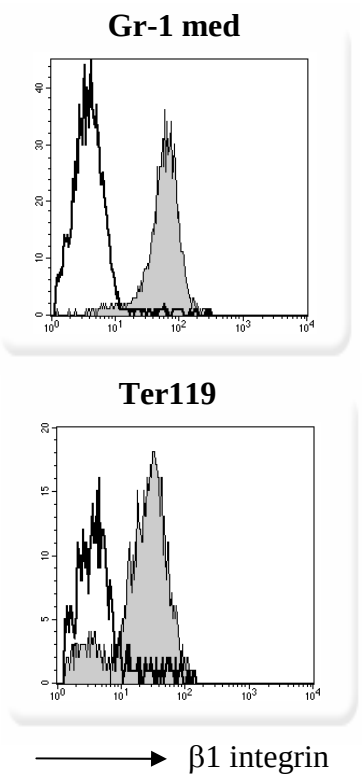


Figure 27: β1 and β7 integrin are not essential for granulocyte, monocyte or erythroblast development 2 months after knockout induction

Single cell suspensions from BM of β7 mutant and β1β7 mutant BM chimeras two months after polyIC treatment were prepared, stained with antibodies against Gr-1, Mac-1 and Ter119 in combination with antibodies against β1 integrin and analysed in FACS. The relative size of granulocyte, granulocyte/monocyte and erythroblast subpopulations is shown in the table. The β1 integrin expression of immature granulocytes (Gr-1medium) and erythroblasts (Ter119+) of β7 mutant (filled) and the β1 expression of β1β7 mutant BM chimeras (line) is shown in representative histogram overlays (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 4/4).

A

BM	popul. size	
(n=5/5)	β7mutant	β1β7mutant
Gr-1+	61.7±7.3	50.6±17.5
Mac-1+	66.0±6.8	60.6±11.7
Ter119+	24.9±4.8	26.9±10.9

B

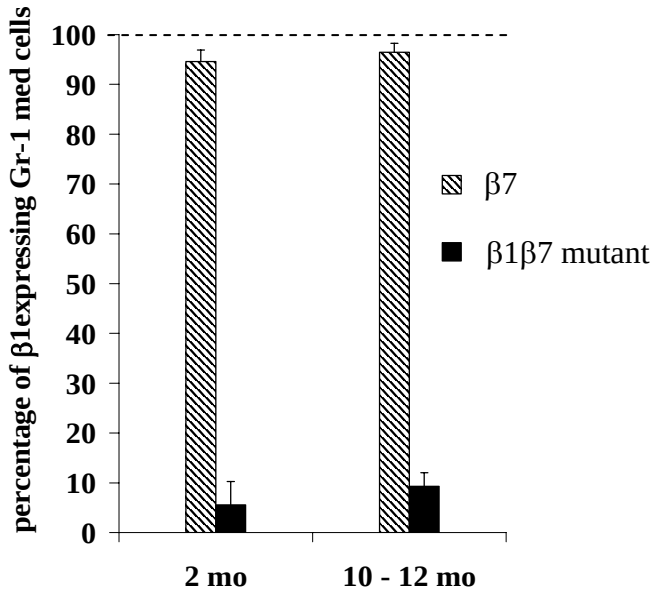


Figure 28: β1 and β7 integrin are not essential for granulocyte, monocyte or erythroblast development 10 to 12 months after the β1 integrin gene deletion

Single cell suspensions from BM of β7 mutant and β1β7 mutant BM chimeras 10 to 12 months after polyIC treatment were prepared.

A: Cells were stained with antibodies against Gr-1, Mac-1 and Ter119 in combination with antibodies against β1 integrin and analysed in FACS. The relative size of granulocyte, granulocyte/monocyte and erythroblast subpopulations is shown in the table. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 5/5).

B: The β1 integrin expression of Gr-1 med cells was assessed by staining for Gr-1 in combination with β1 integrin. This population has very high β1 expression levels and was therefore chosen to monitor the deletion efficiency. Error bars show the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 2 mo (4/4); 10-12 mo 5/5).

3.6.3 Migration of granulocyte/monocyte progenitors to secondary lymphoid organs

During our investigation of the possible functional redundancy of β1 and β7 integrin in the hematopoietic system and the analysis of the blood cell development in the absence of both α4β1 and α4β7 integrin, a function of α4 integrin for the distribution of blood cell progenitors was reported (Scott et al., 2003). The authors of this study generated α4 conditional knockout mice with a Mx-cre mediated deletion, but did not restrict the gene deletion to the hematopoietic system by transplanting BM into lethally irradiated wild type mice. In this system, the authors found that CFU-C progenitors, comparable to GM colony forming units, occurred in similar number in the BM (52.488±7.543 per femur in α4 mutants versus 49.554±3.615 per femur in controls), but were more numerous in the PB of α4 mutant mice leading to an increase from 142±13 CFU-C/ml in control mice to 1.177±71 CFU-C/ml in mice two weeks after the induction of the gene ablation. The increased amount of progenitors in the periphery of the α4 deficient mice therefore did not correspond to a decrease of progenitors in the BM. At that time the number of progenitor cells within the spleen was moderately increased from 29.538±2.549 in controls to 39.994±3.680/spleen in deletion-induced mice. 6 months after the gene deletion the number of CFU-C was elevated by approximately 30% in BM and 160% in spleen compared to control animals.

To test whether there are similar alterations in β1β7 mutant BM chimeras compared to β7 mutant controls, the frequency of GM progenitors (equivalent to CFU-C) was assessed in BM, PB and spleen 2, 6 and 10 months after the β1 integrin gene deletion (Fig. 29).

These data indicate that after deletion of β1 and β7 integrin in BM chimeric mice the number of GM progenitors in the BM increases after 2 months roughly two-fold, but becomes normal again at 6 months and 10 months. Correspondingly, GM progenitor numbers in PB were increased more than two-fold 2 months after ko induction, but normal at 6 or 10 months. In spleen, the number of GM progenitors was unchanged 2 months after the induction of the β1 gene deletion, but decreased in β1β7 mutant BM chimeras after 10 months.

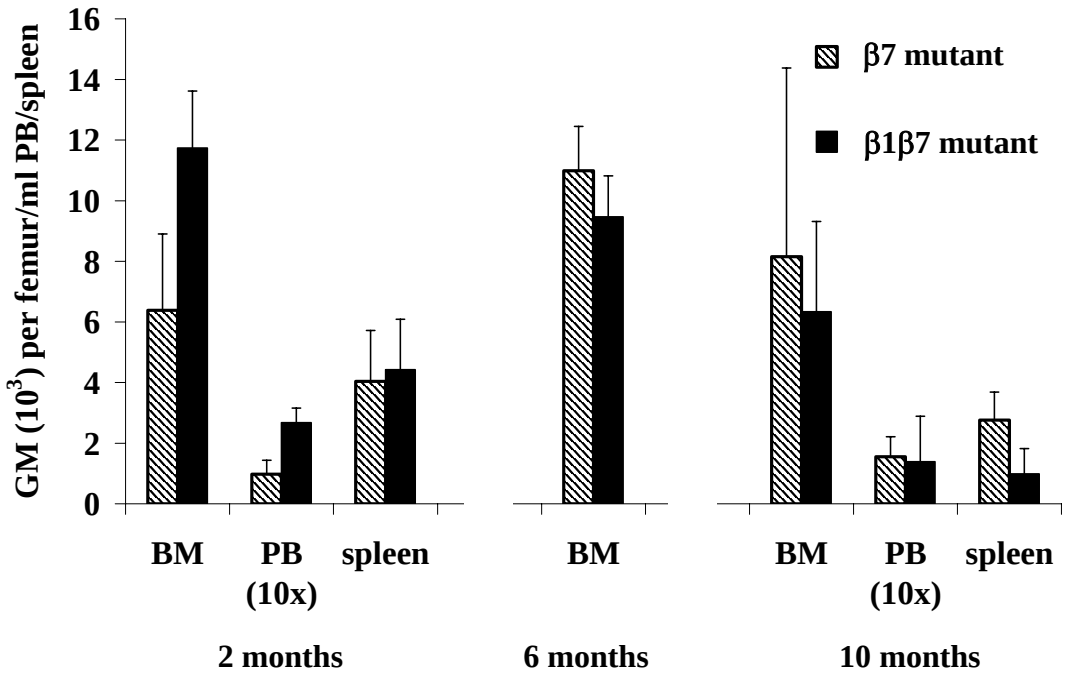


Figure 29: Distribution of granulocyte/monocyte progenitors

Single cell suspensions were made from BM and spleen and erythrocytes were lysed of blood from β7 mutant and β1β7 mutant BM chimeras at indicated times after the gene deletion. 180.000 BM cells, 3.600.000 splenocytes and 250µl PB were seeded into MethoCult GF M3534 medium and counted seven days later. Total numbers of colonies per femur, spleen and ml PB are shown. Error bars show the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 2 months 5/5, 6 months 4/4, 10 months 3/3).

These data would be consistent with a model where combined loss of β1 and β7 integrin leads to increased production of progenitors in the BM, a proportionally increased release of progenitors to the blood and inefficient migration of these progenitors to the spleen. Compared to the published data the increase of progenitors in PB is lower in β1β7 mutant BM chimeras and no increase of progenitors was observed in the spleen.

One explanation for these differences between the non-BM transplanted α4 integrin deficient mice and the β1β7 BM chimeras could be the different technical systems used. In order to address this question, the frequency of GM progenitors was determined in PB of β1β7 mutant mice that were not BM-transplanted. There the number of progenitors in PB increased about 8 fold from 95±33 in β7 mutant controls to 764±400 in β1β7 double mutant mice four weeks after induced gene deletion (n=5), which is more similar to the reported 11-fold increase from

100±46 in controls to 1150±120 in α4 deficient mice (n=4). These results suggest that the different systems used to investigate the role of α4β1 and α4β7 integrins in hematopoietic progenitor distribution might explain in part the difference in the results obtained.

3.7 Surface expression of α4 integrin in the absence β1 and β7 integrins

The more severe hematopoietic phenotype observed in α4 null somatic chimeras compared to β1β7 mutant BM chimeras prompted us to test whether β1β7 mutant BM chimeras might still express α4 integrin on the cell surface which could compensate for the loss of α4β1 and α4β7 integrins. We therefore checked by FACS, whether there are cells that express α4 integrin but not β1 and β7. Analysis of BM, PB, thymus and spleen revealed that only in BM of β1β7 mutant mice such a population of α4+β1- β7- cells exists (Fig. 30).

This cell population was found independently of the time after the induction of the β1 gene deletion only in the BM and accounted for 5%-20% of total BM cells in β1β7 mutant mice.

To exclude that this antibody staining was obtained due to unspecific cross-reaction, different anti α4 antibody clones were used in FACS (Fig. 31). Of these monoclonal antibodies 9C19 and R1-2 were reported to recognize different epitopes (Kinashi et al., 1994, Lin et al., 1995).

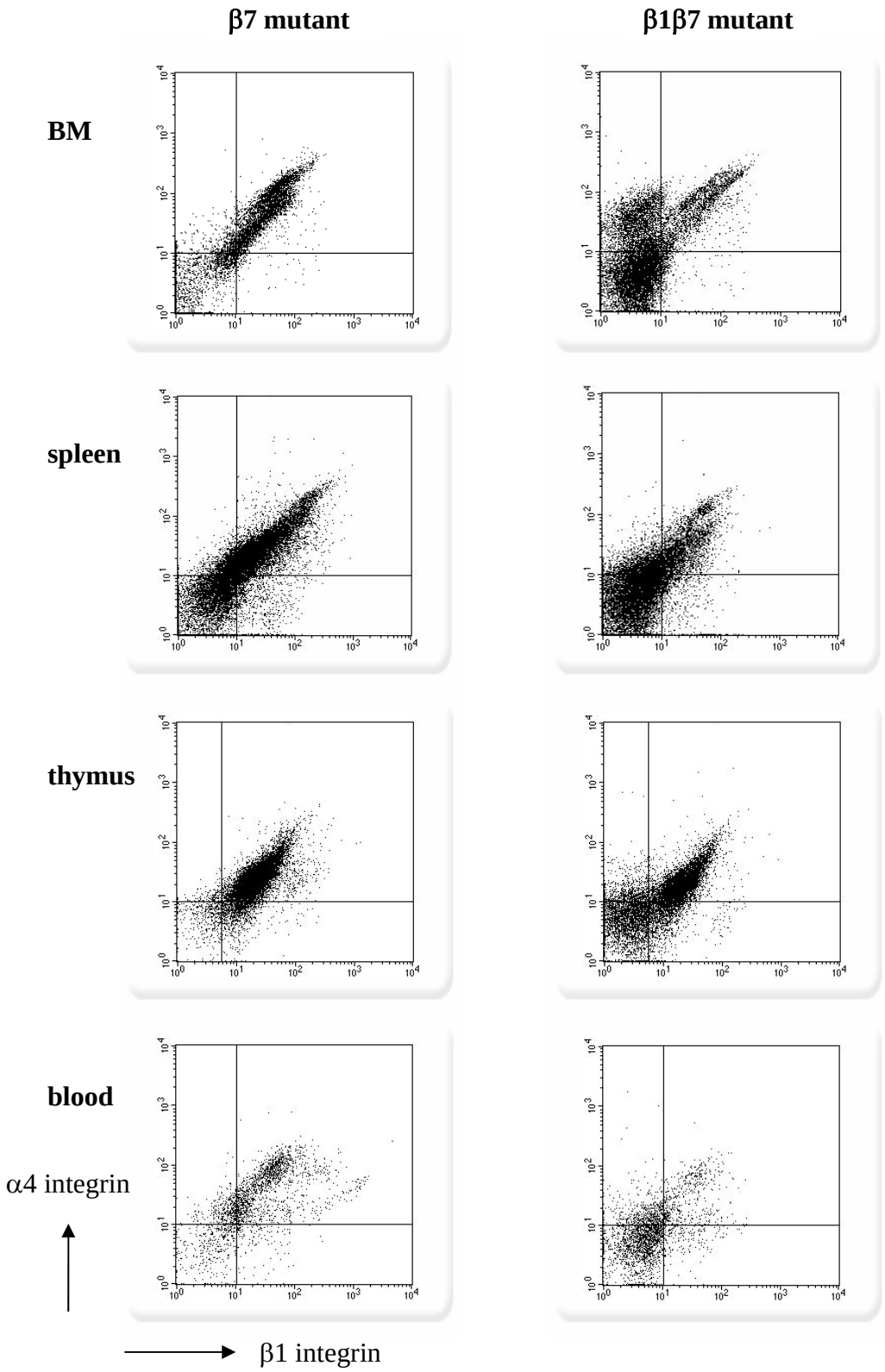


Figure 30: Cells that are negative for β1 and β7, but do express α4 integrin on the cell surface in the BM of β1β7 mutant BM chimeras.

Peripheral blood and single cell suspensions from BM, spleen, thymus of β7 mutant and β1β7 mutant BM chimeras were prepared, stained with antibodies against α4 integrin and β1 integrin and analysed by FACS.

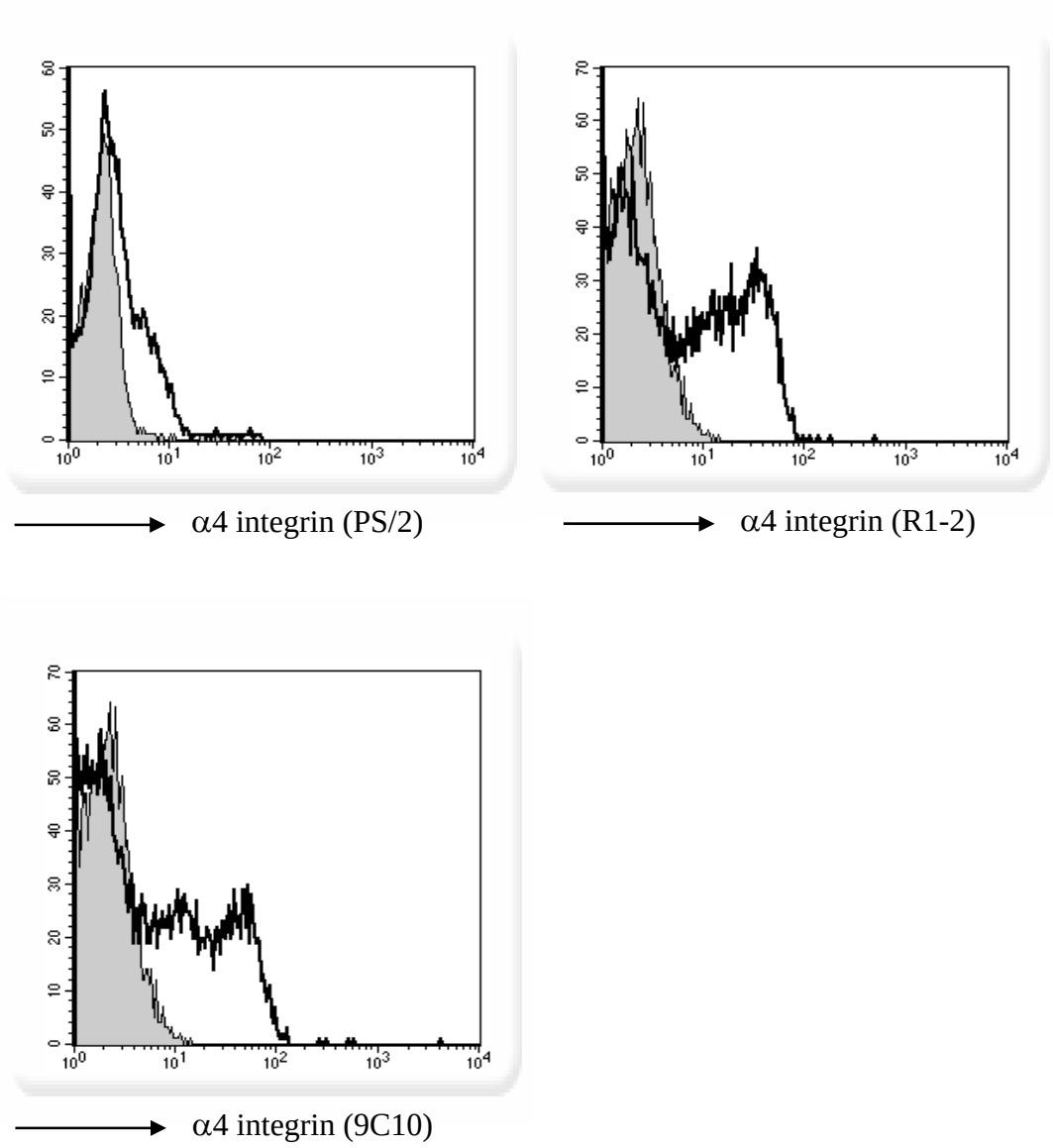


Figure 31: Three different clones of anti α4 antibodies recognise the α4 integrin on the surface of erythroblasts in the absence of β1 and β7 integrins

Single cell suspension from β1β7 mutant BM was prepared, stained with Ter119 and β1 and β7 integrin in combination with different clones of α4 integrin antibodies and subsequently analysed by FACS. The histograms show the α4 integrin expression of Ter119+, β1- and β7- integrin cells as assessed by the indicated clone. The filled curve represents the background and the line the antibody staining. A donkey anti rat Cy5 labelled secondary antibody was used to detect the clone PS/2. The clones 9C10 and R1-2 were directly PE-labelled.

While the clones 9C10 and R1-2 gave comparable signals, the clone PS/2 gave a weaker signal but was clearly different from the background staining. This experiment shows that different monoclonal antibodies can detect the α4 integrin chain on the cell surface in the absence of β1 and β7. Therefore it can be excluded that the signal obtained is due to some non-specific binding of one clone. Furthermore, this result indirectly suggests that the α4 subunit is correctly folded, since it is not likely that all tested monoclonal antibodies recognise a denatured α4 integrin.

To further characterize this α4+ β1- β7- cell population, BM cells from β1β7 mutant BM chimeras were stained for α4 and β1 integrin and additionally for lineage affiliated markers (Fig. 32).

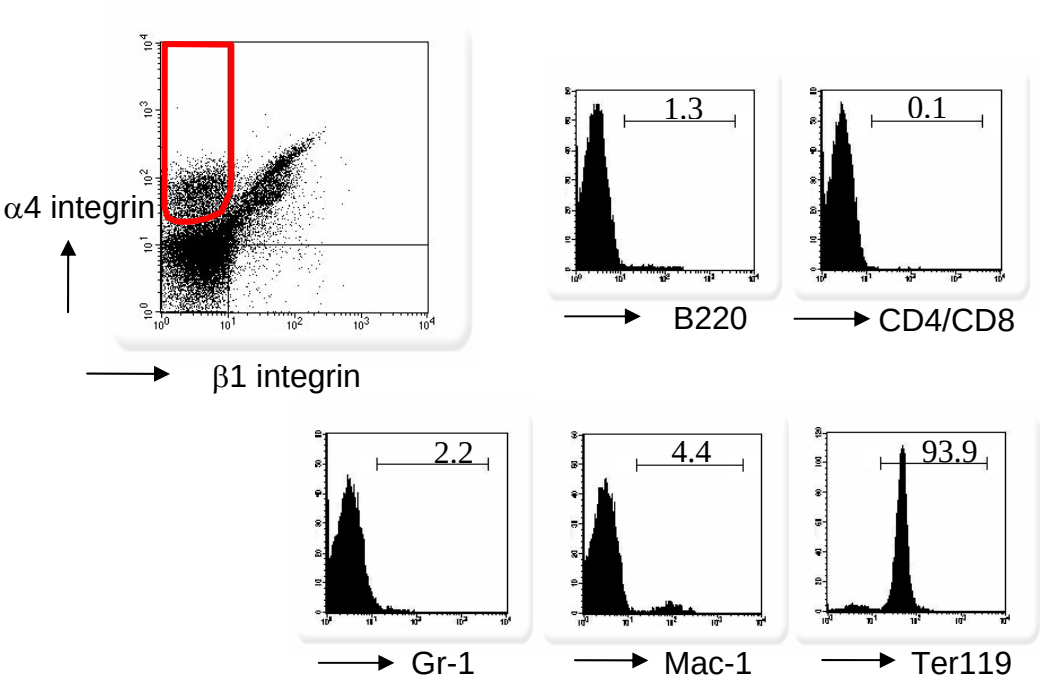


Figure 32: The majority of β1β7 negative, α4 positive cells in the BM are Ter119+ erythroblasts

Single cell suspensions from BM of β1β7 mutant mice 6 months after polyIC treatment was prepared, triple stained with antibodies against α4 integrin, β1 integrin and lineage markers and subsequently analysed by FACS. Histograms show only α4+, β1-, β7- cells gated out as shown in the α4/β1 integrin dotplot.

The majority of cells (~90%) expressing α4 integrin, but not β1 and β7 appeared to be erythroblasts. 4-5% of the α4+ β1- β7- cells were Mac-1+ monocytes, while B cells, T cells and granulocytes were hardly detected.

It is not clear, whether also erythroblasts of wild type or β7 mutant mice express surface α4 not bound to β1or β7, since this particular cell population might be hidden in the vast amount of cells positive for α4 and β1 integrin (Fig. 33).

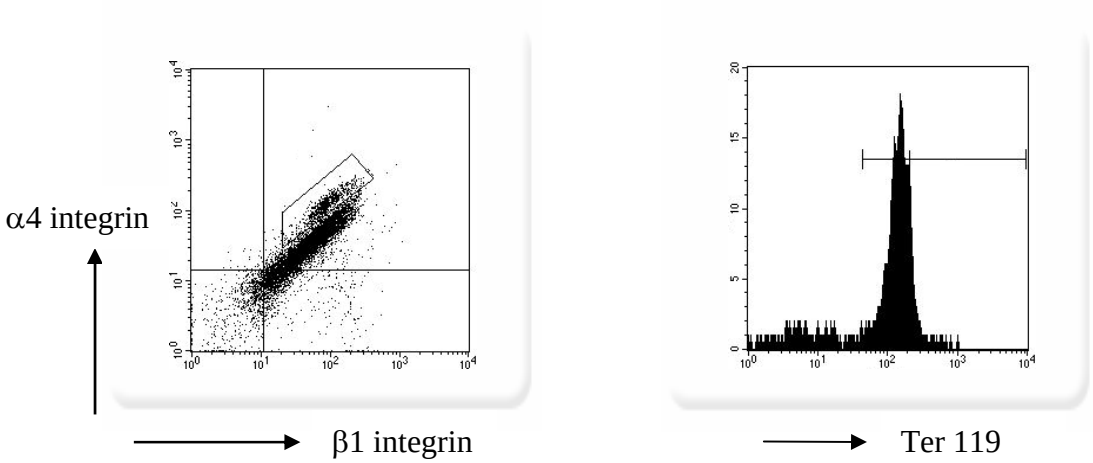


Figure 33: BM cells with high α4 integrin levels are predominantly erythroblasts.
Single cell suspensions from BM of β7 mutant BM chimeras was prepared, stained with antibodies against α4 integrin and β1 integrin and analysed by FACS.

However, also in β7 mutant mice erythroblasts express the highest amounts of α4 integrin.

To test whether a certain developmental stage of erythroblasts contributes more than another to the α4+ β1- β7- cells in β1β7 mutant BM chimeras a Ter119 - CD71 double staining was performed that allows the separation of BM erythroblasts into five different maturation stages (Socolovsky et al., 2001) (Fig. 34). The α4 and the β1 integrin expression of each of these stages were assessed in β7 mutant and β1β7 mutant mice.

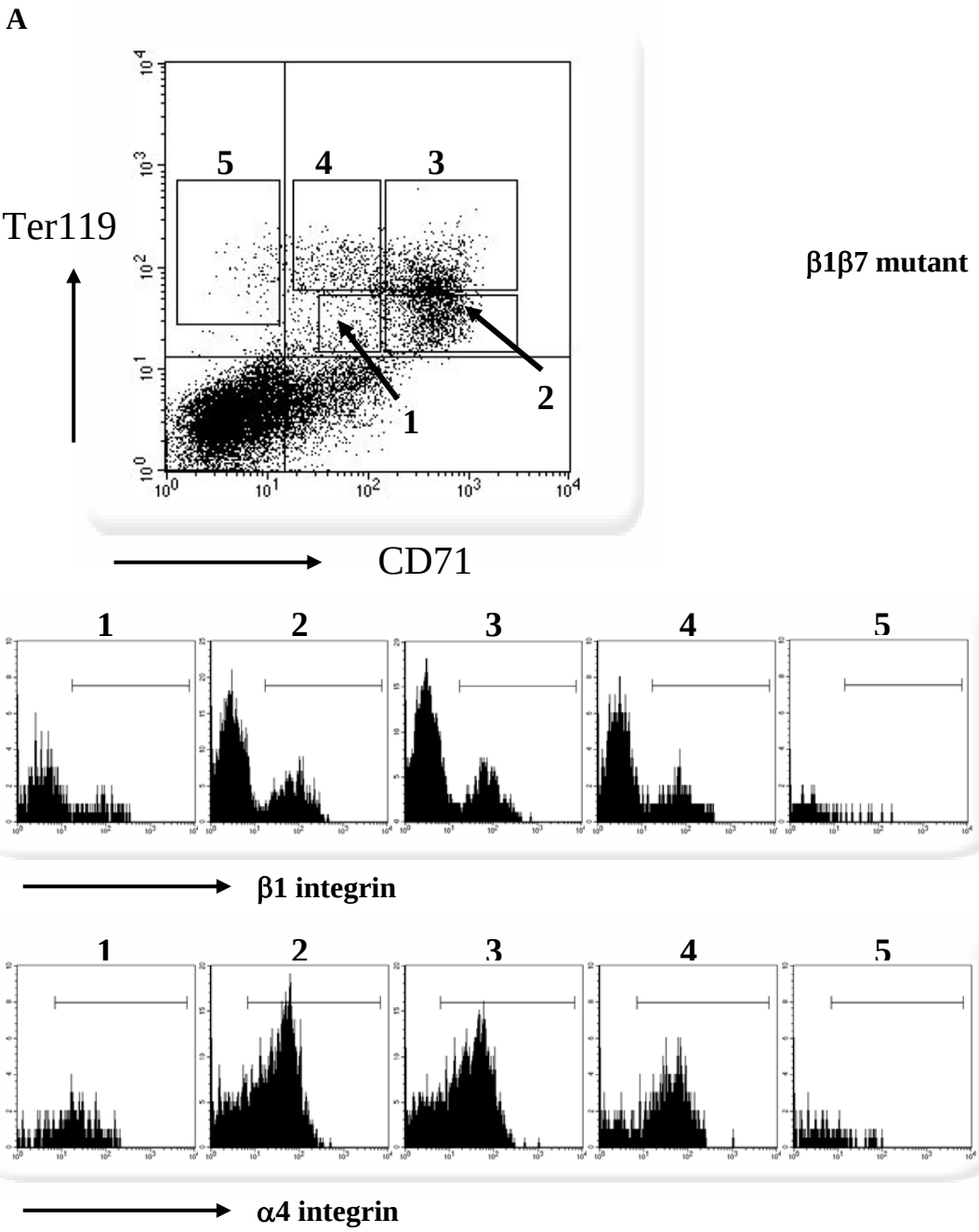


Figure 34 A: All developmental stages of erythroid cells contribute to the α4+ β1- β7- population.

BM single cell suspensions from β1β7 mutant BM chimeras that were phenylhydrazine (PHZ) treated were prepared. Cells were stained with antibodies against α4, β1 integrin, CD71 and Ter119 and subsequently analysed by FACS. (Ter119-CD71 staining distinguished 5 different developmental stages.) The α4 and β1 expression was plotted for each developmental stage of erythroid cells.

B

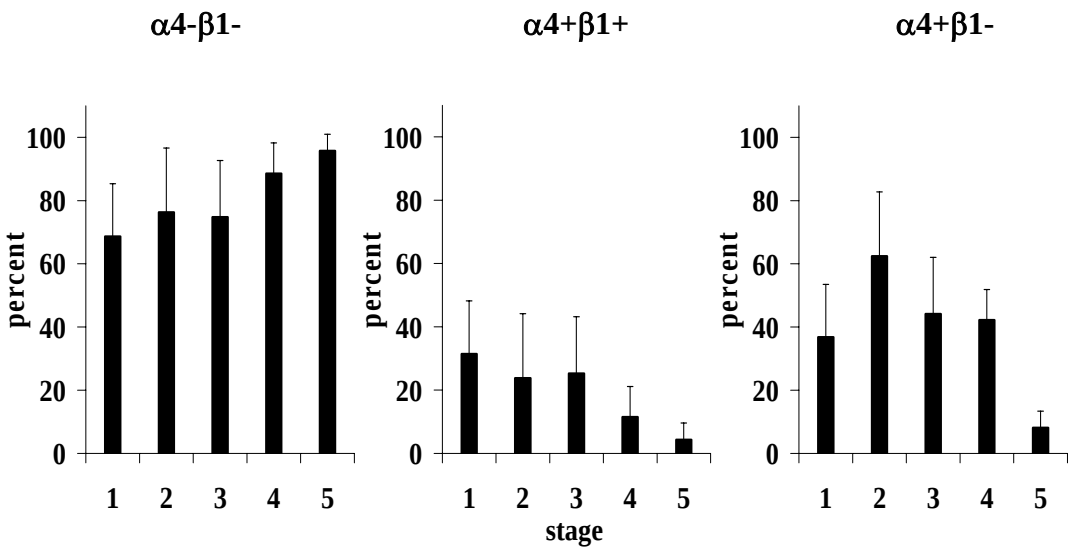


Figure 34 B: All developmental stages of erythroid cells contribute to the α4+ β1- β7- population.

The bar graphs show the percentage of cells that are α4-β1- (all α4- cells), α4+β1+ (all β1+ cells) and α4+β1- (remaining cells), respectively. Error bars show the standard deviation. (n (β7 mutants)/(β1β7 mutants): 3/3).

The analysis revealed the presence of α4+β1- cells in each stage of erythroid development. The percentage of α4+β1- cells varies between 40% and 60% in the first 4 stages and decreases drastically in stage 5, because erythroblasts downregulate integrin expression during final maturation.

α4 integrin was reported to be important for the retention of HPC cells within the BM (Papayanopoulou et a., 1995; van der Loo et al.,, 1998). To test whether also HSCs and HPCs express α4 integrin in the absence of β1 and β7, we gated β1β7 mutant BM cells for lineage marker deficient (lin-), β1- and c-kit+ cells. This population was plotted for Sca-1 and α4 integrin. HSCs are lin-, c-kit+ and sca-1+, while HPCs are lin-, c-kit+ and Sca-1- (Fig. 35). The analysis of three mice 10 months after the β1 integrin gene deletion revealed that almost all of the HSCs (88.6%±4.1%) and HPCs (93.7±1.5%) were α4 integrin positive in β7 mutant BM chimeras, as expected. In β1β7 mutant BM chimeric mice, however, no significant subpopulation of HSCs (14.4%±2.5%) was found to express α4

integrin, but 51.1%±4.7% of the HPCs expressed α4 in the absence of β1 and β7 integrin.

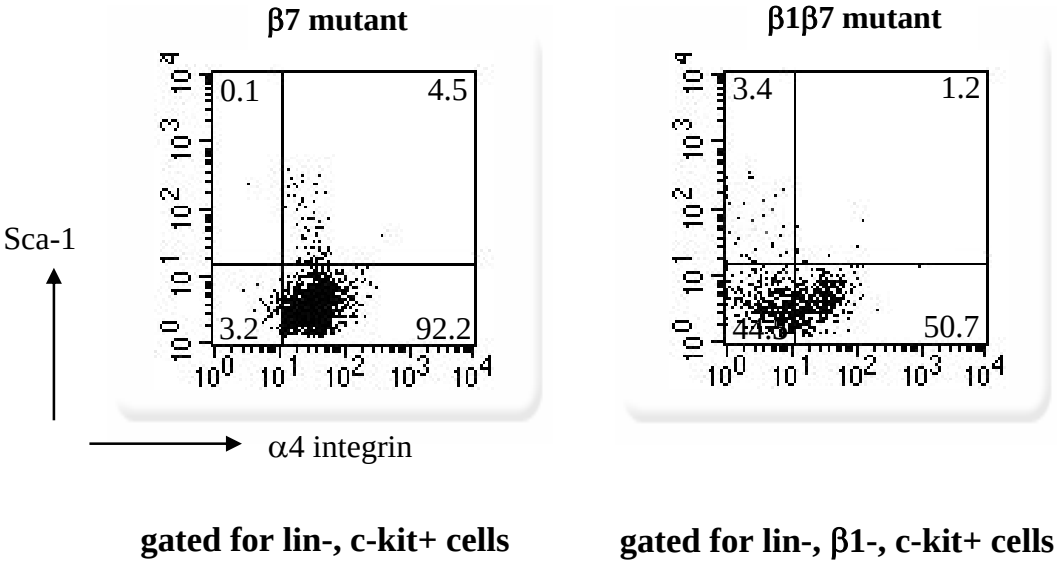


Figure 35: Approximately 50% of all HPCs, but hardly any HSCs express α4 integrin in the absence of β1 and β7 integrin in β1β7 mutant BM chimeric mice.

Single cell suspensions from β7 mutant and β1β7 mutant BM 10 months after the β1 integrin gene deletion was prepared, stained with a cocktail of lineage markers, c-kit and Sca-1 in combination with α4 integrin and subsequently analysed by FACS. The dot blots show the α4 integrin expression of lineage-, c-kit+ and Sca-1- cells (HPCs) and lineage-, c-kit+ and Sca-1+ cells (HSCs). In case of the β1β7 mutant BM, β1 integrin positive cells that might occur due to an incomplete gene deletion are excluded from the analysis.

3.8 Functional analysis of α4+ β1- β7- cells

Although the previous experiments demonstrated surface expression of α4 integrin in the absence of β1 and β7 integrin, they gave no information about a possible function of this α4 subunit present either as a monomer or complexed with other molecules. Therefore we tested whether cells positive for α4 and negative for β1 and β7 integrin can bind VCAM-1 which is a known ligand of α4 integrins. BM cells with an incomplete deletion of β1 integrin were incubated with fluorescently labelled VCAM-1 and then stained for α4 and β1 integrin in

the presence of EDTA or in the presence of Mn^{2+}. Mn^{2+} induces a high affinity conformation of integrin heterodimers by binding to the β subunit. Mn^{2+} was added here in case α4 integrin is heterodimerizing with another β subunit than β1 and β7. In the presence of EDTA integrin are in a low affinity conformation. α4+β1+ cells bound VCAM-1 in a Mn^{2+} dependent manner, while α4+, β1- cells did not (Fig.36).

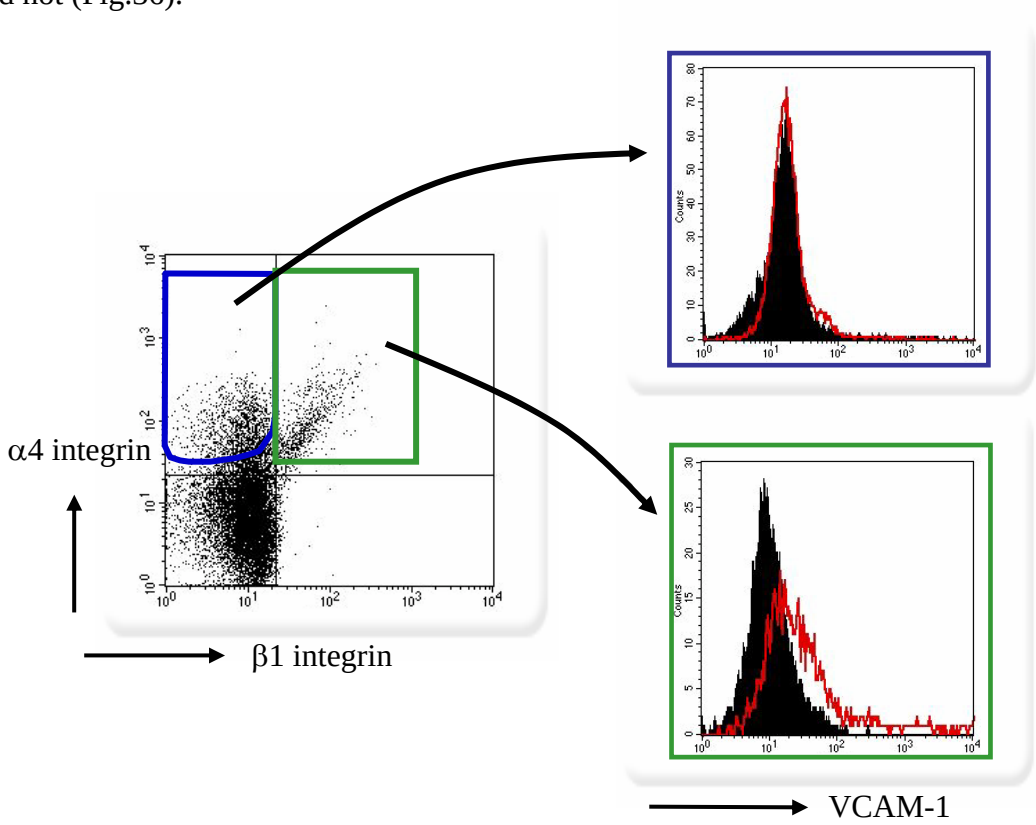


Figure 36: α4+β+-β7- BM cells bind VCAM-1 in a Mn^{2+} inducible manner, while α4+β1-β7- BM cells do not.

Single cell suspensions from β7 mutant and β1β7 mutant BM were prepared, incubated with fluorescently labelled VCAM-1 in either EDTA or Mn^{2+} containing buffer, stained for α4 and β1 integrin and analysed by FACS. α4+β1- and α4+β1+ subpopulations were gated out and analysed for autofluorescence (filled) and VCAM-1 binding (line).

3.9 Expansion of erythrocyte precursors after anaemia

After phenylhydrazine (PHZ) induced lysis of erythrocytes *in vivo*, the erythroid precursor cells have to expand in order to compensate the loss of erythrocytes. In

addition, haemolytic anaemia promotes extramedullary erythropoiesis leading to proliferation of progenitors in the spleen (Nijhof et al., 1983). Since in α4 conditional knockout mice the ability of erythroblasts to expand in response to a PHZ induced haemolytic anaemia was reduced (Scott et al., 2003), we investigated whether combined loss of β1 and β7 integrin have a similar effect. For better comparison with the non BM-transplanted α4 mutant mice we used non BM-transplanted β1β7 mutant mice. Therefore we analysed the number of erythrocytes, erythroblasts and the total number of CFUe after two daily injections of 60mg of PHZ per kg mouse weight. In addition we tested, whether the α4+β1-β7- erythroblasts expand differently and whether they can be found in the spleen under these conditions. The effect of the PHZ treatment was followed by measuring the amount of erythrocytes identified by their FSC/SSC characteristics among Ter119+ cells using FACS (Fig. 37).

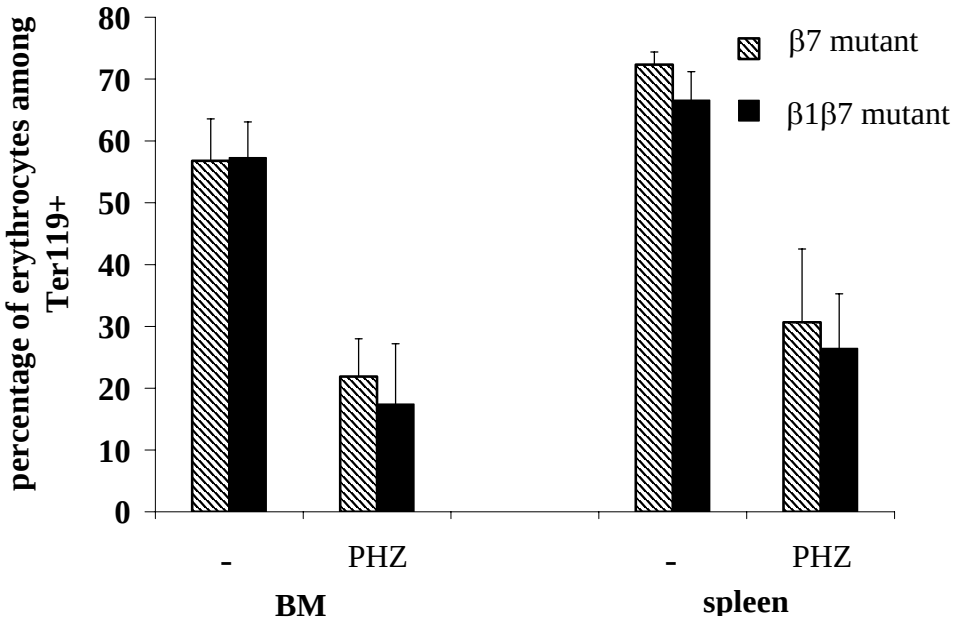


Figure 37: PHZ treatment leads to a relative reduction of erythrocytes in BM and in spleen

Single cell suspensions from β7 mutant and β1β7 mutant BM and spleen were prepared, stained for Ter119 and analysed by FACS. Erythrocytes were identified among Ter119+ cells by their FSC/SSC characteristics. Error bars show the standard deviation. (n (β7 mutants)/(β1β7 mutants): BM and spleen untreated 3/3; BM and spleen PHZ treated 5/5).

In BM and spleen of all mice the PHZ treatment led to a severe reduction of the amount of erythrocytes as compared to untreated mice indicating that PHZ treatment was effective. No differences were observed in the effect of PHZ on erythrocytes between β1β7 mutant mice and β7 mutant controls.

In contrast to erythrocytes, erythroblasts numbers in the BM increased after PHZ treatment (Fig. 38). No significant difference was observed between β1β7 mutant mice and β7 mutant controls. These data are in contrast to data from the α4 mutant mice, where loss of α4 corresponded to a 33% reduction in erythroblast numbers in BM.

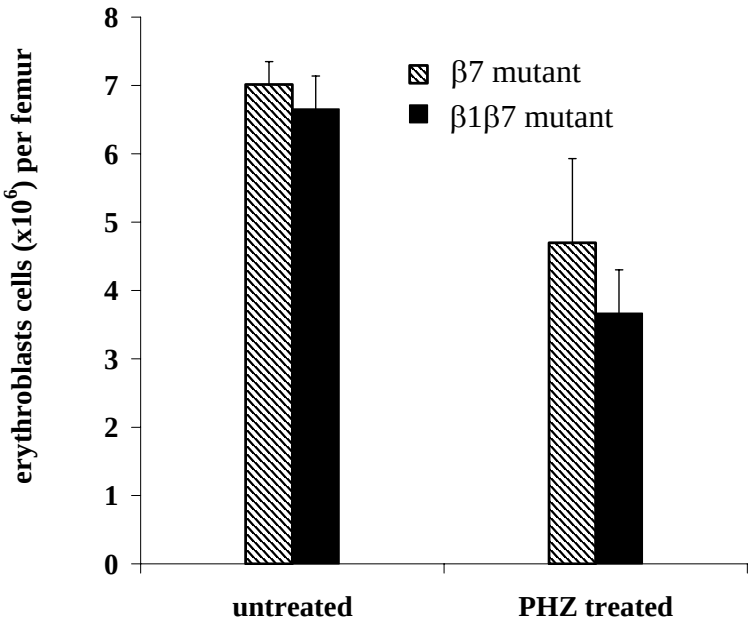


Figure 38: β1β7 mutant mice do not display a delayed recovery from hemolytic anaemia as compared to β7 mutants

Single cell suspensions from BM of β7 mutant and β1β7 mutant mice (no BM transplantation) untreated and treated with PHZ were prepared, stained with Ter119 antibody and subsequently analysed by FACS. The total amount of Ter119+ erythroblasts per femur is shown. Error bars show the standard deviation. (n (β7 mutants)/(β1β7 mutants): 5/5).

In the spleens of β1β7 mutant mice the number of erythroblasts after PHZ treatment tended to be higher (48.2±13.4) than the amount in β7 mutant controls (35.7±9.5), but this difference was not significant. The results indicate that β1β7

mutant and β7 mutant erythroblasts have similar capacity to expand in order to compensate for the reduced erythrocyte number.

The amount of CFUe after PHZ treatment was similar in the BM of β7 mutant controls and β1β7 mutant mice but was strongly decreased in the spleen of β1β7 mutants compared to β7 mutant controls (Fig. 39), whereas for α4 mutant mice a decrease in the BM but no change in the spleen as compared to control mice was reported.

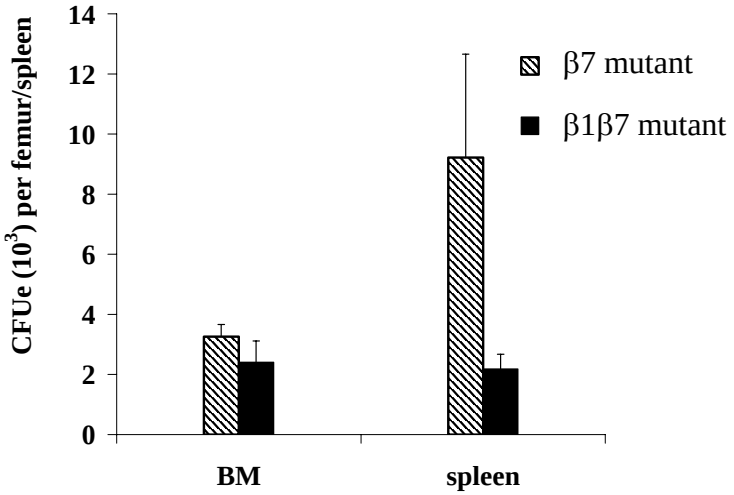


Figure 39: Severely reduced CFUe after PHZ treatment in spleens of β1β7 mutant mice

Single cell suspensions were made from BM and spleen from β7 mutant and β1β7 mutant mice (no BM transplantation). BM cells and splenocytes were seeded into MethoCult 3334 medium and counted three days later. Total numbers of colonies per femur and spleen are shown. Error bars show the standard deviation. (n (β7 mutant BM chimera)/(β1β7 mutant BM chimera): 3/3).

These data suggest that β1β7 mutant mice do not display a delayed recovery from PHZ induced anaemia in BM as compared to β7 mutant controls. The decreased frequency of CFUe in the spleen, however, points to a defect of PHZ treated mice of β1β7 mutant erythroid precursors to colonise this organ leading to a decrease of the total amount of all CFUe per mouse in the β1β7 mutant mice compared to β7 mutant controls.

BM cells and splenocytes of PHZ treated mice were stained for α4 and β1 integrin to test whether α4+β1-β7- cells can be mobilised under conditions of acute haemolysis. However, significant amounts of α4+β1-β7- cells were only

encountered in the BM of β1β7 mutant mice. These amounts were comparable to those of untreated β1β7 mutants (Fig. 40).

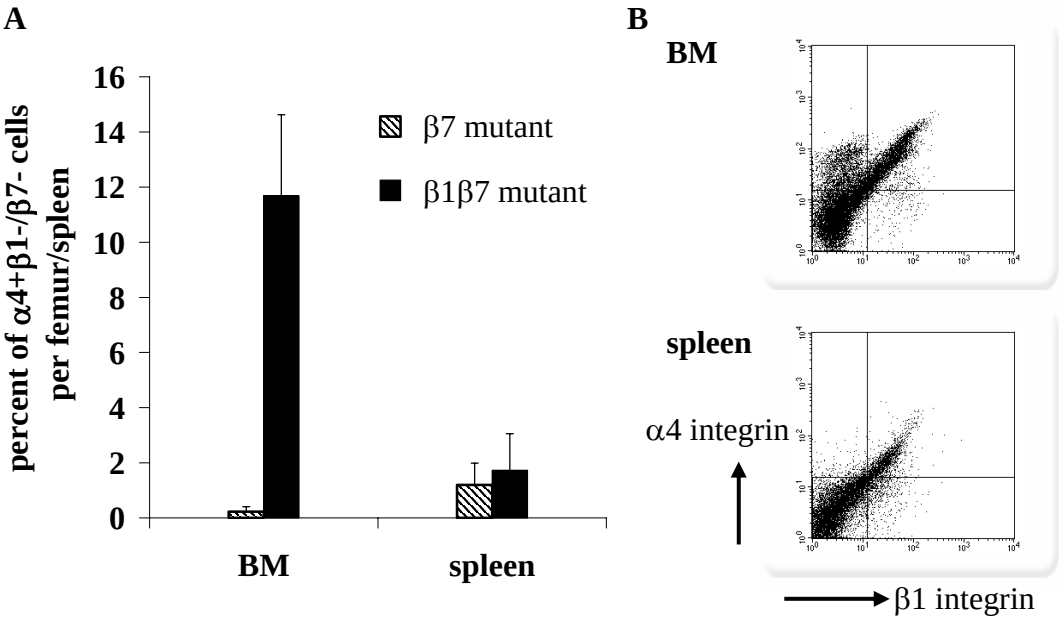


Figure 40: α4+β1-β7- cells occur also under conditions of haemolytic recovery only in the BM of β1β7mutant mice.

Single cell suspensions from BM and spleen of β7 mutant and β1β7 mutant mice (no BM transplantation) after PHZ treatment were prepared, stained with α4 and β1 integrin antibodies and subsequently analysed by FACS.

A: The percentage of α4+β1-β7- cells is shown. Error bars show the standard deviation. (n (β7 mutants)/(β1β7 mutants): 3/3).

B: A representative example of an α4 β1 integrin staining of β1β7 mutant BM cells and splenocytes.

3.10 Attempts to identify α4 associated cellular protein in the absence of β1 and β7 integrin

3.10.1 Co-immunoprecipitation using an anti α4 integrin antibody

To clarify whether α4 integrin is associated with another molecule or reaches the cell surface as a monomer immunoprecipitations (IPs) were conducted. First

$5x10^7$ BM cells from wild type mice were isolated and radiolabelled using ^{125}I. Cells were lysed and IPs were performed using an anti α4 integrin antibody and as a control experiment using an anti α_M integrin antibody. The supernatants and the precipitates were electrophoretically separated on a SDS-PAGE and analysed by autoradiography (Fig. 41).

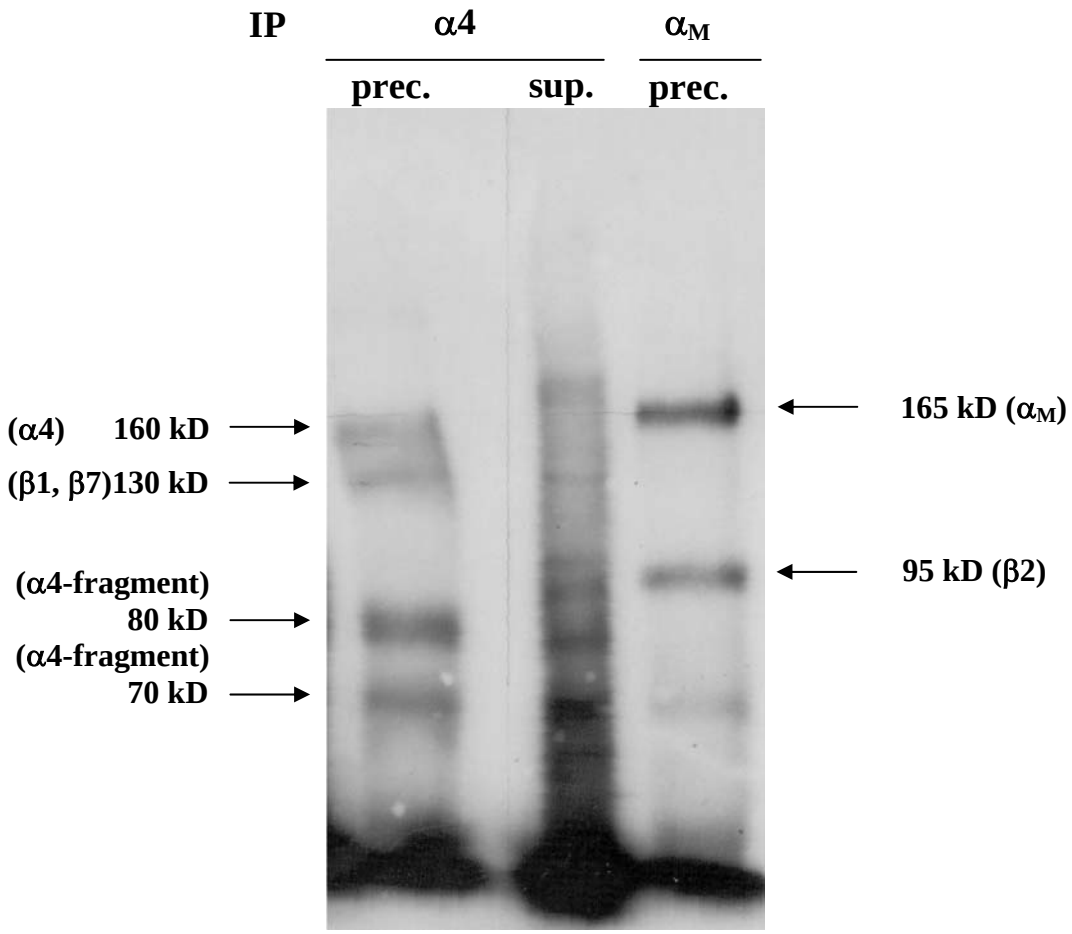


Figure 41: Using wild type BM β1 and β7 integrins could be co-immunoprecipitated with α4 integrin Single cell suspension from BM of wild type mice was prepared, surface radiolabelled using ^{125}I and lysed. The Lysate was immunoprecipitated (prec.) using anti α4 and anti α_M antibodies, separated on a polyacrylamide gel and autoradiographically analysed. For the α4-IP, also supernatant (sup.) of the IP is shown as a control.

In the SDS-PAGE of the α_M immunoprecupitate a strong band at around 165kD appeared, that corresponds well to the α_M integrin chain. In addition, a band of approximately 95kD was visible corresponding to the molecular weight of β2 integrin and suggesting that β2 integrin was co-immunoprecipitated with α_M. In the α4 integrin IP a 160kD band corresponding well to the size of α4 integrin and

a 130kD band fitting to the size of β1 and β7 integrin suggesting that β1 and β7 integrin were detected, can be co-immunoprecipitated with α4. Additional bands around 80kD and 70kD correlate very likely to known degradation products of α4 integrin (Altevogt et al.,, 1995).

Using the same approach, we wanted to test whether in the absence of β1 and β7 integrin α4 integrin can bind to intracellular ligands such as paxillin, which was described to bind to α4 integrin directly (Liu et al., 1999). Using $3x10^7$ wild type BM cells an α4 integrin IP was performed to establish the method. The supernatant and the precipitate were electrophoretically separated, blotted on a membrane and probed with anti paxillin antibody (Fig. 42).

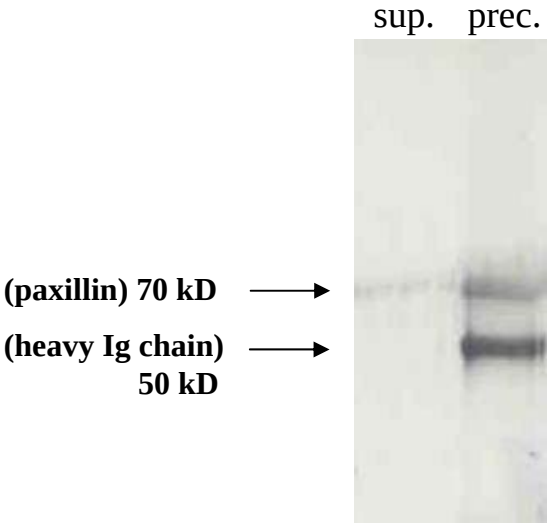


Figure 42: U*sing wild type BM paxillin could be co-immunoprecipitated with α4 integrin*
Single cell suspension from BM of wild type mice was prepared, the lysed and immunoprecipitated using anti α4 antibody. The Precipitate (prec.) and an aliquot of the supernatant were separated on a polyacrylamide gel and probed with an anti paxillin antibody.

Paxillin was clearly enriched in the precipitate as compared to the lysate, indicating that it co-immunoprecipitates with α4 integrin. The second band in the precipitate is most likely due to recognition of the immunoglobulin heavy chain of the α4 antibody used for the IP by the secondary antibody used for paxillin detection.

Using the same techniques we tried to co-immunoprecipitate α4 integrin associated molecules in lysates of surface-iodinated BM cells of β1β7 mutant mice. For each experiment BM of 3 β1β7 mutant mice was combined. However, due to low expression levels of α4 integrin restricted to a subpopulation of BM cells of β1β7 mutant mice, it was very difficult to obtain convincing immunoprecipitations of α4 integrin itself. Based on the intensity of the α4

staining in FACS and considering that the α4+β1-β7- cells account for 5%-20% of total BM cells, the cell number used for one experiment needs to be increased by 25 to 35 fold to obtain similar amounts of α4 molecules. This would mean that BM of approximately 30 mice should be combined. Such an experiment should be carried out in the future.

3.10.2 Analysis of α4 integrin expression on β1β2 mutant BM cells

β2 integrin is expressed on all leukocytes in high amounts. Although it was not described to associate with α4 integrin it might do so in the absence of β1 and β7 integrin, explaining the presence of α4 integrin on the cell surface of β1β7 mutant cells. To test whether β2 integrin can associate with α4 on BM cells, we intercrossed β1 conditional Mx-cre positive mice with β2 integrin null mice (Scharffetter-Kochanek, K. et al., 1998) to obtain β1β2 mutant mice after polyIC treatment. FACS analysis was carried out for α4, β1 and β7 integrin and α4 and β1 expression was tested on BM cells gated for the absence of β7 integrin (Fig. 43).

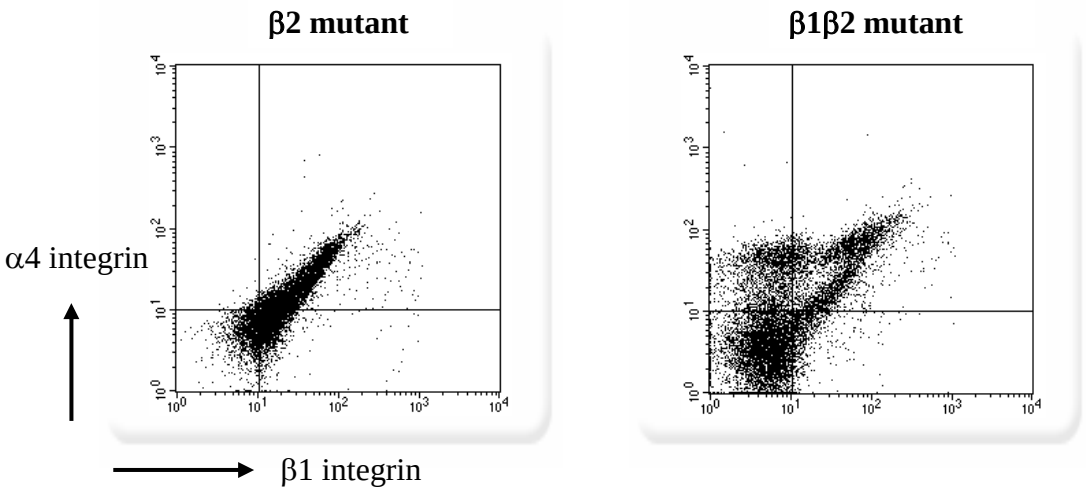


Figure 43: BM cells of β1β2 mutant mice contain cells that are negative for β1, β2 and β7, but do express α4 integrin on the cell surface.

Single cell suspensions from BM of β2 mutant and β1β2 mutant mice were prepared, stained with antibodies against α4, β1, β2 and β7 integrin and analysed by FACS. Shown is the α4, β1 expression of β7- cells. One representative experiment of three is shown.

Also on cells lacking β1, β7 and β2 integrin α4 integrin was found on the cell surface, indicating that β2 integrin is not heterodimerizing with α4 integrin.

3.10.3 Generation and analysis of integrin mutant fibroblastoid cells

To test whether the appearance of α4 integrin on the cell surface in the absence of β1 and β7 is restricted to hematopoietic cells or not we infected fibroblastoid cells lacking β1 integrin (GD25) and corresponding control cells expressing β1 (GD25 β1+) (Wennerberg et al., 1996) with a retrovirus containing cDNA encoding for α4 integrin. We expected these cells to be a suitable model since GD25 cells were deficient for β1 integrin and do not express β7 on the surface as tested previously. FACS analysis revealed, however, that GD25 β1- cells showed α4 integrin expression on the cell surface as well as β7 integrin, while GD25 β1+ cells were positive for α4, β1 and β7 integrin (Fig. 44).

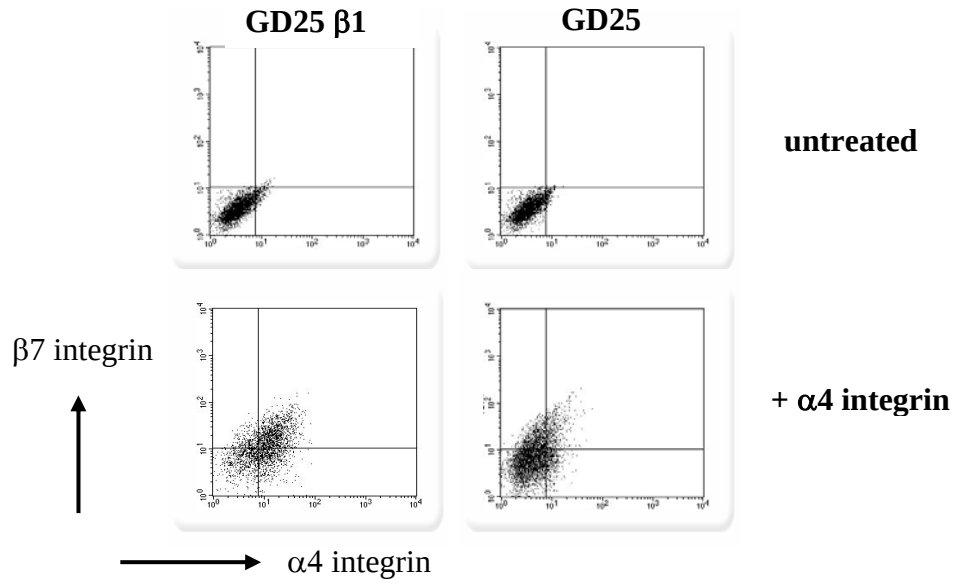


Figure 44: GD25 β1+ and GD25 β1- fibroblastoid cells do neither express α4 nor β7 integrin. Upon infection with α4 integrin cDNA, however, both cell lines express α4 as well as β7 integrin.

GD25 β1+ and GD25 β1- cells were before and after infection with a retrovirus containing α4 integrin cDNA stained with antibodies against α4, β1 and β7 integrin and analysed by FACS. Shown is a representative α4 vs. β7 staining of both cell lines before and after retroviral infection with a virus containing α4 integrin cDNA.

If β7 integrin mRNA is expressed in GD25 cells lacking β1 integrin only after ectopic α4 expression, it could indicate that α4 integrin induces signalling that leads to β7 integrin expression. To test this possibility RT-PCR was carried out for β7 integrin mRNA in α4 transfected and untransfected GD25 and GD25β1 cells (Fig. 45).

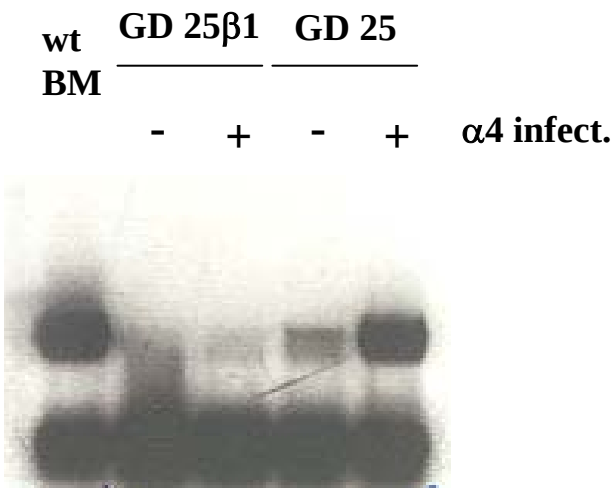


Figure 45: GD25β1 and GD25 fibroblastoid cells do express β7 mRNA prior to α4 infection.

RNA from GD25β1 and GD25 cells was isolated before and after infection with a retrovirus containing α4 integrin cDNA and subjected to β7 integrin RT-PCR analysis as indicated. RNA isolated from wild type BM cells served as a positive control.

Clearly, also in the absence of α4 integrin β7 mRNA is present in GD25 and GD25β1 cells. Whether expression of α4 results in an upregulation of β7 integrin expression should be tested by quantitative RT-PCR or Northern blot.

To circumvent the problem of cytoplasmic β7 integrin in GD25 cells we generated fibroblastoid cell lines from embryos deficient for β2 and β7 integrin carrying a conditional deletion of the β1 integrin gene. By adenoviral cre infection *in vitro* cell lines lacking β1, β2 and β7 were generated. After infection of those cells with α4 integrin cDNA, FACS analysis revealed that also in fibroblastoid cells α4 integrin can reach the cell surface in the absence of β1, β2 and β7 integrins (Fig. 46).

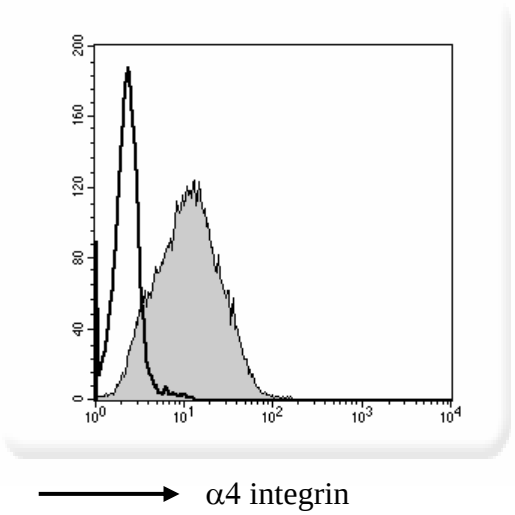


Figure 46: Fibroblastoid cells deficient for β1, β2 and β7 integrin express α4 integrin on the cell surface upon infection with α4 integrin cDNA.

Fibroblastoid cells deficient for β1, β2 and β7 integrin were stained with antibodies against α4 and analysed by FACS before and after infection with a retrovirus containing α4 integrin cDNA. The deficiency of β1, β2 and β7 integrin on these cells was confirmed by FACS analysis. Shown is an overlay of a representative α4 staining of cells before (line) and after retroviral infection with a virus containing α4 integrin cDNA (filled).

4. Discussion

In this PhD work, the functional role of the β1- and β7 integrin receptors for the murine hematopoietic system were studied *in vivo*. Surprisingly, β1- and β7 integrins are not essential for the retention, proliferation and survival of HSC and HPC within the BM. Furthermore, hematopoiesis and lymphopoiesis are not dependent on the adhesive interactions nor signal transduction mediated by these integrins, suggesting compensatory effects of other adhesion receptors. Finally, the analysis revealed cell populations expressing α4 integrin in the absence of β1 and β7. This α4 integrin expression could contribute to the mild phenotype of β1β7 mutant BM chimeras.

4.1 Retention and function of HSC

In adult mammals HSCs are retained in the BM where they self-renew and differentiate into HPCs giving rise to the different blood cell lineages. Integrins are thought to be crucial for the retention and maintenance of HSCs in the BM, although up to now no single integrin receptor could be found that is essential for HSC function in the BM. Several experimental evidences suggested that β1 integrin-mediated adhesion of HSC/HPCs is important for their function in the BM (Prosper and Verfaille 2001). First, β1 integrins were thought to be crucial for the adhesion of hematopoietic cells to stroma cells and to the extracellular matrix (ECM) in the BM. Fibronectin (FN) is a component of the BM ECM and *in vitro* experiments demonstrated that adhesion of HSC and HPC to fibronectin is mediated by α4β1 as well as α5β1 (Williams et al., 1991; van der Loo et al., 1998). Integrin α4β1 also mediates binding to VCAM-1, which is expressed on BM stroma cells (Oostendorp et al., 1995). The importance of these interactions for the retention of HPC and HSC was supported by intravenous injection of FN fragments and antibodies against α4, blocking α4β1 and α4β7, or VCAM-1,

which mobilised HPC into the blood (Papayannopoulou, 1995; van der Loo et al., 1998). Release of HPCs can also be induced by other mobilising agents such as stem cell factor (c-kit ligand), IL-3, granulocyte-macrophage colony stimulating factor and others (Fruehauf, 2003). The α4 integrin expression level on HSCs/HPCs mobilized by G-CSF was shown to be reduced compared to steady-state BM and PB cells (Chen et al. 2003), again suggesting an important role for α4 integrins in HPC retention in the BM. In addition to retention of HSC and HPC in the BM, integrin mediated adhesion might also be crucial for the self-renewal and survival of HSC, since α4β1-mediated attachment of HPC to FN promotes proliferation (Yokota et al., 1998; Schoefield, 1998) and prevents apoptosis (Wang et al., 1998).

At the time this study was initiated, however, several genetic approaches pointed to a less prominent role of these integrin receptors for HSC/HPC retention and function. In β1 integrin mutant mice, there was no evidence for an increased release of HSCs/HPCs into the periphery as the numbers of progenitor cells in PB were normal (Brakebusch et al., 2002). Normal hematopoiesis in these mice, even 12 months after the knockout induction, revealed no functional abnormalities of hematopoietic progenitors in the absence of β1 integrins. Likewise, no defects in the retention of HSC/HPC leading to abnormal hematopoiesis were apparent in β7 knockout mice (Wagner et al. 1996). Furthermore, data obtained with α4-null chimeric mice could not confirm an essential role of α4 integrins as these mice show normal function, retention and maintenance of HSC despite of the absence of α4β1 and α4β7 (Arroyo et al., 1996, 1999). Mice lacking VCAM-1 on endothelial and hematopoietic cells showed a reduced migration of lymphocytes to the BM, but no impaired HSC function (Koni et al., 2001; Leuker et al., 2001; Friedrich et al., 1996). Genetic studies of α5- or αv-null chimeric mice and of mice deficient for α1, α2, α3, α6, α7, α8, α9, α10, αL, αM, β2, β3, β4, β5, β6 and β8-deficient mice revealed that none of these integrins is essential for the retention of HSC in the BM (reviewed by Hynes, 2002).

In the midst of this PhD project an analysis of a conditional knockout of α4 integrin was published which demonstrated a role of α4 in HSC/HPC retention (Scott et al., 2003). In these mice, which lack α4β1 and α4β7 integrins in the hematopoietic system and at least partially in many other tissues, HPC numbers in

the PB were elevated indicating a release from the BM. However, the HPC numbers were not decreased in the BM leading to a higher total amount of HPCs per mouse. This increase could be due to a release of HPCs from the BM which is then compensated by upregulated formation of HPCs. Alternatively, the loss of α4 integrins might lead to an enhanced formation of HPCs, which are then released into the periphery suggesting a role of α4 integrin in HPC expansion apart from its adhesive function.

In contrast to these reported findings, the results presented in this study show that simultaneous loss of all β1 and β7 integrin receptors, including α4β1 and α4β7, did not severely impair HSC retention or function. The mutant mice exhibited normal hematopoiesis and had normal numbers of BM cells. The overall number of progenitors was increased about two-fold in BM and blood, which would fit to an increased release due to increased production of HSCs/HPCs in the BM. The maintenance of β1β7 null HSCs was proven by their high contribution to all hematopoietic lineages even 12 months after knockout induction, as analysed by FACS, Southern blot and genotyping of progenitor colonies. Comparison of the knockout efficiency on myeloid cells in the BM of β1β7 mutant BM chimera 2 and 10 to 12 months after knockout induction showed a slight, but not significant, decrease of the percentage of β1β7 deficient cells. This could indicate that the β1 integrin expressing HSC in β1β7 mutant BM chimeras, which are always present due to the incomplete knockout of the β1 integrin gene, have a growth advantage over β1β7-deficient HSC. Such an advantage could be caused by decreased proliferation or increased apoptosis of β1β7-mutant cells. Whether this subtle difference is indeed caused by a growth advantage of β1 expressing HPC or whether it is caused by mouse-to-mouse variations of the donor BM is not clear. Comparison with the mouse-to-mouse variation of knockout efficiencies on HPC-derived colonies and differentiated cells, however, favour the latter explanation.

One explanation for the reported strong increase in the relative release of HPCs from the BM of α4 integrin conditional knockout mice could be that the deletion of the α4 integrin gene was not restricted to the hematopoietic system. For example, loss of α4 in non-hematopoietic tissues such as vasculature, BM stroma cells or endothelial cells might result in the production of cytokines which favour the mobilisation of HPCs. This explanation is supported by the fact that mice

lacking β1 and β7 integrin with a similar tissue distribution as the α4 conditional knockout mice show a comparable relative release of HPCs into the peripheral blood as was shown in this PhD study using non-BM-transplanted mice. This explanation might also apply for antibody and peptide inhibition studies since these agents can bind to many other integrin-expressing cells where they might induce secondary effects indirectly favouring the release of HSC into the blood before reaching the HSCs in the BM. Cytokine mediated release of HPCs into the PB peaks from day 4 to 7 after administration of the respective agent (Fruehauf 2003). One would expect a more rapid release mediated by blocking antibodies and peptides, however, the earliest time point assessed was 4 days after agent administration. Therefore, the kinetics of blocking agents versus cytokine mediated release should be determined to address the mechanism underlying HPC release into the PB.

In addition, the presence of α4 integrin on the surface of approximately 50% of the HPCs in the absence of β1 and β7 could contribute to the mild phenotype. However, the strong release of HPCs form the BM to the periphery of non-BM-transplanted β1β7 mutant mice indicates that α4 expression in the absence of β1 and β7 is probably not responsible for the HPC retention in β1β7 mutant BM chimeras.

Since HSCs/HPCs are retained and functioning in β1β7 mutant BM chimeras, other HSC adhesion receptors may compensate for the loss of β1 and β7 integrins. The adhesion receptors still present in the β1β7-null BM chimeras might be sufficient for HSC maintenance, function and retention. One possibility is the fibronectin and hyaluronan-binding CD44 molecule which itself is not crucial for HSC retention and maintenance as revealed in mice lacking the CD44 gene (Schmits et al., 1997). Integrins containing the β2 subunit might compensate for the loss of β1 and β7 integrins, although they are normally not expressed on HSC, but on leukocytes (Voura et. al., 1997). It remains to be examined if the expression of other adhesion receptors is upregulated in response to the loss of β1- and β7 integrins. To address this question, HSC should be purified by FACS and tested for the expression levels of different receptors. Quite likely, αv integrins mediate compensating adhesive interactions and prevent HSC release from the BM as all αv integrins bind to FN in a manner similar to α4β7. A triple knockout

of αv, β1 and β7 would elucidate whether this is the case. Alternatively, *in vitro* cultures could be established to assess the adhesion of wild type and mutant HSC to a basic layer of BM stroma cells. Treatment with different antibodies blocking ligand binding to specific adhesion receptors should determine which adhesion receptors mediate the attachment of wild type and mutant HSC to the stroma cells. Although it seems unlikely, it cannot be excluded that the loss of β1 integrin receptors, different from α4β1, rescue the phenotype observed in α4 deficient mice.

4.2 Lymphopoiesis

In adult mice, lymphopoiesis originates in the BM. While B cells mature in the BM, T cell precursors leave the BM and enter the blood stream to colonize the thymus and mature there. Previous *in vitro* experiments indicated a stroma dependent phase for early B cell development and suggested a role for α4β1 integrin (Gisler et al., 1987). The function of α4 integrins has also been studied *in vivo*. To circumvent embryonic lethality of α4-deficient mice, somatic chimeric mice were generated by injecting α4 null ES cells into α4+/+ RAG-1- or RAG-2- blastocysts (Arroyo et al., 1996). Since RAG-1 and RAG-2 deficient mice do not develop any mature lymphocytes, all mature lymphocytes in the chimera should be derived from the α4-/- ES cells. However, also non-lymphoid cells are derived from the α4-null ES cells resulting in a knockout that is not restricted to the hematopoietic system. The analysis of these α4-null chimeric mice, which lack both α4β1 and α4β7 on all cells derived from α4 null ES cells, revealed an important role for α4 integrin in lymphopoiesis (Arroyo et al., 1996; Arroyo et al., 1999). In the absence of α4 integrin, B cell development stopped before the pro B cell stage. In addition, T cell precursors were unable to leave the BM. However, injection of α4 null BM cells into the blood of RAG-2 mice resulted in normal colonisation of the thymus, indicating that α4 integrin is neither important for the homing of T cell precursors to the thymus nor for

intrathymic development, but is important specifically for extravasation out of the BM. Since β7 knockout mice, which lack only α4β7, have normal hematopoiesis (Wagner et al., 1996), it was concluded that α4β1, and not α4β7, plays a crucial role in lymphopoiesis (Wagner et al., 1998). Yet, analysis of β1 mutant BM chimeras demonstrated that β1 integrins, including α4β1, are not essential for lymphopoiesis (Brakebusch et al., 2002). The analysis of B cell development in BM and spleen did not reveal any gross abnormality in the differentiation of these cells. One possible explanation for this finding is that α4β1 and α4β7 might have redundant functions in early hematopoiesis and that only the absence of both molecules impairs lymphopoiesis. To address this question in this study, β1β7 mutant BM chimeras were generated and lymphopoiesis was examined.

Surprisingly, B cell development occurred normally in β1β7 mutant BM chimeric mice. Pre-B cell colony assays revealed that pre-B cell colonies can form *in vitro* in the absence of β1 and β7 integrins and that the knockout efficiency at this stage of B cell development was similar to the deletion efficiency on mature B cells as assessed by Southern blot indicating no developmental defects in between these stages. Immature (B220 medium) and mature (IgD+) β1 and β7 integrin deficient B cells were present in the β1β7 mutant BM chimeras even 12 month after induction of the knockout, demonstrating that B cell development *in vivo* can continue to mature stages in the absence of both β1 and β7 integrins. Clearly, α4β1 and α4β7 are not required for B cell development. One explanation for the contrasting results could be that the loss of β1 integrin receptors in addition to α4β1 rescues the α4-null phenotype. The decrease in transmigration through the BM stroma observed with α4-null progenitor cells could, for example, be due to increased adhesion to fibronectin via α5β1. An alternative explanation is the use of different experimental systems. In somatic chimeras, also non-hematopoietic cells can be α4 integrin deficient including, for example, BM stroma cells. BM stroma cells are known to be important in the regulation of hematopoiesis by producing hematopoietic cytokines and by direct cell-cell contact with HSC and HPC. It might be that loss of α4 integrin on non-hematopoietic cells leads to the production of cytokines inhibiting B cell development. Thirdly, if pre-pro-B cells (B220-, CD19-, IgM-) in β1β7 mutant BM chimeras express α4 in the absence of β1 and β7 integrin; this could result in a rescue of the α4 null phenotype.

However, using a 4-colour FACS we cold not test this directly. Finally, it cannot be excluded that in these somatic chimeras α4 deficient lymphocyte precursors develop, but have a competitive disadvantage against α4 positive RAG-1 deficient cells. Mice lacking α4 integrin in the hematopoietic system and in other tissues were reported to have a slight, but significant reduction in B220+ cells in the BM from 24.9% in control to 20.8% in α4 mutant animals (Scott et al., 2003). However, since no further information about B cell subpopulations was provided, it is not clear whether the reduction in B cell numbers corresponds to a developmental or a migratory defect. Since the α4 deletion was not restricted to the hematopoietic system, the loss of α4 integrin on other cells could attribute to the differing results.

After 10-12 months, BM of β1β7 mutant BM chimeras contained less immature B cells than control animals while the relative amounts of all other B cell subpopulations remained the same. Whether there is a defect in B cell development that is not apparent at 2 months, but only becomes apparent 10-12 months after the β1 integrin gene deletion or whether these variations are due to individual changes in B cell progenitor frequency from one donor mouse to another remains to be determined. The donor mice were outbred, i.e. genetically not identical, and it is known that the genetic background influences the size of hematopoietic cell compartments (Henckaerts et al. 2004). More mice should be analysed to test whether there really is a change in immature B cell numbers.

Several results proposed an important role of α4 integrins in T cell development. First, α4-null T cell precursors are unable to exit the BM. The normal thymus of β7-null mice suggests that the emigration of T cell precursor might be mediated by α4β1. Secondly, the migration of T cell precursors from the blood to the thymus was proposed to be at least partially mediated by α6β1 integrin (Ruiz et al., 1995). Finally, adhesion and migration of thymocytes within the thymus were suggested to be dependent on a coordinated engagement of α4β1 and α5β1 (Salomon et al., 1994; Crisa et al., 1996). In contrast to these models, T cell precursors deficient for β1 integrin can leave the BM, are able to migrate to and even to develop normally within the thymus implying functional redundancy between β1 and β7 integrins (Brakebusch et al., 2002). The analysis of β1β7

mutant BM chimeras in this study revealed that also in the absence of both α4β1 and α4β7, T cell precursors are generated. Furthermore, β1-β7- T cells precursors migrate from the BM to the thymus and mature within the thymus to CD4+ or CD8+ T cells. These findings are in sharp contrast to the expectations raised by the somatic α4-null chimera, which showed a complete block in migration of T cell precursors from the BM. In α4 integrin conditional knockout mice, CD4+ T cells were reduced from 1.7% in controls to 1.0% in α4 mutants in the BM. Albeit no specific developmental block was reported, these data indicate that T cell precursors can exit the BM in the absence of α4 integrins. Whether there is a defect in T cell development or migration, however, is not clear. Loss of α4 integrin on non-hematopoietic cells could be the reason for the reduced number of T cells in the BM that we could not detect in β1β7 mutant BM chimeras lacking α4β1 and α4β7 restricted to the hematopoietic system. Furthermore, expression of α4 on β1-β7- T cell precursors migrating from the BM might contribute to these differences.

Alpha4 integrins are therapeutic targets in the treatment of the autoimmune diseases Multiple Sclerosis and Crohn's disease. Inhibitory antibodies against α4 integrin are currently tested in long term studies (Andrian et al. 2003). This treatment, however, does not lead to impaired B or T cell development which is in agreement with the normal lymphoid development observed in β1β7 mutant BM chimeras.

4.3 Lymphocyte migration

Lymphocytes extravasate by weakly binding to and rolling on the endothelium, followed by integrin activation, integrin mediated firm adhesion, and transmigration through the endothelial cell layer and the underlying basement membrane (Moser and Loetscher, 2001). Although β1 integrin is crucial for the adhesion of HSC to the endothelium of hematopoietic organs (Hirsch et al., 1996; Potocnik et al., 2000), it was not known whether it plays a similar role in differentiated lymphocytes. Since antibodies against the basement membrane

component laminin reduced lymphocyte emigration to lymph nodes *in vivo* (Kupiec-Weglinski and De Sousa, 1991) and adhesion to basement membrane laminins correlated with the ability of T cells to transmigrate these barriers (Sixt, 2001). It was also suggested that β1 integrin-mediated binding of extravasating cells to basement membrane components like laminin is crucial for this process. The analysis of BM, spleen, LN and PP of β1-null BM chimeras did not confirm a role of β1 integrin in lymphocyte trafficking because the β1 mutant animals showed normal amounts of lymphocytes in secondary lymphoid organs (Brakebusch et al., 2002). Competitive lymphocyte migration assays with β1-null cells did not reveal significant differences in short term homing of β1-deficient and normal cells to spleen, LN and PP, suggesting that β1 integrin on lymphocytes is dispensable for firm adhesion to and transmigration through the endothelium and the basement membrane. It is possible that the migration through the basement membrane does not require binding to this structure, but rather local protease activity enabling cell movement without specific contact of cellular receptors and basement membrane molecules (Friedl and Brocker; 2000).

In mice deficient for the β7 integrin subfamily the formation of the gut associated lymphoid tissue (GALT) is severely impaired. It was shown that α4β7 integrin expressed on lymphocytes contributes to the rolling, but not to the initial binding of these cells on the high endothelial venules of the gut, which are the entry points for leukocytes into the GALT. In addition, it was suggested that β7-null lymphocytes fail to adhere firmly to the high endothelial venule of the gut (Wagner and Müller et al., 1996). The mucosal counter receptor for α4β7 is MAdCAM-1 and, as expected, mice not expressing MAdCAM-1 show a similar absence of GALT as β7-deficient mice (Pabst et al., 2000).

β1β7 mutant lymphocytes were found in all lymphoid organs besides PP demonstrating that α4β1 and α4β7 are not essential for lymphocyte circulation. A reduced number of B cells were observed in LN 10-12 months after induction of the knockout, however, no defect was observed 2 months after knockout induction. Short-term homing assays should be carried out to verify whether β1 and β7 deficient B cells have an impaired migration to LN. All other lymphoid organs showed similar percentages of β1β7 deficient B cells, suggesting normal

migration of β1β7 mutant B cells to the spleen and normal recirculation to the BM.

The percentage of β1β7-deficient CD4 or CD8 T cells was similar in BM, spleen and lymph nodes 2 and 10-12 months after knockout induction. However, the amount of CD4 T cells observed in LN 10-12 months after induction of the knockout was slightly increased. Since only 3 mice have been analysed, it cannot be definitely concluded whether T cell migration to LN is altered in mice 10-12 months after the gene deletion or whether relative T cell numbers are influenced by the amount of B cells homing into this tissue. It seems, however, that β1 and β7 integrins are dispensable for the circulation of T cells under non-inflammatory conditions.

VCAM-1-deficient mice were reported to have a defective migration of mature B cells to the BM (Koni et al., 2001; Leuker et al., 2001). The cell surface receptor VCAM-1 can bind to α4β1, α9β1 and α4β7. However, ablation of all three receptors in the β1β7 mutant BM chimeras did not reproduce the phenotype described for the VCAM-1-deficient mice, indicating an additional VCAM-1 binding partner responsible for it or an indirect mechanism not involving a direct interaction of VCAM-1 on endothelial cells with a counter receptor on lymphocytes.

4.4 Development of myeloid cells

In vitro experiments demonstrated that antibodies against α4β1 delay myeloid development *in vitro* (Miyake et al., 1991). In accordance with this data a low steady state level of differentiated myeloid cells was described for α4 deficient chimeric mice (Arroyo et al., 1999). In the BM of one month old somatic chimeric mice the contribution of α4-deficient myeloid cells was lower than 10% compared to an average chimerism of more than 30% in all other tissues. Since no developmental defect for myeloid cells was described for β7 integrin deficient

mice (Wagner et al., 1996) the defects found in α4 null chimeric mice were suggested to be caused by the loss of α4β1 integrin.

α9β1 integrin, which is highly expressed on human neutrophils and can bind to VCAM-1, was shown to promote transendothelial migration of neutrophils *in vitro* (Taooka et al., 1999). It was therefore possible, that loss of β1 integrin leads to an accumulation of neutrophils in the blood. In contrast to this hypothesis, β1 mutant BM chimeras showed a completely normal development of myeloid cells and displayed no accumulation of neutrophils in the peripheral blood (Brakebusch et al., 2002). Since also β1β7 mutant BM chimera displayed normal myelopoiesis, there is no evidence for a redundant function of α4β1 and α4β7 with respect to differentiation of this blood cell lineage. A closer look at the deletion efficiency of β1 integrin in the β7 null background among immature granulocytes shows that the amount of β1 and β7 integrin deficient cells decreases from 94.4%±4.7% 2 months after the gene deletion to 90.7%±2.7% after 10-12 months after the gene deletion. However, different mice were mice were tested at these time points so that this slight difference is most likely due to mouse to mouse variations and not caused by a competitive advantage of β7- over β1-β7- myeloid progenitors.

Although α4 integrin is not important for erythropoiesis in colony assays *in vitro*, it was reported to be absolutely required in erythropoiesis *in vivo* since in adult α4-null somatic chimeras no erythrocytes derived from α4-null HSC could be found. In β1β7 mutant BM chimeras, erythroblast formation was not impaired. Erythrocyte numbers in BM and spleen were normal, but it was not possible to confirm in this system that the erythrocytes are derived from β1- β7- progenitors or, although rather unlikely, from the low number of β1 expressing precursor cells. Normal numbers of Ter119+ erythroblasts were also reported in the conditional α4 knockout mice, however, the origin of the erythrocytes was not investigated (Scott et al., 2003).

4.5 Surface expression of α4 integrin in the absence β1 and β7 integrins

Integrins are heterodimeric cell surface receptors that are composed of an α and β subunit (Hynes 2002). So far, no integrin receptor has been reported to function as a monomer. Furthermore there is no evidence from the mouse genome database that there are more β integrin subunits than the 8 already known. However, in studies using B cell or T cell lymphoma cell lines α4 and α6 integrin were observed on the cell surface in the absence of β1 and β7 integrin (Crowe et al., 1994, Stroeken et al., 1998). Since no β subunit or any other molecule could be co-immunoprecipitated with these α chains, it was concluded that α4 and α6 could be expressed on the cell surface as a monomeric subunit. Alternatively, α4 or α6 might dimerize with a molecule different from β1 and β7 rather weakly, so that the association will be lost under the lysis conditions used. With the presence of α4 integrin on the cell surface in β1β7 mutant mice we observe now for the first time in untransformed cells an integrin subunit in the absence of its known dimerisation partners. To date, it is not clear whether α4 is part of a new heterodimer or whether it can reach the cell surface as a monomer. Furthermore, no function could yet be ascribed to this α4 chain as α4+ β1- β7- cells do not bind VCAM-1 in a Mn^{2+} inducible manner. Mn^{2+} inducible binding, however, would require a β subunit associated to α4. Therefore this assay does not exclude the capability of α4, as part of a heterodimer or as a monomer, to bind extracellular ligands. This is difficult to test as the ligand preferences might be different from α4β1 or α4β7 integrins. The surface expression of α4 integrin in the absence of β1 and β7 integrin was mainly found on erythroblasts, but not on other differentiated cells. Since erythroblasts of wild type mice express the highest levels of α4 integrin of all hematopoietic cells, α4 expression on β1β7-null cells might be due to inefficient intracellular sorting and degradation of the large amounts of α4 lacking a conventional partner.

Also, non-hematopoietic fibroblastoid cells deficient for β1 and β7 can express α4 on the surface. Whether α4 is present as a monomer or in a complex and whether this is similar to the hematopoietic system was not yet investigated. In any case

the availability of stable, fibroblastoid α4+β1-β7-, cell lines will facilitate the further analysis as they allow the generation of very high cell numbers for biochemical analysis without any contaminating wild type cells present in conditional knockout mice.

4.6 Conclusion

In this study the role of β1 and β7 integrins for the hematopoietic system has been elucidated using BM chimeric mice in which these two integrins were absent only in hematopoietic organs. B cells, T cells, granulocytes and monocytes as well as erythrocytes were found to develop normally in these mice despite the absence of α4β1 and α4β7. These results were unexpected since α4 null somatic chimeric mice have severe hematopoietic defects. Integrin α4 conditional knockout mice show milder but significant impairments in lymphopoiesis. Furthermore, α4 integrin expression on HSCs/HPCs was supposed to be crucial for their retention in the BM. Our data suggest that inhibition of α4 integrin on non-hematopoietic cells can contribute significantly to the release of HPCs from the BM indirectly affecting HPC retention maybe by cytokines. These data completely change the previous view of integrin function in blood cell development and contribute to a new understanding of the role of these molecules during hematopoiesis.

The analysis of β1β7 mutant BM chimeras revealed cells expressing α4 in the absence of β1 and β7 integrin. To which extent the existence of such cells account for the relatively mild phenotype is not yet clear. The focus in the future will be on the biochemical analysis of an established α4+β1-β7- fibroblastoid cell line to uncover potential binding partners and intra- and/or extracellular ligands and to test the importance of these findings for the α4+β1-β7- cells observed in β1β7 mutant BM chimeric mice.

5. Zusammenfassung

Integrine sind im Blutsystem von entscheidender Bedeutung für die Besiedlung von hämatopoietischen Organen, für die Entwicklung und Verteilung der Vorläuferzellen und für die Extravasation von Leukozyten in entzündete Organe. Studien mit somatisch chimären Mäusen mit einer gezielten Inaktivierung des Gens für α4 integrin, das mit β1 und β7 heterodimerisieren kann, zeigten, dass α4 integrin notwendig für normale Hämatopoiese ist. In diesen Mäusen wurden nur wenige, α4 defiziente B Zellen gefunden und T Zell-Vorläufer konnten das Knochenmark nicht verlassen um im Thymus heranzureifen. Die Entwicklung der Erythrozyten war in Abwesenheit von α4 Integrin blockiert und die Expansion der myeloiden Vorläuferzellen eingeschränkt.

Mäuse mit einem Genverlust von entweder β7 oder β1 Integrin nur im hämatopoietischen System, zeigten jedoch eine normale Blutzellentwicklung. Um zu überprüfen, ob α4β1 und α4β7 funktionell redundant sind, wurden Tiere erzeugt, denen beide Untereinheiten, β1 und β7, gleichzeitig im Blutsystem fehlen. Dazu wurden Mäuse mit einer β7 Nullmutation mit Mäusen verpaart, die eine konditionale Gendeletion für β1 Integrin tragen. Der Verlust der funktionellen Gene wurde durch eine Knochenmarkstransplantation auf das hämatopoietische System beschränkt. Die resultierenden Knochenmarkschimären wurden nach der Repopulation der hämatopoietischen Organe mit polyIC behandelt um die Gendeletion zu induzieren. Überraschenderweise konnten keine Defekte in der Entwicklung von Blutzellen gefunden werden. Die Entwicklung von B Zellen, T Zellen, Blutplättchen, Erythrozyten, Granulozyten und Monozyten wurde untersucht und war unabhängig von β1 und β7 Integrinen. Da die Zellularität der lymphatischen Organe von β1β7 mutanten Knochenmarkschimären nicht verändert war im Vergleich zu β7 mutanten Knochenmarkschimären, die als Kontrollen verwendet wurden, gab es keinen Hinweis auf defekte Leukozytenmigration. 12 Monate nach der Induktion der β1 Integrin Gendeletion wurde keine Expansion der β1 exprimierenden Zellen festgestellt, was darauf hindeutet, dass β1+ hämatopoietische Zellen keinen kompetitiven Vorteil in Entwicklung und Expansion haben. Die Expansion der hämatopoietschen Zellen war sogar nach induzierter Anämie unbeeinträchdigt.

Bei der Analyse der β1β7 mutanten Knochenmarkschimären wurden hämatopoietische Vorläuferzelln und Erythroblasten gefunden, die α4 exprimieren, aber negativ sind für β1 und β7 Integrin. Die funktionelle Bedeutung dieser α4 Expression ist jedoch unklar, da keine Bindung an VCAM-1 nachgewiesen werden konnte. Obwohl noch unbekannt ist, ob α4 Integrin auf der Zelloberflächefläche mit einem Protein assoziiert ist oder alleine existiert, kann durch einen gentetischen Ansatz ausgeschlossen werden, dass es mit β2 Integrin heterodimerisiert, welches sehr stark auf hämatopoietischen Zellen exprimiert wird.

6. Abstract

In the hematopoietic system, integrins are crucial for the colonisation of hematopoietic organs, the development and distribution of progenitor cells, and for the extravasation of leukocytes into inflamed tissues. Using somatic chimeric mice carrying a targeted disruption of the gene for α4 integrin, which is known to dimerize with either integrin β1 or β7, α4 integrins were shown to be crucial for normal hematopoiesis. In these mice only few α4-null B cells were detected and T cell precursors could not leave the BM for maturation in the thymus. Furthermore, erythrocyte development was blocked and expansion of myeloid progenitors was reduced in the absence of α4 integrins.

Mice with a knockout of β7 or β1 integrin restricted to the hematopoietic system, however, have no defects in hematopoiesis. To test whether α4β1 and α4β7 integrins have redundant functions β1 and β7 double knockout mice were generated by intercrossing mice with a constitutive β7 knockout and a conditional ablation of β1. To restrict the deletion to the hematopoietic system, BM chimeric mice were generated. The deletion of the β1 integrin gene was induced after repopulation by repeated injections of polyIC. Surprisingly, no defects in the hematopoietic development of β1β7 mutant BM chimeras were noted. Development of B cells, T cells, platelets, erythrocytes, granulocytes and monocytes was unimpaired in the absence of β1 and β7. Normal cellularity of lymphoid organs indicated no obvious defect in leukocyte migration. No expansion of β1 expressing cells was noted 12 months after the induction of the β1 integrin gene deletion, suggesting no competitive advantage of β1+ hematopoietic cells. Finally, normal expansion of hematopoietic cells was observed after acute haemolysis.

Unexpectedly, HPCs and erythroblasts were found which express α4 integrin on the surface in the absence of β1 and β7 integrin. This α4 integrin was not able to mediate binding to VCAM-1 and it is therefore unclear, whether it is of functional importance. Using a genetic approach we could show that this α4 integrin is not heterodimerizing with β2 integrin, which is strongly expressed on hematopoietic cells.

These data indicate that α4β1 and α4β7 are not required for hematopoiesis and do not have essential overlapping functions. The severe phenotype reported for α4 null somatic chimeric mice might be caused by the loss of α4 integrin on non-hematopoietic cells, while in our system the ablation of β1 and β7 integrin is restricted to the hematopoietic system.

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