Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

The roles of integrin α4β1, α4β7 and the small GTPase RhoH during hematopoiesis and autoimmunity

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aus

Eutin

2008

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. Reinhard Fässler betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am 20.02.2008

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(Martina Bauer)

Dissertation eingereicht am 22.02.2008
1. Gutachter: Prof. Dr. Reinhard Fässler
2. Gutachter: Prof. Dr. Markus Sperandio
Mündliche Prüfung am 24.04.2008

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2 List of Publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-V):

I. Sixt, M., **Bauer, M.**, Lämmermann, T., Fässler, R. β1 integrins: zip codes and signaling relay for blood cells. *Current Opinion in Cell Biology* **18**, 482-490 (2006).

II. Bungartz, G., Stiller, S., **Bauer, M.**, Müller, W., Schippers, A., Wagner, N., Fässler, R., Brakebusch, C. Adult murine hematopoiesis can proceed without β1 and β7 integrins. *Blood* **108**, 1857-1864 (2006).

III. Dorn, T., Kuhn, U., Bungartz, G., Stiller, S., **Bauer, M.**, Ellwart, J., Peters, T., Scharffetter-Kochanek, K., Semmrich, M., Laschinger, M., Holzmann, B., Klinkert, W.E., Straten, P.T., Kollgaard, T., Sixt, M., Brakebusch, C. RhoH is important for positive thymocyte selection and T-cell receptor signaling. *Blood* **109**, 2346-2355 (2007).

IV. **Bauer, M.**, Sixt, M., Coisne, C., Brakebusch, C., Wekerle, H., Engelhardt, B., Fässler, R. Extravasation of autoreactive T cells into the central nervous system is controlled by β1 integrins. Manuscript in preparation.

V. Montanez, E., Piwko-Czuchra, A., **Bauer, M.**, Li, S., Yurchenco, P., Fässler, R. Analysis of integrin functions in peri-implantation embryos, hematopoietic system, and skin. *Methods in Enzymology* **426**, 239-289 (2007).

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3 Abbreviations

aa	amino acid
ADP	adenosine diphosphate
AGM	aorta-gonad-mesonephros
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
APL	altered peptide ligand
ATP	adenosine triphosphate
BBB	blood brain barrier
BCR	B cell receptor
BDNF	brain derived neurotrophic factor
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CFA	complete Freund´s adjuvant
CLA	cutaneous lymphocyte antigen
CNS	central nervous system
c/pSMAC	central/peripheral supra-molecular activation cluster
CS-1	connecting segment-1
DC	dendritic cell
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECM	extracellular matrix
EDSS	Expanded Disability Status Scale
ES	embryonic stem
E	embryonic day
FAK	focal adhesion kinase
FDA	Food and Drug Adminstration
FucT	fucosyltransferase
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GSK3β	glycogen-synthase kinase-3β
GTP	guanosine triphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEV	high endothelial venule
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILK	integrin-linked kinase
IPP	ILK-PINCH-parvin
JCV	JC virus
LAD	leukocyte adhesion deficiency
LAT	linker for activation of T cells
LDV	leucine-aspartic acid-valine
LFA	lymphocyte function-associated antigen
LPAM	lymphocyte Peyer's patch adhesion molecule
LPS	lipopolysaccharide
mAb	monoclonal Ab
Mac	macrophage adhesion molecule
MAdCAM	mucosal addressin cell adhesion molecule
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MS	multiple sclerosis
NF-κB	nuclear factor-κB
PAS	para-aortic splanchnopleura
PINCH	particularly interesting new cystidine-histidine-rich protein
PIP2	phosphatidylinositol (4,5) bisphosphate
PIPKIγ	phosphatidylinositol phosphate kinase type Iγ
PKB/AKT	protein kinase B
PKD1	protein kinase D1
PLP	proteolipid protein
PML	progressive multifocal leukoencephalopathy
PNAD	peripheral node addressin
PPMS	primary progressive MS
PSGL	P-selectin glycoprotein ligand
RAPL	regulator of cell polarization and adhesion enriched in lymphoid tissues
RGD	arginine-glycine-aspartic acid
RIAM	Rap1 GTP-interacting adapter molecule
RRMS	relapsing-remitting MS
RTK	receptor tyrosine kinase
SCF	stem cell factor
SPMS	secondary progressive MS
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TNBS	2, 4, 6-trinitrobenzene sulfonic acid
TNF	tumor necrosis factor
T_{reg}	regulatory T cell
T_H1 cell	$CD4^+$ T helper type 1 cell
T_H2 cell	$CD4^+$ T helper type 2 cell
VCAM	vascular-cell adhesion molecule
VLA	very late antigen

4 Summary

Members of the integrin family of adhesion molecules are transmembrane proteins that provide a link between the cytoskeleton and the extracellular matrix (ECM) or neighboring cells. Thereby they control the shape, proliferation and survival of cells and modulate various intracellular signaling pathways. They are heterodimers, consisting of α and β subunits. Integrins and intracellular effector molecules of integrin signaling, such as small Rho GTPases that regulate the actin cytoskeleton, play important roles during hematopoiesis and immune responses.

Firstly, we examined the role of α4 integrins in adult murine hematopoiesis (Paper II), since previous experiments investigating the function of the integrin α4 subunit and its two association partners β1 and β7 provided controversial results for hematopoietic development. We analyzed the hematopoietic system of bone marrow chimeric mice with an inducible knockout of the β1 subunit and a constitutive deletion of the β7 gene. We found only subtle or transient alterations in the number and distribution of progenitor cells, while the maintenance and retention of hematopoietic stem cells (HSC) was not altered. The development of T and B lymphocytes and their adhesion molecule expression profiles were normal. Furthermore, the development of myeloid and erythroid cells was unchanged. In summary, both α4β1 and α4β7 integrins are dispensable for adult hematopoiesis indicating that previously observed severe hematopoietic defects in somatic chimeric $\alpha4^{-/-}$ mice are likely due to the loss of α4 expression on non-hematopoietic cells.

Secondly, we analyzed the role of the small GTPase RhoH in the hematopoietic system (Paper III). Previous publications implicated RhoH in the development of B cell lymphomas, proliferation and survival of HSCs as well as migration and adhesion of lymphocytes and HSCs. Since RhoH is expressed only in hematopoietic cells we introduced a constitutive RhoH gene disruption in the germline of mice and analyzed their hematopoietic development. RhoH-null mice had no remarkable changes in myeloid, erythroid or B lymphocyte populations. In contrast T cell development was impaired at two distinct steps: (i) during late stages of development of $CD4^-CD8^-$ double negative cells, and (ii) during the transition of double positive $CD4^+CD8^+$ T cells to $CD4^+$ or $CD8^+$ single positive cells. These defects could be attributed to: (i) defective pre-TCR signaling to T cells that generated functional TCR β-chains during β-selection, and (ii) compromised positive selection. Both defects resulted from impaired pre-TCR and TCR signaling to the scaffold protein linker for activation of T cells (LAT) and its associated molecules downstream of the tyrosine kinase ZAP70. In conclusion, RhoH

deficient mice have no defect in the maintenance of HSCs, but the differentiation of T lymphocytes is severely disturbed due to reduced pre-TCR and TCR signaling.

Finally, we examined the functions of β1 integrins during the development of autoimmunity (Paper IV). Previous investigations pointed to a crucial role of α4 integrins in the development of multiple sclerosis (MS) in man and experimental autoimmune encephalomyelitis (EAE) in rodents. Antibodies that block the α4 subunit became an efficient new medication for the treatment of MS and other inflammatory diseases, such as inflammatory bowel disease (IBD). Although these antibodies are already approved for the treatment of MS, their exact working mechanism, their target cells and their precise target integrin (α4β1 and/or α4β7) are not clear. Mice with hematopoietic cells that lack integrin β1 expression provided the opportunity to analyze particularly the role of α4β1 during activation, proliferation and extravasation of encephalitogenic T cells. Furthermore, we examined whether β1 integrins play a role on other immune cells during the pathogenesis of EAE. We found that encephalitogenic T cells rely on β1 integrins to accumulate in the CNS, a defect that could not be rescued by the presence of α4β7. This implies that α4β1 is the main target of anti-inflammatory therapies directed against the α4 integrin subunit. The antigen-specific proliferation and cytokine response of $\beta1^{-/-}$ T cells *in vivo* was not significantly altered. In contrast, the firm adhesion of β1 integrin deficient T lymphoblasts to postcapillary venules of the spinal cord white matter was greatly reduced *in vivo*; indicating that the failure of $\beta1^{-/-}$ T cells to enter the CNS and the main effect of the antibody therapy is due to impaired extravasation. Furthermore, we could demonstrate that active EAE development is significantly delayed in mice lacking β1 expression specifically on T lymphocytes. This result, together with the observation that lack of β1 expression on granulocytes and macrophages does not alter active EAE development, indicates that the main targets of the antibody therapy are the α4β1 integrins on T lymphocytes.

5 Introduction

5.1 The integrin family of adhesion and signal transduction receptors

5.1.1 Integrins in general

Integrins are heterodimeric, type I single-span transmembrane glycoproteins consisting of non-covalently associated α and β subunits. The extracellular domain of each subunit is large and contains around 1000 amino acids (aa) or 750 aa for α or β subunits, respectively, whereas the short intracellular parts consist of only 20 to 50 aa[1]. The genome of mouse and man contains 18 α and 8 β subunits, which form together at least 24 heterodimers with different binding and signaling properties (Figure 5.1)[2]. The extracellular domains of integrins bind to a wide variety of extracellular matrix (ECM) molecules, soluble proteins or to counter receptors on other cells. The N-termini of α subunits are folded into 7-bladed β propellers and the distal ends of the β subunits are folded into I/A domains. Together these globular head domains form the ligand-binding domain that is separated from the plasma membrane in both subunits by a long stalk[1,3]. Accordingly the ligand binding specificity of a heterodimer is dependent on both subunits. The cytoplasmic tails are connected by adaptor proteins to the actin cytoskeleton and to various signal transduction pathways. An exception is the β4 subunit, which has an unusually long 1072 aa cytoplasmic domain, which recruits intermediate filaments[3]. Therefore integrins bridge the gap between the cytoskeleton and the ECM or other cell adhesion molecules. Apart from mediating mere adhesion to substrates and cells integrins have important functions as signaling molecules and thereby regulate processes as diverse as proliferation, cell survival, immune responses and cell shape leading to cell polarity and motility[4]. Although the integrin family is huge several studies indicate that they have overlapping as well as specific functions. Genetic inactivation of single integrin subunits[5-9], for example leads to distinct phenotypes (ranging from early and severe peri-implantation lethality in β1 integrin-deficient mice lacking 12 different integrins (Figure 5.1)[10] to subtle effects in other knockouts), while compound mutants uncovered novel functions[5].

5.1.2 The four classes of integrin ligands

Extracellularly most integrins bind to several different ligands and most integrin ligands are recognized by several members of the integrin family. Nevertheless the different integrins can be grouped into four main classes, based on their ligand-binding specificity.

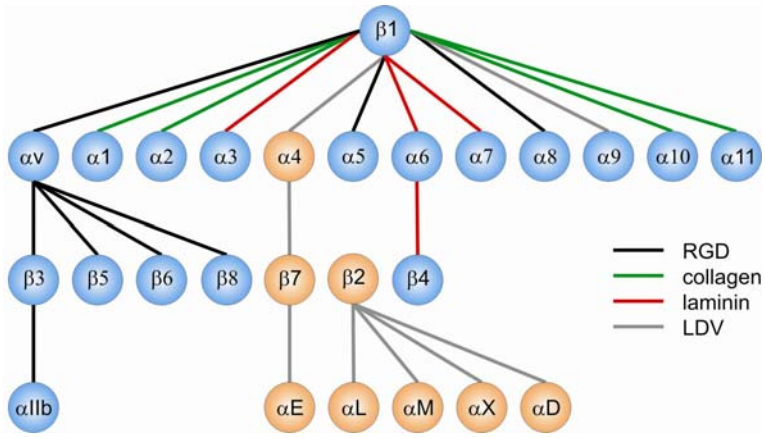

Figure 5.1: The family of integrin heterodimers. Shown are all known combinations of integrin subunits in mammalian cells. The color code of the connecting lines between the subunits indicates the major ligand binding specificity of the respective heterodimer. Black: RGD-binding integrins, green: collagen-binding integrins, red: laminin-binding integrins, grey: integrins binding the LDV motif and related or structurally similar sequences. Note that these are only the main ligands, and that most integrins bind to additional molecules[2]. The subunits that are depicted in orange are expressed exclusively on leukocytes.

The first group (connected by black lines in Figure 5.1) contains all αv integrins, the platelet integrin αIIbβ3 and some β1 heterodimers and binds RGD-containing ligands. RGD (arginine-glycine-aspartic acid) is a tripeptide binding motif that is present in soluble and ECM molecules, for example fibronectin, vitronectin, fibrinogen, thrombospondin, osteopontin, and laminins[11]. The α1, α2, α10 and α11 chains dimerize exclusively with the β1 subunit (connected by green lines) and form the collagen-binding integrins. The heterodimers that are connected by red lines are laminin-binding integrins. While the α6 subunit-containing integrins bind only laminin, the α3β1 integrin also binds a large number of additional ligands. The fourth subclass of integrin receptors include the leukocyte-specific subunits (orange subunits connected by grey lines) and recognize the LDV or structurally related motifs. LDV (leucine-aspartic acid-valine) is an acidic tripeptide that can be found for example, in the alternatively spliced connecting segment-1 (CS-1) fragment of fibronectin. Other typical ligands of this integrin subfamily including vascular-cell adhesion molecule-1 (VCAM-1), mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and intercellular adhesion molecule-1 (ICAM-1), contain binding motifs that have a different aa sequence but are structurally related to the LDV motif[2].

5.1.3 Inside-out and outside-in signaling of integrins

Binding of integrins to their ligands depends on the composition of the heterodimer and on its activation state. A striking example is the platelet integrin αIIbβ3

that is amongst other integrins present on all circulating thrombocytes. In its active state, αIIbβ3 binds ligands such as fibrinogen, fibrin, von Willebrand factor and fibronectin, resulting in platelet adhesion to subendothelial cells, plug formation and sealing of injured vessels[12]. If αIIbβ3 integrins are constitutively active they trigger pathologic thrombus formation causing strokes, myocardial infarction and other embolic events. Therefore, αIIbβ3 is in a low-affinity state on all circulating platelets but can be activated instantly if plug formation is required. Activation of αIIbβ3 is achieved via agonists such as thrombin, collagen, adenosine diphosphate (ADP), and adenosine triphosphate (ATP) that signal through different types of receptors[12]. The various signal transduction pathways converge as a final step in the activation and recruitment of several molecules including kindlins and talins to the cytoplasmic tail of integrin β subunits[13]. Recruitment of kindlin and talin is followed by the separation of the membrane-proximal α-helical regions of the two short cytoplasmic tails that are in close proximity in the inactive conformation and interact via hydrophobic and electrostatic interactions. Tail separation leads to conformational changes characterized by a switchblade-like opening of the bended, inactive into an extended, highly active integrin (Figure 5.2)[4]. Thus, the cytoplasmic domains alter the activation state of the extracellular domains and consequently, this process was named inside-out signaling. Integrins are therefore unique as they act as classical signal transduction receptors transferring signals from the extracellular space to the cytoplasm (outside-in signaling) and at the same time regulate their activation state by inside-out signaling. Notably, it has also been shown that signaling by one integrin heterodimer can alter the activation state of other integrins on the same cell. A good example for such an integrin crosstalk is the activation of β2 integrins on leukocytes upon engagement of α4β1[14]. It has also been shown that the avidity of integrin binding is regulated. Binding of extracellular ligands leads to clustering of integrins and to the recruitment of signaling and adapter proteins to the intracellular tails resulting in increased binding strength or avidity. These integrin clusters are called focal complexes when they are developing, and focal adhesions when they have matured to their final size[4].

Since integrins do not have enzymatic activity themselves the focal complex proteins are needed to connect them with signal transduction pathways and the actin cytoskeleton. Talin for example, mediates integrin activation by inside-out signaling[13] and participates in integrin clustering and linkage to the actin cytoskeleton. Furthermore, talin recruits proteins that are involved in outside-in signaling such as the non receptor tyrosine kinase focal adhesion kinase (FAK) and phosphatidylinositol phosphate kinase type Iγ (PIPKIγ), which is producing the second messenger

phosphatidylinositol (4,5) bisphosphate (PIP2)[4]. An essential role of talin in outside-in signaling was corroborated in talin1-deficient platelets. In platelets, integrin ligand-binding leads via activation of Src family kinases to cell spreading, a process that is completely abolished in platelets lacking talin-1.[13]

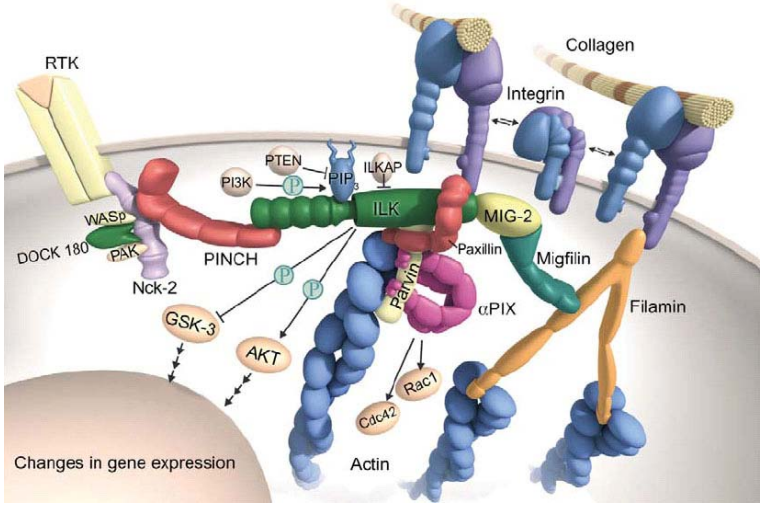

Figure 5.2: Adaptor proteins binding directly and indirectly to the cytoplasmic tail of integrin heterodimers and linking them to the actin cytoskeleton and to signal transduction processes. Note the two conformations of the heterodimers: the bent, inactive form and the extended, active form with separated cytoplasmic tails. The ILK-PINCH-parvin complex represents a large multiprotein complex at the cytoplasmic tail of activated β1 and perhaps β3 integrins that links integrins to the actin cytoskeleton and to signal transduction pathways. RTK, receptor tyrosine kinase. The cartoon is taken from Grashoff et al.[15].

The proteins binding intracellularly to integrins are organized in several multiprotein complexes. One example for such a protein complex is the ILK-PINCH-parvin (IPP) complex consisting of integrin-linked kinase (ILK) and different isoforms of the adaptor proteins particularly interesting new cystidine-histidine-rich protein (PINCH) and parvin (Figure 5.2). ILK is binding to the β1 subunit of integrins[16], and it has also been shown to interact with β3 subunits[17] but the recruitment of the IPP complex to focal adhesions requires an interaction with other adaptor molecules, including paxillin (Figure 5.2)[18]. The resulting multiprotein complex mediates binding to F-actin, actin polymerization and turnover, and modulates signals of a wide variety of signaling pathways[4,15]. Despite of its name there are doubts, whether ILK acts as a kinase *in vivo*. ILK possesses a domain that has a high homology to serine/threonine kinases but it lacks several residues thought to be essential for kinase activity[18]. It has been demonstrated that ILK is phosphorylating the protein kinase B (PKB/AKT) and glycogen-synthase kinase-3β (GSK3β) *in vitro*, but *in vivo* current data support as well as object an ILK kinase activity[18-20]. There is consensus in the community that a major

function of ILK *in vivo* is linking integrins to the actin cytoskeleton and modulating actin reorganization[4].

5.1.4 The Rho family of small GTPases

Rho GTPases represent an important class of signaling machines downstream of integrins. The >20 mammalian Rho GTPases are small signaling molecules that exist in two conformational states, the active, guanosine triphosphate (GTP) bound state, and the inactive guanosine diphosphate (GDP) bound state. The switch between these two conformations is carefully regulated positively by guanine nucleotide exchange factors (GEFs) and negatively by GTPase-activating proteins (GAPs). The C-terminus of most Rho GTPases is prenylated, and can therefore target the proteins to plasma membranes. Another level of regulation occurs through guanine nucleotide dissociation inhibitors (GDIs) that bind inactive GDP bound GTPases and extract them from the plasma membrane (Figure 5.3)[21,22].

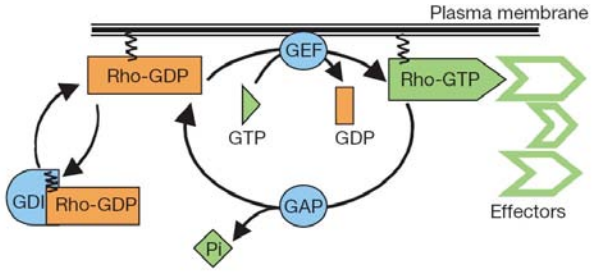

Figure 5.3: GEFs mediate the exchange from the GDP to the GTP bound state and thus activate Rho GTPases. GAPs enhance the hydrolysis of GTP that is catalyzed by the GTPases themselves, and thereby inactivate Rho GTPases. GDIs bind inactive Rho GTPases and sequester them from the cell membrane and block the nucleotide exchange. The cartoon is taken from Etienne-Manneville et al.[21].

The three best studied members of the family of Rho GTPases RhoA, Rac1 and Cdc42 exemplify that - despite structural and functional similarities - each GTPase performs distinct functions. At the leading edge of cells both Rac1 and Cdc42 promote actin polymerization resulting in the formation of lamellipodia and filopodia, respectively. At the rear end of a migrating cell RhoA regulates contraction of the cell body by inducing the assembly and contraction of actomyosin filaments[23].

Another member of the Rho GTPase family is RhoH, which is expressed exclusively in hematopoietic cells including HSCs, differentiated myeloid cells and B and T lymphocytes, particularly T cells with a T_H1 phenotype[24-27]. RhoH inhibits activation of the mitogen-activated protein kinase (MAPK) p38 and the transcription factor nuclear factor-κB (NFκB), which are both activated by GTPases such as RhoA, Rac1 and Cdc42[24]. In HSCs RhoH negatively regulates proliferation and survival[25]. Furthermore, RhoH is an atypical GTPase since it is GTPase deficient and exists only

in the active, GTP-bound state[24], and is kept in check through interaction with all three classical GDIs[24]. Besides it was proposed that RhoH is regulated on the transcriptional level. In T cells it was demonstrated that RhoH transcription is rapidly downregulated upon T cell activation[24]. In B cell lymphomas RhoH is affected by mutations in non-coding regions of the gene and by chromosomal translocations[26,28,29]. Both types of mutations likely result in deregulated expression of RhoH. Moreover, RhoH was implicated in lymphocyte adhesion and migration since downregulation of RhoH expression increases integrin αLβ2-mediated adhesion of a Jurkat T cell clone[30]. Finally, overexpression of RhoH leads to defective assembly and polarization of F-actin and thereby impairs the migration of HSCs towards a chemokine[25]. In order to assess the function of RhoH *in vivo* we analyzed the hematopoietic system of RhoH$^{-/-}$ mice (Paper III).

5.1.5 The α4 subfamily of integrin receptors

The α4 integrins are mainly expressed on cells of the hematopoietic system. The α4 integrin subunit can dimerize with two β chains: β1 and β7[31]. In the hematopoietic compartment, α4 is expressed by lymphocytes[32], HSCs[33], monocytes[34], eosinophils, basophils[35] and natural killer cells[36]. Neutrophils express little or no α4[35], although the expression is upregulated under chronic inflammatory conditions[37]. Integrin α4 is also expressed on HSCs of the adreno-gonad-mesonephros (AGM) region (Figure 5.5), the fetal liver and the adult bone marrow[38]. There is evidence that the α4β1 heterodimer on those cells is in a high affinity state triggered by the stem cell factor (SCF) through c-kit binding[39].

α4β1 was first described as an activation-dependent receptor on hematopoietic cells and is therefore also called very late antigen 4 (VLA-4) whereas α4β7 is also called lymphocyte Peyer`s patch adhesion molecule-1 (LPAM-1) due to its ability to home lymphocytes to high endothelial venules of Peyer`s patches. Both heterodimers belong to the family of LDV motif binding integrins. The main ligand for α4β1 is VCAM-1 whereas the main ligand for α4β7 is MAdCAM-1. Both receptors bind to the alternatively spliced CS-1 fragment of fibronectin (Table 5.1). Interestingly the α4 chain has the unique property to exist in two different forms: either as single molecule with a molecular weight of 150 kDa or as cleaved molecule consisting of two non covalently linked fragments of 70 and 80 kDa[40]. The cleavage is thought to increase upon T cell activation but is neither altering the adhesion to VCAM-1 nor to fibronectin[41]. In addition to the classical ligands VCAM-1 and MAdCAM-1 many other interaction partners for α4 integrins have been described.

Table 5.1: The α4 integrins and some of their described interaction partners.

integrin ligand	properties	α4β1 (VLA-4; CD49d/CD29)	α4β7 (LPAM-1; CD49d/β7)
VCAM-1	Ig superfamily protein expressed on vascular endothelial cells of inflamed tissues	main ligand[42,43]	binding[44]
MAdCAM-1	Ig superfamily protein expressed in HEVs of gut-associated lymphatic tissues and endothelial cells of the lamina propria	binding[2]	main ligand[42,45]
fibronectin	protein of the ECM, isoform containing the alternatively spliced CS-1 region	binding[44]	binding[44]
osteopontin	phosphorylated acidic matrix glycoprotein that is secreted by osteoblasts, monocytes and macrophages; contains an RGD-sequence and hence is bound by several different integrins	binding[2,46]	binding[2]
thrombospondin	ECM protein secreted by platelets and endothelial cells in damaged and inflamed tissue	weak binding; α4β1 has to be activated[44,47]	no interaction[2]

HEV, high endothelial venule; Ig, immunoglobulin

The activation state of both α4 heterodimers is tightly regulated and can vary considerably. During leukocyte extravasation into inflamed tissues (Chapter 5.3.1) leukocyte integrins are activated in milliseconds to ensure their adhesion to the vessel wall under high shear. Other processes in the hematopoietic system where integrins possess high affinity include antigen recognition by T and B cell receptors.

The final step of integrin activation is mediated by talin as described in chapter 5.1.3. Upstream of talin are members of the Rap family of small GTPases like Rap1 that mediate talin activation in hematopoietic cells upon stimulation by chemokines, growth factors or activation of the T cell receptor (Figure 5.4)[48]. Rap1 enhances the adhesion of T cells to fibronectin via α4β1 by activation of β1 integrins. Interestingly, Rap1 increases the binding of β2 integrins by increasing their avidity through integrin clustering rather than integrin activation[49]. One important effector molecule of Rap1 during these processes is regulator of cell polarization and adhesion enriched in lymphoid tissues (RAPL). In RAPL-deficient mice the chemokine CCL21 is unable to stimulate adhesion of T cells to VCAM-1 via α4β1 and the homing of T and B lymphocytes and dendritic cells to their target organs is impaired, indicating that RAPL is involved in activation of α4β1 and other integrins[50]. Protein kinase D1 (PKD1) and Rap1 GTP-interacting adapter molecule (RIAM) are other recently identified effector proteins of Rap1 that are mediating inside-out activation of β1, β2 and β3 integrins through the activation of talin[51]. Similar mechanisms of integrin activation take place after B cell receptor stimulation[52].

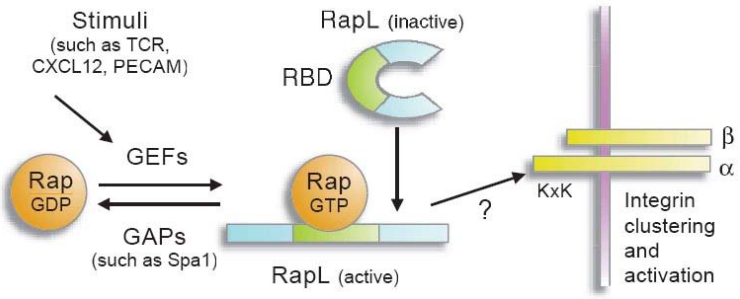

Figure 5.4: The small GTPase Rap and its effector RAPL mediate the rapid activation and clustering of integrins. Upon stimulation of e.g. chemokines or engagement of the T cell receptor Rap1 is activated by the GEF-mediated GDP/GTP exchange, translocates to the cell membrane and binds RAPL. RAPL then mediates integrin activation via talin by an as yet unknown mechanism that might involve the serine-threonine kinase Mst1[53]. The cartoon is taken from Price and Bos[54].

Apart from the control of affinity and avidity, the expression level of VLA-4 on lymphocytes is tightly regulated. Memory T cells that have encountered their specific antigen express much more α4β1 and α4β7 than naïve T cells and therefore show also enhanced ligand binding[55,56].

5.1.6 The role of α4 integrins in embryonic and adult hematopoiesis

The two α4 integrins have many important roles during development and in physiology. Ablation of the α4 integrin gene leads to two waves of embryonic lethality, first around embryonic day (E)10 due to disturbed fusion of the allantois with the chorion and then again between E11.5 and E14 due to severe cardiac hemorrhage[57]. These defects are very similar to those found in mice lacking VCAM-1, indicating that the observed defects are due to an abrogated interaction between α4 integrins and VCAM-1[58,59].

Apart from the early developmental defects in mice lacking the α4 subunit other studies implicated α4 integrins in the development and homing of hematopoietic cells and in inflammation. The interactions between α4β1 and CS-1 of fibronectin or VCAM-1, and between α5β1 and fibronectin, are important for HSC binding to stroma cells of the bone marrow[60], and for lymphopoiesis[33,61-64]. Furthermore, the attachment of HSCs to fibronectin via integrins depends on the cytokine regulated activation state of integrins[65]. Binding of α4β7 to MAdCAM-1 is important for lymphocyte homing to Peyer`s patches and the lamina propria of the intestine[45,66]. Finally, the interaction between α4β1 and VCAM-1 is important for the homing of leukocytes to the inflamed CNS during EAE[67]. Due to the early embryonic lethality of the α4 knockout mice the role of α4 integrins in the hematopoietic system *in vivo* was subsequently studied in somatic α4 chimeras and conditional mutants. Somatic chimeras are generated by

injecting gene deficient embryonic stem (ES) cells into wild-type blastocysts. In this system cells derived from the injected stem cells as well as from the blastocysts will contribute to the development of all tissues of the adult mouse. This often circumvents the problem of early embryonic lethality. Moreover, the absence of mutant cells in a certain tissue indicates a failure or disadvantage for the mutant cells to contribute to its formation.

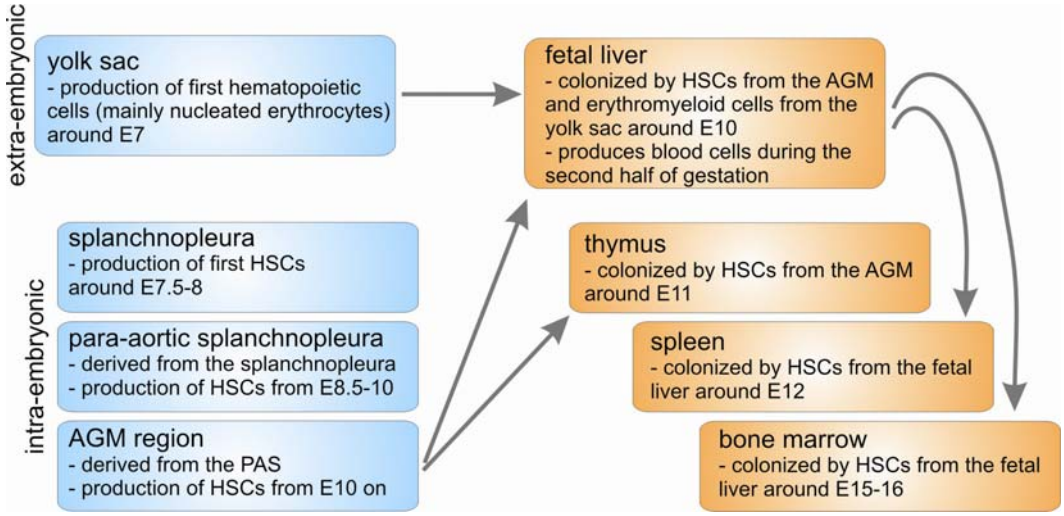

Figure 5.5: Hematopoiesis during murine embryogenesis. Hematopoietic cells are first generated in the extra-embryonic yolk sac (around E7) and the intra-embryonic splanchnopleura that first develops into the para-aortic splanchnopleura (PAS) and then into the AGM region. HSCs from the AGM region are colonizing the fetal liver and thymus and from the fetal liver the HSCs migrate subsequently to the spleen and the bone marrow, the place of adult self-renewing HSCs. Around E8 the fetal circulation starts, allowing an exchange of cells between the yolk sac and the splanchnopleura/AGM region[68].

In order to analyze the role of α4 integrins for hematopoiesis, wild-type blastocysts or blastocysts deficient for RAG-1 or RAG-2 (RAG-1 or RAG-2 knockouts lack mature lymphocytes) were injected with α4-deficient ES cells. These experiments showed that α4 is important for the continuous input of T cell precursors from the bone marrow into the thymus after birth, is crucial for the formation of Peyer`s patches and for the development of B cells in the bone marrow after birth[69]. Fetal liver erythropoiesis is diminished in the chimeric animals although α4 deficient cells are able to differentiate into mature erythrocytes *in vitro*, indicating that those defects are likely due to the α4 deficient microenvironment of the fetal liver (see Figure 5.5 for a description of fetal hematopoiesis). Similar results were obtained with myeloid and B lymphoid progenitors from fetal livers. *In vivo* the α4 deficient progenitors contribute less to the development of mature cells than wild-type progenitors, but in *in vitro* colony formation assays their differentiation potential is not altered, indicating that the reduced differentiation *in vivo*

occurs in a non cell autonomous mode. Post-natally the development of α4 deficient erythrocytes, myeloid cells and B lymphocytes in bone marrow and spleen is almost completely abolished although $\alpha4^{-/-}$ erythroid burst forming colonies develop. *In vitro* it was shown that erythroid cells and B cells have a diminished ability to interact with cultures of bone marrow stromal cells and proliferate less than wild-type cells[70]. The latter result is in accordance with observations that α4 antibodies inhibit the development of B cells and erythroid cells on a stroma cell layer *in vitro* [61,71].

To distinguish cell autonomous phenotypes from defects caused by an α4 deficient microenvironment studies were performed with mice in which the α4 gene was ablated in a conditional manner. Using the inducible Mx1-Cre system all cells of the hematopoietic system lose the α4 gene (Paper V). This circumvents embryonic lethality and excludes side-effects of a knockout in non-hematopoietic cells such as bone marrow stromal cells, for example. The deletion of α4 on HSCs results in a persisting increase of lymphocytes and hematopoietic progenitor cells in peripheral blood and a continuously increasing number of progenitor cells in the spleen, but the steady-state hematopoiesis and erythropoiesis are still normal. To study the role of α4 integrins during erythropoiesis after anemia an acute hemolysis was induced by treating the mutant mice with phenylhydrazine. α4 knockout animals show a delayed regeneration of erythroid cells. Furthermore, the colony forming units in the bone marrow recover with a delay from 5-fluorouracil induced killing of hematopoietic cells. The self-renewal of α4 deficient HSCs and their homing to the bone marrow of lethally irradiated recipient mice is impaired. Accordingly the number of HSCs in the spleen of such animals is slightly higher than in control mice[72,73]. These results are in accordance with experiments showing that the administration of antibodies specific for the α4 chain or VCAM-1 reduces the homing of HSCs to the bone marrow of irradiated recipient mice and leads to an accumulation of HSCs in the spleen. Administration of these antibodies to non-irradiated mice results in an increase of HSC numbers in peripheral blood[74]. The injection of fibronectin fragments also impairs the repopulation of irradiated mice with stem cells and in addition causes their mobilization into the blood[75]. The authors of another study proposed that α4 integrins might be of crucial importance during the fetal development of HSCs. To test this hypothesis they transplanted control or α4 deficient embryonic HSCs isolated from E12.5 AGM regions into irradiated adult wildtype mice. This experimental design bypasses fetal development of HSCs and leads to a comparable reconstitution of recipient mice with control and $\alpha4^{-/-}$ cells. The α4 deficient HSCs contribute stably to hematopoiesis and are able to engraft irradiated recipient mice in secondary transplantations. Only in Peyer`s patches and the peritoneal cavity a relative reduction of α4 deficient myeloid and lymphoid cells could be observed[38]. The

failure to develop Peyer`s patches is in line with observations made in mice that lack the β7 subunit, indicating that Peyer`s patch development is a specific function of the α4β7 subunit. Interestingly, lymphocyte development is not disturbed in β7 deficient animals, which suggests that mainly α4β1 regulates lymphopoesis[76]. In Mx1-Cre induced conditional knockout mice lacking VCAM-1, the counter receptor for α4β1, the retention of B cells in the bone marrow is disturbed while the remaining aspects of hematopoiesis are unaffected[77].

Although the β1 subfamily of integrins and their role in the hematopoietic system are described in detail in Paper I it is important to mention in this context that β1 deficient somatic cell chimeric mice lack β1 deficient cells in liver and spleen[10]. In ES cell chimeric β1 deficient mice hematopoietic stem and progenitor cells develop in the yolk sac and the PAS. These cells are able to differentiate into erythroid, myeloid and lymphoid cells *in vitro*. However, *in vivo* the colonization of the fetal liver, thymus and spleen by HSCs does not occur in the absence of β1 integrins and HSCs accumulate in the fetal circulation[78,79]. In conditional β1 knockout mice HSCs were isolated from the bone marrow and the β1 gene was deleted by retroviral Cre-recombinase expression. The β1-deficient HSCs are unable to home to the bone marrow and the spleen of irradiated recipient wild-type mice and hence accumulate in their blood[79]. In summary, β1 integrins are crucial for HSC homing, but they are neither required for the retention and survival nor for the differentiation of HSCs into the different blood cell lineages. In addition, the homing of the different blood cell lineages to spleen, lymph nodes and Peyer`s patches is β1 integrin-independent[80]. Interestingly, mice with a β1 deficient hematopoietic system show a unique phenotype in the T cell mediated immune response. After immunization with a T cell dependent antigen they secrete highly reduced levels of IgM, whereas the IgG production is increased. After immunization with a T cell independent antigen both IgM and IgG responses are decreased[80]. Since this effect could not be rescued by β1 expressing T or B lymphocytes it might be caused by an impaired function of dendritic cells. Notably, mice lacking VCAM-1, one counter receptor of β1 integrins, in the hematopoietic system also show an impaired humoral immune response after immunization with a T cell dependent antigen[77].

An apparent explanation for the different results regarding the hematopoietic development in α4, β7 and β1 deficient mice would be that α4β1 and α4β7 functions compensate each other. Furthermore it is possible that α4 integrins are important for HSCs during fetal hematopoiesis which would at least partially explain the differences observed between the α4-deficient ES cell chimeras[69,70] and α4-deficient bone marrow chimeras[38,73]. To unravel a possible compensation between α4β1 and α4β7 we

analyzed hematopoietic development in adult mice lacking the β1 and the β7 subunits (Paper II).

5.1.7 The role of α4 integrins in the adult immune system

In addition to the important functions in the developing hematopoietic system and the homing to mucosal lymphoid organs and the inflamed CNS, many functions have been attributed to α4 integrins in the adult immune system. The role of α4β1 in leukocyte extravasation is discussed in detail in chapter 5.3. α4 integrins also regulate the correct positioning of immune cells in different organs: together with αLβ2, α4β1 is important for retaining B cells in the marginal zone of the spleen[81], and for the adhesion of B cells to follicular dendritic cells in germinal centers, which is critical for the maturation of the immune response[82,83] and the prevention of B cell apoptosis[84].

It has already been discussed that the activation state of integrins is highly regulated, and that T cell integrins are rapidly activated upon chemokine stimulation during extravasation. Furthermore the activation state of integrins on T lymphocytes is dependent on the activation state of the T cells themselves. Stimulation of the CD3-T cell receptor (TCR) complex results in signaling to integrins – probably via the tyrosine kinases Lck, Fyn, ZAP-70 and Itk – and subsequently activates β1 integrins[85] ensuring the interaction of T cells with antigen presenting cells and the extracellular matrix. The generation of a stable contact between T lymphocytes and antigen presenting cells is a key event during the generation of an adaptive immune response. β2 integrins mediate adhesive interactions in the immunological synapse that develops between T and antigen presenting cells (Figure 5.6). Recently α4β1 has also been localized to the immunological synapse[86], which is in line with data showing that α4β1 has a costimulatory potential in T lymphocytes.

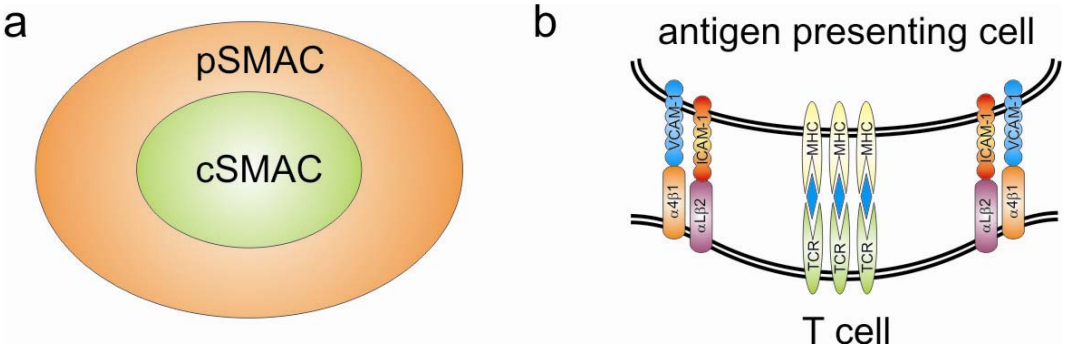

Figure 5.6: Schematic view of the immunological synapse. (a) Top view of the interaction zone. The central supramolecular activation cluster (cSMAC) is surrounded by the ring-like peripheral supramolecular activation cluster (pSMAC). (b) The cSMAC consists of the T cell receptor and associated coreceptors binding to the antigen presented by molecules of the major histocompatibility complex (MHC). The cSMAC is surrounded by

the pSMAC which consists mainly of adhesion molecules. The integrins in the pSMAC are linked by adaptor proteins to F-actin, ensuring proper formation of the pSMAC[87].

The B cell receptor (BCR) on B cells binds soluble antigens and cell-bound antigens such as antigens bound by Fc or complement receptors. The recognition of membrane antigens is also facilitated by interactions between αLβ2/ICAM-1 and α4β1/VCAM-1 that organize into an immunological synapse with the BCR-antigen complexes in the cSMAC and the four adhesion molecules in the pSMAC and enhance BCR signaling[88,89].

5.2 Multiple sclerosis and experimental autoimmune encephalomyelitis

5.2.1 Multiple sclerosis in general

MS is an inflammatory autoimmune disease of the central nervous system (CNS) that can lead to severe disability. With its early onset during the second and third decade of life MS is the most common cause of non-traumatic disability in young adults[90]. In Germany MS has an estimated prevalence of 83 per 100.000[91] and worldwide approximately 2,5 million people are affected[92,93]. Based on the different disease courses three different forms of MS are distinguished clinically. Around 85% of the patients present with relapsing-remitting MS (RRMS) at the beginning. RRMS is characterized by attacks of clinical symptoms followed by periods of complete or partial improvement. After around 10 years RRMS changes into secondary progressive MS (SPMS) in most of the patients[93]. The repeated inflammatory episodes lead to irreversible damage of the CNS and the neurological condition is slowly but continuously worsening over the course of many years. Approximately 15% of the patients suffer from primary progressive MS (PPMS) where the clinical course is progressive with the onset of disease and no periods of relapses can be observed[94]. Since MS is causing many different symptoms and impairments systems like the Expanded Disability Status Scale (EDSS) have been developed to record the disease course of patients, provide a basis for prognosis and validate the efficacy of new treatments. The EDSS is ranging from 0 to 10 in 0,5 intervals with 0 standing for a complete lack of symptoms and 10 being death due to MS[95]. MS is causing characteristic lesions in the patients CNS that can be detected by magnetic resonance imaging (MRI). The two most useful types of imaging are normal T_2-weighted MRI that detects lesions in the white matter and gadolinium-enhanced MRI that detects areas with an increased permeability of the blood-brain-barrier in T_1-weighted scans. These areas of enhancement are often the first detectable change during the development of a new lesion[93].

Up to now, there is no cure for MS, and patients need a lifelong treatment with disease damping drugs. Therefore, MS is severely impairing the quality of life of affected patients and is also causing high social costs.

5.2.2 The etiology of multiple sclerosis

The wide variety of symptoms characteristic for MS such as numbness, weakness, paresis, visual problems, severe fatigue and depression and also intellectual and cognitive impairments are caused by demyelinating lesions in the various myelinated regions of the CNS. This demyelination leads to the loss of saltatory conduction and therefore nerve impulses are not transmitted properly anymore. Furthermore also the axons themselves can be damaged or lost. It is widely accepted that MS lesions are caused by an autoimmune reaction, although the triggers of this inflammatory process remain unclear and complex[96]. Despite the lack of clarity about the etiology of MS several factors show a correlation to increased MS susceptibility.

Genetically there is a clear association with certain human leukocyte antigen (HLA) class II region alleles, particularly HLA-DRB1 and HLA-DQB1[97]. Susceptibility as well as protective alleles have been identified in both regions. In recently published large screens, it could be shown that additional, earlier discovered susceptibility loci for MS are explained by linkage disequilibrium with certain HLA class II haplotypes that seem to have the only valid association with MS risk[98].

In addition to the genetic factors, environmental factors such as the geographic location in which individuals grew up, have been demonstrated to play a role. Furthermore, MS is diagnosed more frequently in individuals that grew up in a temperate climate than in people originating from tropical or subtropical areas[99]. This might be partially explained by the influence of sunlight and UV light-induced formation of vitamin D[96]. There is a clear inverse correlation between vitamin D levels and the risk to develop MS[100].

Furthermore, different infectious agents have been discussed as risk factors for MS. Parasitic infections decrease the risk of developing MS and other autoimmune diseases, supposedly due to a T_H2 deviation of the T helper cell response[101]. On the contrary, strong evidence supports an increased MS risk for individuals with Epstein-Barr virus (EBV) infection[102]. The relation between infections and MS can be explained by several mechanisms. In a process called molecular mimicry potentially self-reactive T cells are activated by foreign antigens that share similarities with self-antigens. Epitope spreading is a process where epitopes other than the initial dominant epitope are recognized by the immune system. Upon the first inflammatory process the ongoing inflammation leads to tissue destruction which in turn releases new epitopes

which can be different peptides from the initial protein (intramolecular epitope spreading) or dominant epitopes of neighboring proteins (intermolecular epitope spreading). Therefore, an inflammatory response to viruses present in the CNS could lead to immune reactions against CNS antigens. Moreover, autoreactive T cells can be activated by the pro-inflammatory milieu during an ongoing infection, a process called bystander activation.

Another - albeit moderate - environmental risk factor is smoking. There is no conclusive explanation for this association, although many hypotheses are discussed. Substances like nicotine can increase leakiness of the blood brain barrier (BBB), other cigarette smoke components like cyanide are directly toxic for the CNS and smoke components might also interfere with antigen-dependent signaling in T lymphocytes[103].

Finally, there is a clear sex discrepancy in MS development: females have a risk more than twice as high as males[104], an observation that holds also true for other autoimmune diseases like rheumatoid arthritis and is ascribed to different hormone status and immune responses between men and women[105].

In summary, although many risk factors for MS have been identified the complex interplay between them and the exact mechanisms that lead to the development of MS are not clear and need to be better understood in order to develop specific treatments and perhaps preventive measures for MS.

5.2.3 The pathology of multiple sclerosis

Although the disease pathology of MS is better understood than the etiology the involved processes are complex as well. An acute MS lesion is a region in the white matter of the brain or spinal cord where myelin is focally lost. These lesions contain many $CD4^+$ and $CD8^+$ T cells, macrophages, activated microglial cells and some B cells and plasma cells, which are the key players of the autoimmune process[106,107].

The presence of T cells that are reactive to self-antigens is a prerequisite for all autoimmune diseases and is caused by the escape of some autoreactive T cells from negative selection in the thymus[108]. Self-reactive T cells are present in all individuals and are normally kept in check by several additional mechanisms that maintain self-tolerance[109]. Firstly, antigens have to be presented by MHC molecules and recognized in the context of costimulation to excite T cell activation. Secondly, antigens can be separated by their target cells via anatomical barriers such as the BBB in the CNS. In the meantime, however, it has been shown that also the CNS undergoes constant immunosurveillance[110]. Finally, the autoreactive T cells present in healthy individuals are tightly controlled by regulatory T cells[108]. This has been impressively demonstrated in mice carrying TCR transgenic T cells specific for myelin antigens that escape

spontaneous EAE[111,112]. It is not clear, why these safety mechanisms fail in MS patients and autoreactive T cells are induced, persist and proliferate in the CNS. The association between MS and certain infectious agents such as EBV suggests that molecular mimicry for example, might break the self-tolerance, possibly in the context of certain HLA alleles and other predisposing factors.

Autoreactive $CD4^+$ T cells that enter the CNS mediate the first steps of the disease process. It has been shown by gadolinium-enhanced MRI that early MS lesions are associated with a breakdown of the BBB that most likely facilitates entry of T cells into the CNS. Furthermore the expression of VCAM-1 on endothelial cells is upregulated under inflammatory conditions which allows increased binding of activated T cells to endothelial cells via integrins[113]. After firm adhesion and crossing of the endothelial cell layer T cells have to penetrate the basement membrane of the BBB that is composed of type IV collagen. T cells use the matrix metalloproteinases-2 (MMP-2) and 9 for penetrating through basement membranes[114,115].

After extravasation T cells are reactivated upon recognizing their antigen on perivascular antigen-presenting cells in the CNS. It is well established that the extravasation of activated T lymphocytes into the brain and spinal cord occurs independent of their antigen-specificity. Nevertheless only T cells that recognize their target antigen in the perivascular area can persist and proliferate in the CNS[116]. *In vitro* studies revealed that astrocytes and microglia are capable of efficiently presenting antigen upon stimulation with cytokines like granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-γ (IFN-γ). *In vivo* microglia cells are required as well for the development of EAE[117]. In addition blood-borne $CD11c^+$ dendritic cells (DCs) are required and sufficient to present antigen to invading encephalitogenic T lymphocytes[107]. Furthermore, it has been shown in mouse models of MS that epitope spreading takes place in the CNS rather than in the periphery and is mediated by local antigen-presenting cells, possibly DCs[118]. This process most likely plays an important role in the chronic progression of MS[118,119].

It is not resolved which antigens are the targets for MS-inducing $CD4^+$ T cells but there is evidence that the classic myelin proteins that are also used to elicit EAE in model animals such as myelin basic protein (MBP), myelin proteolipid protein and myelin oligodendrocyte glycoprotein (MOG), are also targets in human MS. This is underscored by the fact, that most of these proteins are restricted by the HLA class II molecules associated with MS[120]. In addition the small heat-shock protein αB-crystallin, that is upregulated in MS lesions has been identified as a major target antigen in MS[121]. αB-crystallin has neuroprotective and anti-inflammatory properties, which makes its loss especially fatal[122]. Beside $CD4^+$ T cell mediated damage a

humoral immune response by autoantibodies can lead to demyelination in EAE and MS. In the murine EAE model the myelin protein MOG is the only known protein that elicits a demyelinating autoantibody response. Although it is unlikely that MOG is the only protein evoking such responses in humans, it is likely that proteins recognized by demyelinating autoantibodies are membrane proteins which expose their epitopes in the extracellular space[123,124].

Until recently it has been thought that $CD4^+$ T cells initiating MS have a T_H1 phenotype, characterized by the production of IFN-γ, interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and lymphotoxin and mediating cellular immunity[125]. Now there is increasing evidence, mainly from the EAE model, but also from studies in MS patients that these $CD4^+$ T cells represent a subset distinct from the classical T_H1 biased cells. Instead they are expressing IL-17 which gave this new subset the name T_H-17. They are dependent on IL-23 for their development[120,126] (Figure 5.7). T_H17 cells migrate easier through a layer of human brain-derived microvascular endothelial cells than T_H1 cells *in vitro,* and IL-17 together with IL-22 were shown to contribute to the breakdown of the BBB by binding to their respective receptors on brain microvessel endothelium in MS lesions and disrupting tight junctions. Furthermore, stimulation with IL-17 and IL-22 induces expression of the chemokine CCL2 in endothelial cells, which promotes the recruitment of further $CD4^+$ T cells[127].

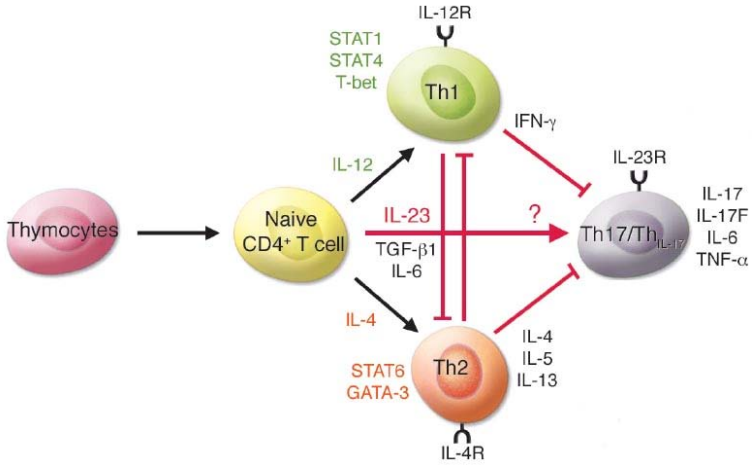

Figure 5.7: Naïve, $CD4^+$ T helper cells can differentiate into three different classes of effector T cells that are characterized by the expression of distinct cytokines. T_H1 cells are induced by the cytokine IL-12 which activates the transcription factors signal transducer and activator of transcription 1 (STAT1), STAT4 and T-bet. IL-4 leads to the activation of STAT6 and GATA-3 and induces T_H2 cells. IL-23 promotes via other unknown transcription factors the development of T_H17 cells that express high levels of IL-17. T_H1 and T_H2 cells antagonize each other via the expression of IL-4 and IFN-γ, which at the same time both inhibit the differentiation of T_H17 cells. The cartoon is modified from Iwakura and Ishigame[126].

Antigen-specific stimulation of the infiltrated $CD4^+$ T cells results in the production of various pro-inflammatory molecules. They increase the permeability of the BBB, lead to upregulation of adhesion molecules on vascular endothelial cells and the recruitment of more lymphocytes into the CNS. Pro-inflammatory cytokines that amplify CNS inflammation include IFN-γ, TNF-α, TNF-β, lymphotoxin, IL-1α, IL-2, IL-6 and IL-12[125].

Although $CD4^+$ T cells mediate the early events during the establishment of a new MS lesion the actual damage to the myelin sheath, oligodendrocytes and axons is mediated by multiple mechanisms and cell types. The main target of the autoimmune process is the myelin sheath, which can principally be repaired by remyelination. The alternating destruction and repair processes lead to the clinical picture of RRMS. During long-term disease the myelin sheath is destroyed and in addition, more and more axons are damaged. The transection of an axon within a demyelinating lesion causes the subsequent loss of the whole distal axon by Wallerian degeneration. Since axonal loss is irreversible this leads to persisting disability and neurological symptoms after a threshold of axonal damage is reached[128,129].

It has been demonstrated for MS patients that the mechanisms of tissue destruction are very heterogeneous between different patients and that at least four different types of MS lesions (called type I to type IV patterns) can be distinguished histologically[128]. The mechanisms of tissue destruction are summarized in Figure 5.8. Tissue destruction by activated macrophages is found in type I lesions. The pro-inflammatory cytokines released by the infiltrated $CD4^+$ T cells lead to macrophage recruitment and activation of macrophages and microglia cells. The activated macrophages and microglia produce many factors leading to demyelination and axon damage including TNF-α, reactive oxygen species, matrix metalloproteinases and other proteases. Another event that leads to both neuron and oligodendrocyte destruction is excessive glutamate production by activated lymphocytes and microglia (Figure 5.8). This causes excitotoxicity mediated by a massive Ca^{2+} influx[130]. Type II lesions also contain T cells and macrophages but tissue destruction is mediated by a humoral immune response. Myelin-specific antibodies bind to and damage myelin through opsonization and complement activation. In most MS patients immunoglobulins can be detected in the cerebrospinal fluid. Since there is no concomitant increase of antibody levels in the serum they are likely produced in the CNS by plasma cells derived from infiltrating B cells[131]. An observation that underscores the significance of destruction by humoral immunity is the fact that patients unresponsive to glucocorticoid treatment during a relapse often benefit from plasma exchange and have type II lesions[132].

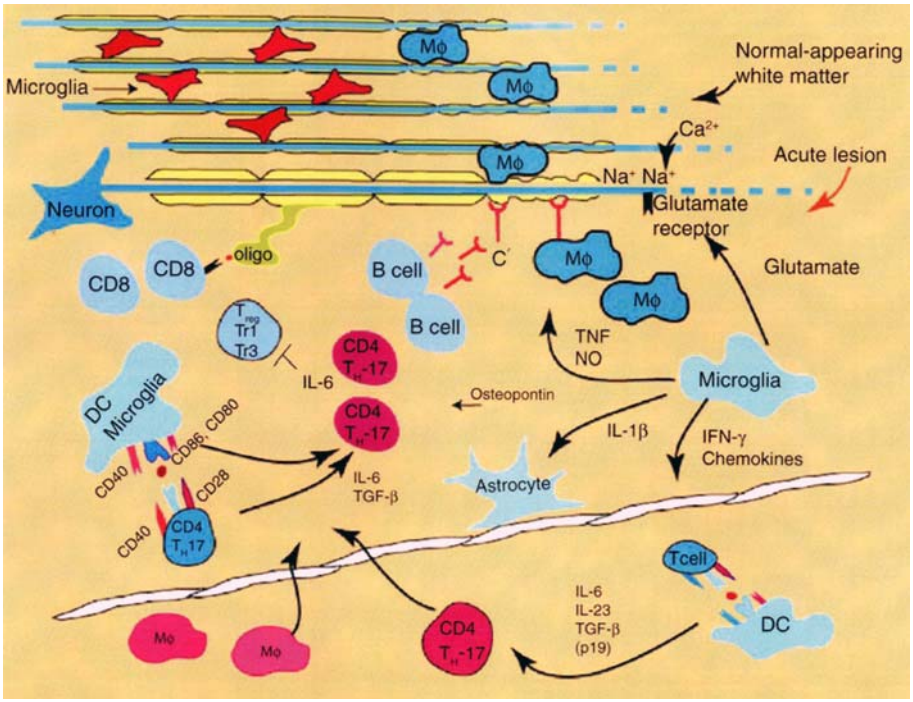

Figure 5.8: Mechanisms of myelin and axonal destruction in MS. CD4$^+$ T cells cross the blood brain barrier and become reactivated in the perivascular space by cells presenting myelin antigens. Activated, IL-17 expressing T cells recruit macrophages (MΦ) from the circulation that lead to tissue destruction by the production of proinflammatory factors and activation of microglial cells. In some cases B cells cause a humoral immune response and deposits of complement and antibodies. Furthermore CD8$^+$ T cells can damage oligodendrocytes directly. The cartoon is taken from McFarland and Martin[120].

Lesion patterns III and IV are characterized by apoptotic or non-apoptotic loss of oligodendrocytes, respectively. The loss of oligodendrocytes leads to destruction of the myelin sheath and secondary axonal loss due to the missing trophic support. Another cell type mediating tissue destruction in active MS lesions are CD8$^+$ T cells which outnumber CD4$^+$ T cells[133]. CD8$^+$ T cells induce cell death, aided by the upregulation of MHC I molecules on target cells under inflammatory conditions[134].

Up to now, no clear correlation between the different histological and clinical manifestations of MS has been shown. Great efforts are made to identify the types of tissue destruction in patients since this knowledge has important implications for prognosis and therapy[128].

Surprisingly at first glance, immune cells also play a role during neuroprotective processes such as remyelination. CD4$^+$ T cells can produce neurotrophins including brain derived neurotrophic factor (BDNF) that promote remyelination. Furthermore it has been shown in EAE that CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (T_{reg}) can inhibit the inflammatory processes[135].

5.2.4 Treatment and treatment trials of multiple sclerosis

The most frequently prescribed treatments for RRMS are corticosteroids, IFN-β and glatiramer acetate. Corticosteroids are administered during an acute attack of MS to treat the symptoms like weakness, visual and gait impairments. IFN-β and glatiramer acetate are used as long-term disease modifying drugs to prevent relapses.

Corticosteroids like methylprednisolone act anti-inflammatory and immunosuppressive. They act by inhibiting transcription factors including NF-κB and AP-1 which mediate the expression of many pro-inflammatory cytokines including IL-1, IL-2 and IL-6 and also adhesion molecules such as ICAM-1. Glucocorticoids interfere also with T cell proliferation by inhibiting IL-2 synthesis[136].

The type-1 interferons, IFN β1-b (trade name: Betaferon) and IFN β1-a (trade name: Avonex, Rebif) used for the treatment of RRMS are cytokines that are normally produced by fibroblasts to combat viral infections. Interferons bind to a dimer of interferon receptor 1 and interferon receptor 2 which leads to phosphorylation of tyrosine kinases including Jak-1. The tyrosine kinases subsequently activate transcription factors like STAT, which trigger an anti-inflammatory response by inducing expression of the anti-inflammatory T_H2 cytokine IL-10 and IL-1 receptor antagonist. Furthermore they suppress IL-1β and TNF-α expression and antagonize IFN-γ induced MHC II upregulation on antigen presenting cells (APCs). IFN-β reduces T cell proliferation and extravasation into the CNS[137], increases the amount of soluble vascular cell-adhesion molecules[138], and reduces the VLA-4 expression on T cells in peripheral blood[139]. Furthermore IFN-β reduces expression of the MMPs 2 and 9, that are crucial for disintegrating collagen type IV and increases the expression of MMP inhibitors. Unfortunately interferons evoke a variety of adverse side effects such as systemic flu-like symptoms with fever, chills and headache that last for 24 hours[140] and they can be neutralized by antibodies impairing or decreasing efficacy of this therapy[141].

Glatiramer acetate (trade name: Copaxone) is a mixture of synthetic random copolymers of four basic amino acids (alanine, glutamic acid, lysine and tyrosine) that were initially designed to mimic MBP. Glatiramer acetate is thought to antagonize the binding of MBP to the TCR and MHC class II antigens and to induce suppressor T cells[142]. A recent study showed that glatiramer acetate is not effective in treating PPMS[143]. Another problem of both glatiramer acetate and IFN-β treatment is that the administration route of subcutaneous or intramuscular injection is cumbersome for many patients.

In patients that fail to respond to IFN-β or glatiramer acetate treatment or that suffer from progressive MS, administration of the chemotherapeutic mitoxantrone is a novel treatment option. Mitoxantrone was primarily used to treat malignancies, especially leukemias. It interferes with deoxyribonucleic acid (DNA) synthesis by intercalating into DNA and inhibiting the topoisomerase II[136]. These unspecific cytotoxic effects lead to a general immunosuppression. Furthermore, mitoxantrone induces apoptosis in APCs[144]. Common side effects are myelosuppression and potentially severe cardiotoxicity. Therefore, the lifetime cumulative dose for mitoxantrone is reached after only two to three years[136,145]. Currently about 10% of all MS patients are treated either with mitoxantrone or with immunosuppressants such as cyclophosphamide, azathioprine and methotrexate although all of these drugs show considerable toxicity and have a high long-term risk[145]. Further treatments based on immunosuppression include total lymphoid radiation, infusion of intravenous γ-globulins and treatment with cytotoxic drugs combined with autologous bone marrow transplantation.

The severe side effects and limited effectiveness of the commonly prescribed drugs underscore the need for new treatments of MS. Some of these attempts are described below. Alemtuzumab is a humanized monoclonal antibody (mAb) against the leukocyte surface molecule CD52. The proposed action is the removal of lymphomononuclear cells. It showed considerable success in the treatment of patients with progressive MS but in early studies 30% of them developed Grave´s disease, an autoimmune disorder of the thyroid gland. Other possible monoclonal antibody therapies are rituximab, which is approved for the treatment of non-Hodgkin lymphomas. Rituximab is an anti-CD20 mAb that depletes B lymphocytes by binding complement and mediating B cell lysis. Another mAb is the anti-CD25 Ab daclizumab that has shown promising results in phase II clinical trials[146,147], although the mechanism of action is unclear.

A very interesting new substance that is currently tested in phase III clinical trials and can be administered orally is FTY720 or fingolimod. Fingolimod is a chemical derivative of the fungal product myriocin. It is an agonist of the sphingosine 1-phosphate receptor on lymphocytes and thymocytes, which is crucial for the egress of T and B lymphocytes from secondary lymphoid tissues[148]. Upon fingolimod binding the receptor is internalized trapping lymphocytes in secondary lymphoid organs and preventing their migration to peripheral target tissues[149].

Statins were initially developed as drugs that lower serum cholesterol levels[136], but they also have immunomodulatory effects[150]. Statins inhibit the proliferation of lymphocytes, reduce the expression of adhesion molecules on activated T lymphocytes

and shift their cytokine expression profile towards a T_H2 phenotype[151]. They also downregulate the expression of MMP-9 and chemokine receptors on T and B lymphocytes[150]. Statins are interesting drugs for MS therapy since they can be taken orally, show promising results and are well tolerated[152].

5.2.5 Experimental autoimmune encephalomyelitis: an animal model for MS

EAE is a very well characterized animal model that resembles many aspects of MS. The phenomenon of EAE was discovered already in 1933, when it was observed that injections of brain tissue emulsions, and even more so mixtures of brain emulsions with killed *Mycobacterium tuberculosis* and paraffin oil induce a paralyzing disease in rhesus macaques and rabbits[153,154]. The mixture of heat-inactivated *M. tuberculosis* and mineral oil was termed complete Freund´s adjuvant (CFA) after its inventor and is used until today to induce so-called active EAE[155]. Subsequently it was found that myelin components, especially myelin basic protein were even more potent in inducing EAE, and that EAE can be induced in many different species including guinea pigs, rabbits, goats, mice, rats, hamsters, dogs, sheep, marmosets, and chickens[153]. Later it was shown, that EAE could not only be induced by immunization with various myelin peptides in CFA but also by adoptive transfer of encephalitogenic lymphocytes, a strategy that was named passive or adoptive transfer EAE[156,157]. Initially the induction of EAE was more robust and reproducible in guinea pigs and rats compared to mice. However it was discovered that EAE induction in mice can be facilitated by the injection of pertussis toxin, an effect that is mediated by several mechanisms. Pertussis toxin activates APCs[158] and steers them to promote a T_H1-biased immune response[159]. It also promotes P-selectin upregulation on brain endothelial cells in a toll-like receptor 4 dependent way and increases thereby T cell recruitment. Interestingly it could also be shown that the increased permeability of the BBB that is discussed since a long time as an effect of pertussis toxin treatment is a result of the rolling and adhesion of T lymphocytes and not vice versa[160].

Since the use of pertussis toxin, mice became an invaluable tool for investigating disease mechanisms in EAE, largely due to the many possibilities of genetic manipulation and the abundant immunological agents available. The clinical course and the susceptibility to EAE vary considerably between different mouse strains and the genetic background of the mice also requires the use of different peptides to induce the disease. For instance EAE induction in C57BL/6 mice is done with the MOG_{35-55} peptide and induces chronic-progressive disease, whereas EAE in SJL mice can best be induced by immunizing with a proteolipid protein peptide ($PLP_{139-151}$) and the disease course is relapsing[123,161].

With the help of EAE models many mechanisms regarding human autoimmune disease have been unraveled. The role of T lymphocytes for MS pathology was confirmed[157], although most mouse EAE models underestimate the role of $CD8^+$ T cells, which are more important in MS than in EAE. The importance of a T_H1 or T_H17-biased cytokine profile of T lymphocytes and secondary macrophage recruitment for the development of autoimmune diseases has been demonstrated in the EAE model as well[162]. The need for a humoral immune response for the development of demyelinating lesions in MS patients was also deduced from EAE. Finally, three of the currently approved treatments for MS have been developed in the EAE model: glatiramer acetate, mitoxantrone and natalizumab[67,163,164].

5.2.6 Limitations of experimental autoimmune encephalomyelitis

Although many insights were gained from EAE models, they have limitations. Firstly, EAE is not a spontaneous disease but requires induction by adjuvants like heat-inactivated mycobacteria and pertussis toxin. Secondly, many EAE models exist that reflect specific aspects of MS, but no model shows all the hallmarks of human MS. For instance, MOG_{35-55} induced EAE in C57BL/6 mice reflects the T cell mediated inflammation and the subsequent recruitment of deleterious macrophages but shows no demyelination mediated by a humoral immune response. Furthermore, no EAE model with a primary progressive disease course exists[123]. Thirdly, treatment trials in EAE have sometimes a poor predictive value for MS. Although the above mentioned therapies were developed in EAE, other EAE-based therapeutic strategies showed disappointing results in man. For example, altered peptide ligands (APL) with contact residues to the TCR specific for encephalitogenic peptides have been examined in human trials[165]. APLs are thought to interfere with TCR signaling by acting as partial agonists or antagonists, to induce anergy or a T_H2 shift in T cells and to activate regulatory T cells. Indeed, APLs ameliorated MS in some patients, but in other patients they led to an exacerbation of MS and evoked allergic-type hypersensitivity reactions. Therefore, the trials were abandoned[166]. Similarly, interference with several cytokines worked well in the mouse model but not in humans. The blockade of TNF-α for example, ameliorated mouse EAE[167,168] but neither mAbs against TNF-α[169] nor treatment with a recombinant p55 TNF receptor immunoglobulin fusion protein[170] alleviated human MS. Nevertheless, blockade of TNF-α represents an effective treatment of other autoimmune diseases such as rheumatoid and psoriatic arthritis and Crohn´s disease[171]. Similar complications halted the treatment trials of IFN-γ for MS patients. Recombinant IFN-γ administration led to a significant increase in immune-mediated disease exacerbations in MS patients[172], although increased IFN-γ

production is correlated with EAE suppression in mice[173]. It is still unclear how these contrasting results can be explained.

5.3 Leukocyte extravasation in EAE and MS

Progress in understanding the paradigm of leukocyte extravasation promoted the development of drugs that interfere with integrin function.

5.3.1 The extravasation cascade

The general concept of leukocyte extravasation from the circulation into tissues is an extensively studied and well described multistep process[42] (Figure 5.9). First, the cells slow down their movement within the blood stream by short, transient contacts between selectins on leukocytes and carbohydrate ligands on the blood vessel endothelium. The so-called tethering decreases the speed of leukocyte movement and permits selectin-mediated rolling on the vessel wall. Rolling can also be mediated by α4 integrin binding to VCAM-1 or MadCAM[174]. The reduced speed of rolling leukocytes facilitates the interaction of G-protein coupled chemokine receptors on hematopoietic cells with chemokines presented on the luminal surface of endothelial cells. Chemokine-triggered signals induce integrin inside-out signaling which leads to the activation of leukocyte integrins. This step is mediated by $G\alpha_i$ proteins and hence can be inhibited with pertussis toxin. The activated integrins bind counter-receptors on endothelial cells resulting in firm adhesion, cell arrest and spreading followed by transmigration through the endothelium.

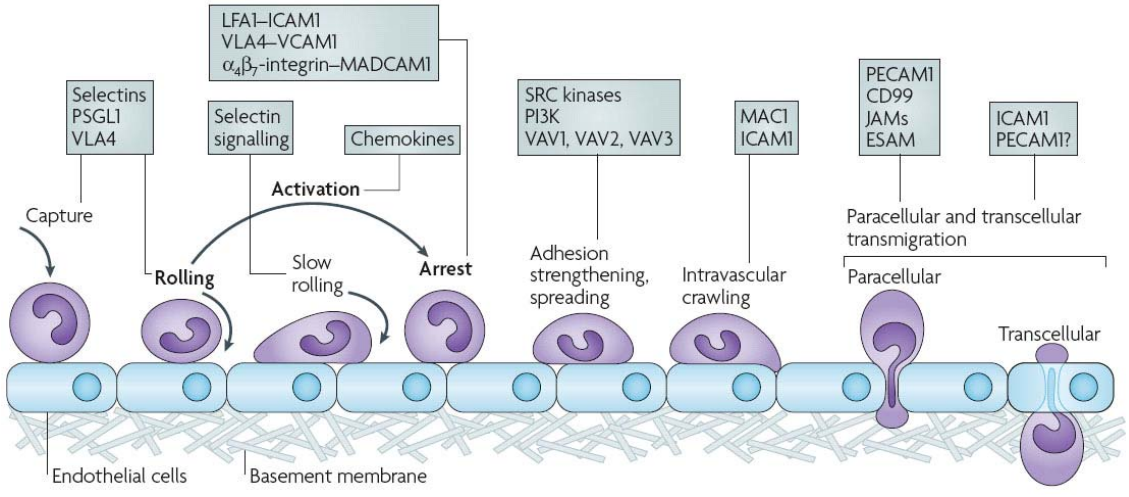

Figure 5.9: Crucial steps during leukocyte extravasation. Leukocytes are captured or tethered by transient interactions and start to roll on the endothelium. Integrins become activated by chemokine receptor signaling which leads to their firm arrest. Upon adhesion strengthening and spreading cells crawl on the vessel wall, supposedly seeking an appropriate spot for transmigration. The subsequent transmigration through the endothelium and the underlying basement membrane occurs either directly through

an endothelial cell in a process called emperipolesis or between two adjacent endothelial cells. For each step the main molecular players are shown in the blue boxes. The cartoon is taken from Ley et al.[175].

Although this general concept holds true for a wide variety of tissues the three receptor events – selectins and carbohydrate ligands, chemokines and chemokine receptors and integrins and their counter-receptors – vary in different extravasation processes and for different leukocyte subtypes to grant specificity[116]. The tissue specific mechanisms of extravasation have been compared with zip codes allowing the highly organized and tightly restricted extravasation of certain cell types into certain organs (Paper I)[42]. Table 5.2 summarizes some typical extravasation processes and the involved adhesion molecules and chemokines. Interestingly, the different extravasation processes also require different time frames. Whereas the extravasation of lymphocytes to peripheral lymphoid tissues via HEVs takes place within minutes the extravasation of lymphocytes into the CNS requires hours[176].

Table 5.2: List of adhesion molecules, chemokines and chemokine receptors executing extravasation processes. The table is modified from Ransohoff et al. and Engelhardt[116,177].

Process	Endothelium	Tethering/ Rolling/ Capture	Activation	Adhesion
recruitment of naïve lymphocytes to peripheral lymphoid tissues[178]	HEVs of secondary lymphoid organs	L-selectin – PNAD	CCR7 – CCL21	αLβ2 – ICAM-1 (and ICAM-2)
T_H1 cell recruitment to inflamed skin[179,180]	dermal blood vessels	CLA and PSGL-1 – E- and P-selectin	CCR4 – CCL17	αLβ2 – ICAM-1
lymphocyte recruitment to the mesenteric lymph nodes and Peyer´s patches[178]	HEVs of mesenteric lymph nodes and Peyer´s patches	α4β7 – MAdCAM-1	CCR7 – CCL21	αLβ2 – ICAM-1
T lymphocyte recruitment to the CNS[176,181]	postcapillary venules of the spinal cord white matter	α4β1 - VCAM-1	CCR7 – CCL19 and CCL21	α4β1 - VCAM-1

CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CLA, cutaneous lymphocyte antigen; PNAD, peripheral node addressin; PSGL-1, P-selectin glycoprotein ligand 1

5.3.2 Extravasation of leukocytes into the CNS

Apart from the longer time that lymphocytes require to extravasate into the CNS the process is unique in several additional aspects. Firstly, the extravasation of

leukocytes into normal, non-inflamed CNS is carefully controlled by the BBB. The extravasation of autoreactive T cells into the CNS parenchyma mainly takes place at postcapillary venules where the BBB consists of endothelial cells tightly associated with each other through tight junctions, and their basement membrane. Secondly, transmigrating leukocytes have to penetrate a thin layer of leptomeningeal cells originating from invaginations of the arachnoid and the pial membranes. Finally, the leukocytes have to overcome the so-called glia limitans that covers the CNS vasculature and consists of the endfeet of astrocytes and their basement membrane[182]. Although previous views of leukocytes being unable to enter the CNS have been dismissed, the CNS is an immunoprivileged site with a low influx of immune cells[110]. The very low expression of adhesion receptors on brain endothelial cells seems key to the low leukocyte influx.

Already the first transient interactions during the extravasation process seem to be different from the classical extravasation cascade. *In vivo* imaging of the microcirculation of the white matter of the cervical spinal cord of healthy SJL/N mice revealed that freshly stimulated encephalitogenic T cells did not roll at the capillary endothelium but instead were instantly captured [176]. The abrupt capture was almost abrogated by pretreatment with antibodies either against α4 or VCAM-1 but not with pertussis toxin. VCAM-1 is upregulated during EAE on vessels of the CNS white matter[183], and blockade of VCAM-1 ameliorates the course of EAE[184]. The results suggest that encephalitogenic T cell blasts are captured in the microvasculature of the CNS white matter without prior rolling and that the T cell capture is mediated by an interaction between α4 integrins and VCAM-1 without the requirement of G-protein mediated activation of the integrins. The reduced permanent firm adhesion of T lymphocytes to the endothelial vessel wall in pertussis toxin treated mice, however, indicates that firm adhesion critically depends on G-protein mediated signaling and integrin activation. T lymphoblast recruitment to the uninflamed BBB endothelium without prior rolling is probably important during the initiation of the inflammatory autoimmune process. During later stages of the disease the recruited leukocytes cause a proinflammatory milieu by producing various cytokines. Under these conditions lymphocytes roll on the endothelium which is also mediated by α4 integrins[174,185]. Therefore α4 integrins can mediate both the immediate capture without prior rolling and capture after rolling during lymphocyte extravasation into the inflamed CNS.

Although ICAM-1 and ICAM-2 are detectable on uninflamed microvessels of the CNS and become further upregulated upon inflammation[181,183] there is accumulating evidence that the interaction between ICAM-1 and αLβ2 does not contribute to the capture and firm adhesion of activated lymphocytes to the inflamed endothelium of the

healthy uninflamed spinal cord white matter. In accordance with a role of ICAM-1/αLβ2 interaction during diapedesis the stable firm adhesion of the T lymphocytes to the endothelial cells after two hours and the transendothelial migration of T cells was slightly reduced after anti-αL antibody treatment[181].

5.3.3 The role of selectins during leukocyte extravasation into the CNS

Although it has been shown that lymphocytes can be captured by the CNS microvasculature without prior rolling[176] there is a controversy about the involvement of selectins in tethering and rolling of encephalitogenic T cells. The selectin family contains three members, of which P- and E-selectin are highly expressed on inflamed endothelial cells and L-selectin on circulating leukocytes[42].

In vitro E- and P-selectin expression is upregulated in cultured cortical endothelial cells after IL-1β and TNF-α treatment[186]. *In vivo*, one study failed to detect E- and P-selectin in brain vessels of healthy and EAE mice[187], whereas others detected them in CNS vessels of EAE mice[188,189].

Interestingly, ligands for both selectins were found on a subset of encephalitogenic T cells[187]. PSGL-1, the main ligand for P-selectin is expressed on encephalitogenic T lymphocytes. However, antibodies directed against PSGL-1 do not influence the course of passive EAE in SJL mice. Furthermore, active EAE in PSGL-1 knockout mice is indistinguishable from control mice.[190] CLA, another selectin ligand, is derived from PSGL-1 by a fucosyltransferase-VII (FucT-VII)-mediated carbohydrate modification. Rolling and adhesion of FucT-VII$^{-/-}$ T cells is reduced, indicating that under inflammatory conditions CLA is important for both rolling and adhesion of T lymphocytes in the CNS grey matter[191].

All these findings, however, are in contrast to intravital microscopy studies that analyzed the interaction between encephalitogenic lymphocytes and endothelial cells. Superficial cerebral vessels were observed through the intact skull of young mice[191]. Rolling or firm adhesion of activated T cells to the endothelium was only observed in recipient mice that were treated either with lipopolysaccharide (LPS) or with TNF-α. The rolling interactions were dramatically reduced by preincubation of the T cells with antibodies to PSGL-1, P- and E-selectin. Notably, antibodies against α4, integrin αLβ2, VCAM-1 or ICAM-1 reduce the rolling only by about 50%. The firm arrest of encephalitogenic T cells was blocked almost completely with antibodies to PSGL-1 and P-selectin and considerably reduced by antibodies to αLβ2, ICAM-1 and E-selectin.[188] In another intravital microscopy study, Kerfoot and Kubes imaged rolling of all endogenous leukocytes in superficial vessels of the brain during active EAE in

C57BL/6 mice. The rolling interactions were reduced with antibodies to α4, and the remaining rolling interactions were abrogated by additional administration of antibodies to P-selectin.[189] Antibodies to P-selectin alone reduce rolling completely[160,192].

Importantly, neither genetic deficiency of P-selectin or PSGL-1 in C57BL/6 mice or P-selectin and/or E-selectin antibody treatment of SJL/J mice change the clinical course of active EAE[187]. Furthermore the development of active EAE in both C57BL/6 and SJL mice that are deficient for E- and P-selectin is indistinguishable from control mice[193].

One explanation for these conflicting results might be that the described intravital microscopy studies analyzed superficial CNS vessels of the grey matter, where the mechanisms of lymphocyte recruitment might differ when compared with those in the white matter. Furthermore, Kerfoot and Kubes[189,192] did not distinguish between distinct cell types in their analyses. Finally, it was shown that the efficacy of antibody blockade of α4 and/or P-selectin differs between C57BL/6 and SJL mice and depends also on how EAE was induced[192].

Regarding the recruitment of T cell blasts to the CNS the role of L-selectin is clear. Activated T cells do not express L-selectin[194], and treatment of mice with antibodies directed against L-selectin does not influence lymphocyte rolling on CNS vessels[188] or EAE development[194]. Notably L-selectin-deficient mice are protected from EAE, which is explained by an impaired effector function of macrophages[195].

5.3.4 The role of β2 integrins for EAE development

In addition to the α4 integrin family and selectins, leukocyte-specific integrins of the β2 family of adhesion molecules and their interaction partners have been reported to influence the course of EAE and possibly MS. The β2 integrins consist of four different members: αLβ2 or lymphocyte function-associated antigen-1 (LFA-1), αMβ2 or macrophage adhesion molecule-1 (Mac-1), αXβ2 and αDβ2. The β2 integrins are expressed on a wide variety of hematopoietic cells. LFA-1 is expressed on B cells, macrophages and T cells, Mac-1 on macrophages, neutrophils and natural killer cells, αXβ2 on dendritic cells, macrophages, monocytes, natural killer cells and activated T cells and αDβ2 on macrophages and neutrophils[196-198]. The β2 integrins play a vital role for the recruitment of neutrophils and other leukocytes (for example T_H1 cells that home to the inflamed dermis) into inflamed tissues and genetic dysfunction of the β2 subunit causes leukocyte adhesion deficiency (LAD) characterized by severe, recurrent bacterial infections[197]. As described already above, β2 integrins are important during formation of the immunological synapse[199,200].

Treatment of passive EAE in rats and mice with antibodies against the αL or the αM subunit ameliorates disease severity[194,201,202]. In another independent study, active EAE in rats could only be inhibited by treatment with antibodies against both LFA-1 and its counterreceptor ICAM-1[203]. Treatment of murine EAE with antibodies directed against LFA-1 produces opposing results ranging from no effect to severe exacerbation[204,205]. Mice lacking the αL gene develop delayed and less severe active EAE. This effect is most likely due to the impaired migration of lymphocytes to the draining lymph nodes as well as disturbed lymphocyte proliferation[206]. Blockade of ICAM-1, the main counterreceptor of LFA-1, interferes mainly with antigen-induced T cell proliferation; antibody blockade of ICAM-1 ameliorates active EAE but fails to prevent the passive transfer of EAE with T cells that are already activated[203,204,207,208]. T cells that lack all ICAM-1 isoforms have an impaired antigen-specific proliferation capacity and cannot transfer EAE in wild-type recipients[209]. Interestingly, one group found that passive transfer EAE in rats could be delayed profoundly with an anti-ICAM-1 antibody[184]. The reason for the contrasting results is not clear.

Genetic deletion of αM results in delayed and ameliorated active EAE and reduces leukocyte infiltration and demyelination of the spinal cord. When wild-type T cells are passively transferred into Mac-$1^{-/-}$ mice the onset of EAE is delayed and when Mac-$1^{-/-}$ T cells are transferred into wild-type mice, they do not develop signs of EAE. Mac-$1^{-/-}$ T cells have no significant reduction of antigen-specific proliferation *in vitro* but draining lymph nodes of Mac-$1^{-/-}$ mice contain fewer blasts, indicating a disturbed proliferative response *in vivo.* Mac-$1^{-/-}$ T cells have a T_H2 shifted cytokine response. Taken together these results suggest that during EAE αM plays a role on T cells and also on other cell types, presumably macrophages[210]. Mac-1 is the main macrophage receptor for the recognition and uptake of myelin, therefore it has been suggested that blockade of Mac-1 interferes mainly with macrophage-mediated tissue destruction during the effector phase of EAE[211,212].

A genetic deficiency of the αx subunit results in milder active EAE and both the passive transfer of αx-deficient T cells into wild-type mice as well as vice versa results in an ameliorated disease course indicating a role for αx on both T cells and other inflammatory cells[213]. Genetic deletion of the fourth β2 heterodimer αDβ2 does not influence the development of active EAE, leukocyte infiltration of the CNS and the cytokine response and proliferation of T cells[214].

5.3.5 Blockade of the α4 subunit in EAE and MS

Natalizumab (brand name: Tysabri) is a humanized monoclonal IgG4 antibody directed against the α4 subunit of the integrin heterodimers α4β1 and α4β7. IgG4

antibodies fail to activate complement and have a longer serum half-life than other isotypes[215]. Natalizumab is an effective treatment of MS and significantly reduces the number and severity of clinical relapses. Natalizumab was approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency as a monotherapeutic treatment for relapsing MS.

The development of natalizumab began in 1992 when it was noted, that antibodies directed against the α4 integrin subunit prevent leukocyte accumulation in the CNS and impair the development of passive EAE in Lewis rats. Since antibodies against other β1 or β2 integrin subunits showed little or no influence on lymphocyte and monocyte binding to inflamed blood vessels, it was concluded that α4β1 and/or α4β7 are the key adhesion molecules for leukocyte homing to the inflamed CNS[67]. This was further supported by the observation that the expression level of α4 integrin on T cells correlates with their encephalitogenic potential[184,216].

α4β1 and α4β7 can bind VCAM-1 and the CS-1 fragment of fibronectin[43,55] (Table 5.1). Since only antibodies against α4 or β1 that interfere with VCAM-1 adhesion blocked the adhesion of T cells to inflamed vessels, VCAM-1 is the most likely counter-receptor for α4 integrins during leukocyte adhesion[67]. In line with this finding, VCAM-1 is upregulated on the endothelium of microvessels in MS lesions[113,217] and venules of mice suffering from EAE[218]. Furthermore, α4 integrins and VCAM-1 are important for the adhesion but not the diapedesis of encephalitogenic T cells through brain endothelial cells[218].

Since then many publications demonstrated a positive effect of anti-α4 antibodies *in vivo* in both active and passive rodent models of EAE. In a murine passive EAE model the development of transfer EAE was dependent on the expression of high levels of α4 on the encephalitogenic T cells and could be inhibited by an antibody versus the α4 integrin subunit[184]. Although another study could not find this clear correlation between α4 expression and encephalitogenicity they also found α4 expression on all encephalitogenic T cell lines that they tested, and prophylactic administration of antibodies to α4 prevented passive EAE elicited by those encephalitogenic cells[194]. In active guinea pig EAE treatment with an anti-α4 antibody before the onset of clinical symptoms results in a delayed onset of disease. The delay can be maintained as long as blocking antibodies are still present in the circulation. Furthermore treatment after development of clinical symptoms ameliorates them significantly. Under both treatment regimens the antibody reduces the number of infiltrated T lymphocytes and monocytes in the CNS and prevents demyelination[219]. Similar results were obtained by treatment of PLP-induced EAE in CSJLF1 mice with

an antisense nucleotide specific for the integrin α4 subunit. Antisense nucleotide treatment led to a significant reduction of α4 mRNA and expression in cell lines and primary lymphoid cells, alleviated EAE when administered before and reduced the severity when given after disease onset. Like with the antibody treatment the antisense oligonucleotides reduced the numbers of infiltrated $CD4^+$ T cells and macrophages in the CNS[220]. Treatment with an anti-α4 antibody or a small molecule antagonist to α4 in PLP-induced active relapsing EAE in SJL mice resulted in delay and amelioration of the disease. Surprisingly, treatment after onset of clinical symptoms led to increased T cell accumulation in the CNS, increased relapse rates, augmented T_H1 responses to the priming peptide and supported epitope spreading[221,222]. It is possible that the observed exacerbation is specific for this disease model, since it was neither observed in other EAE models nor during clinical trials in humans.

5.3.6 Blockade of integrin α4β7 by natalizumab

A role for α4 integrins in the recruitment of inflammatory cells has been shown for various additional disease models including rheumatoid arthritis, contact hypersensitivity, type I diabetes mellitus, nephritis[223] and allergic airway responses[44,224]. α4 integrins also play a crucial role for lymphocyte recruitment in the gut associated lymphoid tissue. During IBD the recruitment of lymphocytes is mediated by the interaction of α4β7 with MAdCAM-1[225,226], whose expression is restricted to venules in the gut and high endothelial venules of the gut-associated lymphatic tissues (Peyer´s patches, mesenteric lymph nodes, and the appendix). Therefore, blockade of integrin α4β7 with natalizumab is also effective for the treatment of ulcerative colitis and Crohn´s disease[227-229]. Interestingly, the effect of natalizumab on IBD is not as pronounced as the efficacy in MS suggesting that additional adhesion molecules are required for the development of IBD. The predominant leukocytes in Crohn´s disease are indeed neutrophils, which extravasate mainly with the help of β2 integrins. Therefore, it is assumed that natalizumab interferes with the recruitment of T cells in IBD like in MS, and indirectly diminishes the secondary recruitment of neutrophils[230].

Although the role of a4β7 in the recruitment of lymphocytes to the gut is well established, its role in EAE is controversial. Theoretically natalizumab could block both a4β1 and α4β7 and both integrins are expressed on encephalitogenic T cells[56]. In SJL/N mice blockade of α4β7 via antibodies directed to β7 or α4β7 does not influence the development of passive EAE[56]. Furthermore, a small molecule inhibitor that is specific for the α4β1 heterodimer is sufficient to inhibit active EAE in Lewis rats which suggests that α4β7 plays no important role in this model[231]. On the contrary, it was shown in passive EAE in C57BL/6 mice that the administration of anti-β7 antibodies at

the peak of disease caused a partial disease remission and reduced the clinical score. β7 gene-deficient T cells are less encephalitogenic when transferred into wild-type mice and surprisingly also β7 gene-deficient mice show delayed and reduced clinical symptoms upon transfer of wild-type T cells[232]. Subsequently, the authors showed that an anti-MAdCAM-1 antibody prevented the development of MOG_{35-55}-induced EAE in C57BL/6 mice or, administered during ongoing chronic disease, supported remission of the disease[233]. It could be that these different findings are due to differences of disease models such as relapsing EAE in SJL mice or chronic, non-remitting EAE in C57BL/6 mice.

5.4 α4-integrin blockade for the treatment of MS

5.4.1 Clinical trials for Natalizumab

After the promising results from the animal studies and several small clinical trials[234-236] two randomized, double-blind, placebo-controlled, parallel-group, multicenter study phase III trials of natalizumab for the treatment for relapsing-remitting MS either alone (AFFIRM; Safety and Efficacy of Natalizumab in the Treatment of Multiple Sclerosis) or in combination with IFN-β (SENTINEL; Safety and Efficacy of Natalizumab in Combination With Avonex in the Treatment of Multiple Sclerosis) were initiated[237,238]. Already the natalizumab monotherapy proved to be extremely effective as it reduced the rate of clinical relapses per year by 68% and the accumulation of new or enlarging T_2-weighted lesions over two years by 83%. The mean number of gadolinium-enhancing lesions after both one and two years was reduced by 92%. The risk of a sustained progression of disability, which was defined as an increase of the EDSS of 1.0 or more that lasted at least for 12 weeks, was decreased by 42%. Importantly, no rebound effects were observed after discontinuation of natalizumab treatment[238]. In comparison, the current standard treatment with glatiramer acetate or IFN-β is reducing the rate of clinical relapses per year by about 30%[239,240]. In patients that were already treated with IFN β-1a additional therapy with natalizumab decreased the risk of a sustained progression of disability by 24% and the annualized rate of relapses by 55%. Very similar to the natalizumab monotherapy over two years the number of new or enlarging T_2-weighted lesions was reduced by 83% and the mean number of gadolinium-enhancing lesions by 89%[237].

Three patients participating in natalizumab trials developed progressive multifocal leukoencephalopathy (PML). This serious side effect is discussed in detail below. Besides PML only a few additional side effects were observed during the clinical trials. Neither of the two studies showed a significantly different incidence of opportunistic infections between the natalizumab and the control group indicating that natalizumab

specifically acts on CNS inflammation and displays no classic immunosuppressive action. Apart from that, natalizumab probably does not impair neutrophils, the bodies mainstay in the immune response against bacterial and fungal infections[42]. Neutrophils rely under normal conditions or during an acute inflammation mainly on β2 integrins for their extravasation[37]. A persistent presence of antibodies that occurred in 6% of the treated patients correlated with a loss of clinical efficacy[237,238,241].

Apart from MS, the efficacy of natalizumab was also shown for Crohn´s disease in phase III clinical trials[227,228,242]. The results of a completed phase II clinical trial of natalizumab for rheumatoid arthritis are not available to the public (http://clinicaltrials.gov/show/NCT00083759) but it has been shown in murine and rat models for arthritis that blockade of α4β1 is beneficial[243,244].

Currently alternative ways of blocking α4 by small molecule inhibitors are explored[245]. Small molecule inhibitors were effective in the treatment of rodent EAE[231]. These small molecule inhibitors offer the advantages that they can be orally administered whereas antibodies are always administered intravenously. Furthermore small molecule inhibitors are less likely to elicit binding antibodies in the patients that render the antibody treatment ineffective[230]. And their affinity, specificity and pharmacokinetics can be optimized easier than those of antibodies. Phase II clinical studies with orally available VLA-4 antagonists are currently ongoing (http://clinicaltrials.gov/ct2/show/NCT00484536).

5.4.2 Progressive multifocal leukoencephalopathy

Based on the positive clinical trials natalizumab was approved by the American FDA in November 2004 for treatment of relapsing-remitting MS[246]. Only three months later, in February 2005, natalizumab was voluntarily withdrawn from the market after two patients developed PML under combined treatment with IFN β-1a and natalizumab[247,248]. One of those patients died from PML and another patient in the clinical trial for Crohn´s disease also developed fatal PML[249]. Out of approximately 3000 patients treated on average for 18 month with natalizumab three developed PML[250]. Before the clinical trials with natalizumab, PML was only known to occur in immunocompromised patients, for example organ transplant recipients receiving immunosuppressants, patients with hematologic cancers or patients suffering from acquired immunodeficiency syndrome (AIDS). PML has never been described to be connected with MS. PML is caused by an infection with JC virus (JCV), a polyomavirus. The primary, asymptomatic infection with JCV is usually acquired during childhood. A seroprevalence of 86% was shown in healthy adults [251]. If the latent virus is reactivated oligodendrocytes are infected leading to viral lysis, which in turn causes an irreversible

demyelination of nerve fibers and persisting, severe neurologic deficits. PML has a very high mortality rate and there exists no specific treatment[252].

Under normal conditions JCV is kept in check, mainly by $CD8^+$ T cells. Therefore it is possible that the prevention of normal lymphocyte trafficking by natalizumab causes the reactivation of JCV, especially under conditions with an additional immunomodulation by IFN-β or other medications[253]. It is also possible, that natalizumab has a direct effect on the JCV reactivation itself. JCV is known to remain latent in the kidney, in lymphoid organs and the bone marrow where JCV is associated with B cells[252]. Therefore, it is possible hat natalizumab might release JCV-infected cells from the bone marrow thereby promoting virus replication, distribution to the whole body and entry into the CNS[254-256]. It is also possible that the interplay of both mechanisms is aiding the development of PML in the natalizumab-treated patients[257].

After a careful reexamination of all treated cases and a safety analysis[250] the FDA reapproved natalizumab for the monotherapeutic treatment of relapsing-remitting MS in June 2006 under a special distribution program (TOUCH; Tysabri Risk Minimization Action Plan). According to current consensus natalizumab is not regarded as a first-line treatment but is only considered after treatment with drugs such as glatiramer acetate, IFN-β, cyclophosphamide or mitoxantrone is not successful or poorly tolerated. Natalizumab is used in patients with relapsing-remitting MS and requires intensive monitoring for the occurrence of PML, exclusion of factors that interfere with cell-mediated immunity and normal leukocyte counts before natalizumab therapy[258].

5.4.3 Effects of natalizumab on the immune system

In all phase III clinical trials it was noted that natalizumab is increasing the number of circulating lymphocytes, monocytes, eosinophils and basophils, and sometimes nucleated red cells[227,237,238]. In addition, natalizumab treatment leads to the release and therefore to elevated levels of $CD34^+$ HSCs in the circulation[259,260]. Peripheral blood mononuclear cells from natalizumab-treated patients show a reduced migratory potential across a fibronectin layer in Boyden chamber experiments[261]. T lymphocytes have a reduced surface expression of the α4 subunit[261,262], and leukocytes, particularly $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells and $CD138^+$ plasma cells in the CSF are dramatically reduced, even 6 months after cessation of treatment[263]. Natalizumab treatment also significantly decreases the ratio of $CD4^+$ to $CD8^+$ T cells in the CSF, but only slightly in peripheral blood. It is possible that $CD8^+$ T cells are less affected by natalizumab because they express more α4 integrin and are thus inhibited less efficiently. Interestingly, the $CD4^+/CD8^+$ ratio in the CSF of

natalizumab recipients is reduced to the same extent as in HIV-positive patients, which have a high risk to develop PML[262].

5.4.4 Open questions regarding the natalizumab treatment

Although 15 years passed since the first report describing integrin α4-blockade for the treatment of MS appeared, several questions remain still unanswered.

Firstly, it is not clear which cell types are affected by the natalizumab treatment and thus mediate the main clinical benefit. Interestingly, it has been shown that B cells and monocytes express more α4 than T cells, and $CD8^+$ T cells have a higher α4 expression than $CD4^+$ T cells[261]. In contrast, neutrophils express little or no α4β1. Nevertheless it has been discussed that neutrophils might also utilize α4 integrins for extravasation under chronic inflammatory conditions[37]. One group demonstrated that anti-α4 antibody treated rats suffering from active EAE loose T cell infiltrates whereas macrophages were still detected in the CNS, indicating that the macrophages were not affected by the antibody treatment[264].

Secondly, it is not clear whether both α4β1 and α4β7 integrins have a function during EAE development. Some reports suggest that the β7 subunit has no role for EAE development[56,231], whereas others identified a beneficial influence of α4β7 blockade on the EAE course[232,233] (Chapter 5.3.6).

Thirdly, it is not known whether blockade of the α4β1-VCAM-1 interaction disturbs several immune functions, such as extravasation and/or T cell priming. Transfer EAE in SJL mice is less efficiently ameliorated with antibodies against VCAM-1 than with antibodies against α4 suggesting that α4 blockade either inhibits the interaction with a second ligand such as CS-1 fibronectin, that the α4 blockade interferes not only with lymphocyte adhesion or that anti-VCAM-1 blockade is incomplete. *In vivo* the anti-α4 antibody R1.2 in contrast to the PS/2 antibody is a poor inhibitor of lymphocyte homing to Peyer´s patches[226], but both antibodies have the same clinical potency in ameliorating EAE[56]. This suggests that R1.2 interferes with EAE development by mechanisms different from a mere adhesion blockade. This is in line with the fact that binding of immobilized CS-1 fibronectin, VCAM-1 or antibodies to α4β1 has a costimulatory effect during antigen-dependent activation and proliferation of T lymphocytes[265-268]. Both R1.2 and PS/2 interfere *in vitro* with antigen-specific proliferation of encephalitogenic T cell lines[56]. Antibodies against α4β1 that bind the same epitopes which mediate the binding to fibronectin and VCAM-1 have the highest costimulatory potential[269]. As described above, α4β1 is localized in the pSMAC of the immunological synapse between T lymphocytes and antigen presenting cells[86]. For B lymphocytes it has been shown that α4β1/VCAM-1 binding enhances cell-cell

adhesion, which in turn increases BCR signaling by indirectly strengthening the interaction of the BCR with membrane-associated antigens. The outcome is to facilitate the activation of B cells, especially for antigens that have a low affinity to the BCR[88].

In vitro myelin-specific T cells induce the production of proinflammatory cytokines such as TNF-α and IL-1β in microglia cells. This effect is contact-dependent and can be significantly reduced by treatment with blocking antibodies against integrin α4[270]. It was also shown that the expression of the α4 subunit on an encephalitogenic T cell clone induced MMP-2 and thereby facilitated transmigration through the blood brain barrier[271]. Finally, it was reported that α4 is important for the retention of autoreactive T cells in the parenchyma of the CNS[114]. Another interaction that might be inhibited by natalizumab is the binding of α4β1 to osteopontin[46], an ECM protein that promotes proliferation and differentiation of T cells into the T_H1 lineage. Osteopontin is upregulated in EAE and in CNS lesions of MS patients[272], and mice that lack osteopontin seem to have a decreased EAE severity[272,273].

6 Aim of the thesis

Firstly, we wanted to analyze the role of the two α4 integrins, α4β1 and α4β7, in adult murine hematopoiesis (Paper II). Previous studies with somatic α4 chimeras suggested an important role for α4 integrins in hematopoiesis, especially during retention and maintenance of HSCs in the bone marrow, while deletion of α4 in HSCs caused only mild hematopoietic defects and hematopoiesis of β1 deficient bone marrow chimeras and β7 knockout mice is normal. The following hypotheses were brought forward to explain the conflicting results: (i) α4β1 and α4β7 compensate each other, (ii) a lack of α4 expression in non-hematopoietic cells such as bone marrow stroma cells causes the severe phenotype in $\alpha4^{-/-}$ somatic chimeric mice, and (iii) fetal hematopoiesis depends more on α4 integrins than adult hematopoiesis. In order to test these hypotheses we analyzed adult hematopoiesis in mice deficient for the β7 gene and lacking the β1 gene in the hematopoietic system in an inducible fashion.

Secondly, we wanted to assess the role of RhoH, a downstream effector of integrin signaling, during hematopoiesis (Paper III). The small Rho GTPase RhoH is expressed exclusively in cells of the hematopoietic lineage and it has been implicated in several processes including the maintenance of HSCs, leukocyte adhesion and migration and the development of B cell lymphomas. In order to assess these roles of RhoH *in vivo*, we generated and analyzed mice that are genetically deficient for RhoH.

Finally, we examined the function of integrin β1 in the autoimmune disease model EAE, which resembles in many aspects MS in man (Paper IV). The monoclonal antibody natalizumab directed against the α4 subunit is currently used to treat MS but its exact working mechanism is not clear. The main mechanism seems to be interference with the interaction of extravasating leukocytes with endothelial cells of the blood brain barrier. However, several observations suggest that the antibody is also interfering with additional processes such as T cell proliferation, the production of inflammatory cytokines by activated microglia cells or other, yet unknown functions of the α4 subunit. Furthermore it is unknown, whether the beneficial effects of the antibody treatment rely on the inhibition of α4β1 and/or α4β7 and which cell type is mainly inhibited. Our main goals were to find out: (i) which events are inhibited by the antibody, (ii) which α4 heterodimer is the main target of the antibody, and (iii) which cell types are affected by the treatment. Therefore, we examined the development of EAE in mice lacking the β1 subunit in the hematopoietic system or specifically in T cells. Furthermore, we analyzed the proliferation, cytokine response and adhesion of β1 deficient T lymphocytes.

7 Brief summaries of the publications

7.1 Paper I: β1 integrins: zip codes and signaling relay for blood cells

The functions of β2 integrins - which are specifically expressed on blood cells – in the hematopoietic and immune system have been described in great detail. However, many members of the large family of β1 integrins are expressed on blood cells as well, and fulfill a variety of important functions. In this review we describe the functions of β1 integrins on hematopoietic cells during the various steps of an immune response. The basis of an adaptive immune response is the antigen-specific activation and proliferation of lymphocytes. Upon activation, lymphocytes have to extravasate from blood vessels and subsequently migrate through the endothelium, the underlying basement membrane and the connective tissue. When they finally reach the site of an ongoing inflammation they have to be correctly positioned and retained in the tissue. A role of integrins of the β1 family has been shown for all these steps. Therefore β1 integrins gain more and more importance as targets of anti-inflammatory drugs, which is discussed in the last part of the article.

7.2 Paper II: Adult murine hematopoiesis can proceed without β1 and β7 integrins

We intended to clarify the role of α4 integrins in adult murine hematopoiesis, since previous experiments examining the importance of the α4 subunit and its two association partners, β1 and β7 for hematopoietic development produced controversial results. Therefore, we analyzed the hematopoietic system of bone marrow chimeric mice with an inducible knockout of the β1 subunit and a deletion of the β7 gene.

To compensate each other, both β1 and β7 have to be expressed on the same cell type. Therefore, we assessed the expression of the β7 subunit on hematopoietic cells and found that β7 is indeed expressed together with β1 on HSCs and on many other cells of the lymphoid and myeloid lineage. Analysis of hematopoiesis in bone marrow chimeras with β1/β7 double-deficient HSCs showed that the maintenance of HSCs was not impaired. Furthermore, neither the development of T nor of B lymphocytes was disturbed. To check whether the upregulation of other adhesion molecules compensates the loss of β1 and β7 in B and T lymphocytes, we compared the expression profiles of control and knockout lymphocyte populations with the help of affymetrix microarrays. No compensatory changes in molecules such as other integrins, selectins, VCAM-1 and MAdCAM-1 could be detected. Likewise, myeloid and erythroid cells developed normally in the double knockouts. Hematopoietic progenitor

cells in bone marrow and peripheral blood were slightly and transiently increased. We could show that the dramatic increase of those cells in non-bone marrow chimeric α4 Mx1-Cre mice results most likely from a loss of α4 expression on bone marrow stromal cells. After challenge of erythropoiesis by phenylhydrazine induced lysis of erythrocytes, the number of erythropoietic progenitor cells in the spleen was significantly reduced in β1/β7 double knockouts, suggesting a role for β1 and β7 integrins during recovery of erythropoietic progenitor cells.

In summary, we found that there were only subtle or transient alterations in the number and distribution of progenitor cells, while the maintenance and retention of HSCs was not severely altered in the absence of β1 and β7 integrins. Furthermore lymphoid, myeloid and erythroid cell development were normal. These results indicate that adult hematopoiesis is indeed independent of α4 integrins, and thus imply that there is no need for a compensation between α4β1 and α4β7. This result hence indicates that the observed severe defects in the somatic chimeric $\alpha4^{-/-}$ mice are likely attributed to a loss of α4 expression on non-hematopoietic cells.

7.3 Paper III: RhoH is important for positive thymocyte selection and T-cell receptor signaling

The small Rho GTPase RhoH has been implicated in the development of B cell lymphomas, for the proliferation and survival of HSCs and for the migration and adhesion of lymphocytes and HSCs. In order to elucidate the role of RhoH *in vivo* we analyzed the hematopoietic system of RhoH deficient mice.

First, we generated mice carrying a disrupted RhoH gene. Animals with a homozygous disruption of the RhoH gene were born at Mendelian ratios. Both two and six months after birth mutant mice developed no remarkable changes in myeloid, erythroid or B lymphocyte populations in peripheral lymphoid organs and the bone marrow. In contrast, at both points of time the number of T lymphocytes was dramatically decreased in bone marrow, spleen and lymph nodes. Analysis of thymocytes *in vivo* and *in vitro* revealed that the generation of T lymphocytes was severely affected during the development of late $CD4^{-}CD8^{-}$ double negative thymocytes. Accordingly during the late stages of double negative thymocyte development, apoptosis was increased and proliferation decreased. At this stage T cells that generated functional β-chains receive signals from the pre-TCR and continue their development (β-selection). In addition the transition of double positive cells into single positive cells was impaired *in vivo*, suggesting that also positive selection of T cells that recognize self-MHC molecules was compromised. Therefore we analyzed mice carrying an ovalbumin-specific TCR in addition to the RhoH deficiency. In those

animals we observed an impaired positive selection of thymocytes from double positive into $CD4^+$ single positive cells, a process that requires weak signaling of the TCR. *In vitro* analysis of TCR signaling revealed that signaling downstream of the tyrosine kinase ZAP70 to the scaffolding protein LAT and molecules associated with LAT was diminished in RhoH deficient thymocytes and splenocytes. Since it was proposed that RhoH negatively regulates the adhesive properties of integrin αLβ2 we also tested the adhesion of $RhoH^{-/-}$ thymocytes to ICAM-1, VCAM-1 or endothelial cells. The adhesion of control and RhoH-null thymocytes was indistinguishable. In line with these findings the defective T cell development could not be rescued in RhoH/β2 double knockout mice.

In conclusion, RhoH deficient mice have no defect in the maintenance of HSCs, but the differentiation of T lymphocytes is severely disturbed due to reduced pre-TCR and TCR signaling.

7.4 Paper IV: Extravasation of autoreactive T cells into the central nervous system is controlled by β1 integrins

The aim of this work was to elucidate the role of β1 integrins during the development of an autoimmune disease. Previous investigations had implicated the α4 integrins α4β1 and/or α4β7 in the development of the human disease MS and the animal model EAE. The blockade of the integrin α4 subunit with antibodies is an approved treatment of MS, nevertheless it is not known, which integrin heterodimer and which cell types are mainly targeted and what is the main mechanism of action of the therapy.

To elucidate the role of integrin α4β1 during EAE pathogenesis, particularly during proliferation and extravasation of encephalitogenic T cells, we analyzed EAE development in mice missing β1 expression in all hematopoietic cells or specifically in T lymphocytes. We found that the ability of β1 deficient T cells to accumulate in the CNS of mice during active EAE is severely impaired. This result supports the current opinion that the main α4 integrin implicated in EAE pathogenesis is α4β1 since the presence of α4β7 could not rescue the defective T lymphocyte accumulation. To clarify, whether the reduced accumulation is due to diminished activation and proliferation and/or extravasation of the T cells we first analyzed T cell proliferation. *In vivo* proliferation of $\beta1^{-/-}$ T cells was not significantly altered; furthermore, β1 deficient T lymphoblasts showed a normal cytokine response upon antigen-specific stimulation *in vivo*. Second, we examined the adhesion of T cell blasts to the spinal cord microvasculature by means of IVM. We found that the adhesion of β1 integrin deficient T lymphoblasts to postcapillary venules was greatly reduced indicating that the failure

of β1 knockout T cells to enter the CNS during EAE is mainly caused by their impaired extravasation. Furthermore we could demonstrate that active EAE development is significantly delayed in mice lacking β1 expression specifically on most T lymphocytes whereas the EAE course was not changed in mice with a complete absence of β1 on myeloid cells. These results indicate that the main targets of the antibody therapy are the T lymphocytes.

In summary, we could show in this study that encephalitogenic T cells critically rely on members of the β1 integrin family during the pathogenesis of the autoimmune disease EAE, identifying α4β1 as major target of anti-inflammatory therapies directed against the α4 integrin subunit. Furthermore, we could show that the main effect of the antibody therapy is the blockade of T cell extravasation, whereas the antigen-dependent proliferation of T lymphocytes was not impaired. Finally, we demonstrate that T lymphocytes are the major target cell hit by the treatments with blocking anti-α4 antibodies.

7.5 Paper V: Analysis of integrin functions in blood

This paper was published as part of a review on the “Analysis of integrin functions in peri-implantation embryos, hematopoietic system, and skin”, and focuses on methods permitting to analyze the functions of integrins in the hematopoietic and the immune system in gene targeted mice. The availability of Cre lines that induce a deletion of loxP-site flanked genes facilitates the analysis of integrin functions in basically all branches and cell types of the hematopoietic system. In addition, hematopoietic system-restricted integrin knockouts can be generated with the help of bone marrow chimeric mice. Subsequently flow cytometric methods that are essential for the examination of the hematopoietic system are discussed. Another interesting feature of hematopoietic cells is the fact that most of them can be readily isolated, cultivated and analyzed in detail *in vitro*. Therefore, we describe the generation of dendritic cells and methods to analyze the proliferation of T lymphocytes. In the end, we explain the induction of EAE in mice, since this is an elegant system to test the properties of integrin deficient hematopoietic cells in an autoimmune disease model.

8 Acknowledgements

This thesis would not have been possible without the help of many people and at this point I would like to express my sincere gratitude to:

Prof. Dr. Reinhard Fässler, for giving me the opportunity to take part in advanced research in his department, for his continuous support and encouragement and for the fruitful discussions throughout this work.

Dr. Michael Sixt and Prof. Dr. Cord Brakebusch, for supervising this work with never-ending enthusiasm and for many valuable discussions.

Prof. Dr. Markus Sperandio, who agreed to be the second referee of my thesis and Prof. Dr. Martin Biel, Prof. Dr. Karl-Peter Hopfner, Prof. Dr. Angelika Vollmar and PD Dr. Stefan Weiss, the members of my thesis committee for taking the time to review my work.

Prof. Dr. Britta Engelhardt, Dr. Caroline Coisne and Heidi Tardent, for the fruitful and exciting collaboration and also for the warm welcome during my visits to their lab.

The members of the Department of Neuroimmunology at the MPI of Neurobiology, particularly Prof. Dr. Hartmut Wekerle, Dr. Andreas Holz, Dr. Florian Kurschuß, Dr. Wolfgang Klinkert, Dr. Bernadette Pöllinger and Dr. Gurumoorthy Krishnamoorthy. They always had time to discuss with me and give advice and reagents during the sometimes challenging task of setting up the EAE system in our department.

All current and former members of the department, in particular Anika, Gerd, Hao-Ven, Katrin, Ralph, Sara and Siegfried. Thanks for your friendship, for creating a good working atmosphere and especially for the discussions and the fun during lunch and coffee breaks. Special thanks go to my three bench mates, Tatjana, Ola and Tim – it was always a pleasure to work next to you!

All the people that provided the “infrastructure” for this thesis, especially Dr. Walter Göhring, Sylvia Zehner and the animal care takers, particularly Diana Schmidt.

In the end I would like to thank my parents, Stephanie and Wolfram, for their support and confidence. And I owe great thanks to Frank, for his understanding, love and encouragement, for many discussions about science, but especially for reminding me sometimes that there is more to life than science!

9 Curriculum vitae

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11 Supplements

In the following, papers I to V are reprinted. On the enclosed CD the supplementary figure for paper II, the supplementary methods and figures for paper III and the supplementary movies for paper IV can be found. The supplementary movies are provided in QuickTime Movie format.

Paper I

β1 integrins: zip codes and signaling relay for blood cells

Michael Sixt, Martina Bauer, Tim Lämmermann and Reinhard Fässler

At least eight of the twelve known members of the β1 integrin family are expressed on hematopoietic cells. Among these, the VCAM-1 receptor α4β1 has received most attention as a main factor mediating firm adhesion to the endothelium during blood cell extravasation. Therapeutic trials are ongoing into the use of antibodies and small molecule inhibitors to target this interaction and hence obtain anti-inflammatory effects. However, extravasation is only one possible process that is mediated by β1 integrins and there is evidence that they also mediate leukocyte retention and positioning in the tissue, lymphocyte activation and possibly migration within the interstitium. Genetic mouse models where integrins are selectively deleted on blood cells have been used to investigate these functions and further studies will be invaluable to critically evaluate therapeutic trials.

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Current Opinion in Cell Biology 2006, **18**:482–490

This review comes from a themed issue on
Cell-to-cell contact and extracellular matrix
Edited by Martin Schwartz and Alpha Yap

Available online 17th August 2006

0955-0674/$ – see front matter

DOI 10.1016/j.ceb.2006.08.007

Introduction

To fulfill their surveillance function immune cells continuously patrol the organism, shuttling back and forth between the blood stream, the lymphatic fluid, secondary lymphatic organs and peripheral tissues [1]. This mobile life style requires flexible switching between passive transport and various cell-to-cell and cell-to-extracellular matrix (ECM) interactions to arrest, migrate and become activated.

The current paradigm of cell locomotion within tissues and along cell surfaces involves integrin-mediated adhesion to ECM or cellular counter-receptors, which generates traction forces necessary for translocation of the cell body [2]. Integrins are perfectly suited to this task since they link the cytoskeleton with the extracellular environment. Integrins are heterodimeric cell surface receptors made up of α and β subunits. The combination of 18 known α and 8 β subunits in mammals can give rise to 24 different receptors [3]. Antibody blocking studies, gene targeting approaches in mice and investigation of human diseases have unambiguously revealed that integrins are essential for intact hematopoietic development, homeostasis and inflammation. However, integrin ligand binding can affect several cellular events in addition to adhesion and migration, including cell differentiation, polarization, activation and survival [3]. A drawback of most studies manipulating integrin functions on blood cells *in vivo* is that the cell-biological process affected by the manipulation is not exactly defined. This is especially evident when complex inflammatory models (for example for autoimmune diseases) are studied and clinical symptoms or histological parameters are used as readout. A further critical issue is that within recent years an increasing number of mouse knockout studies have been published that address the *in vivo* function of cytoplasmic proteins involved in integrin signaling without explicitly investigating which integrins are affected.

In this review we focus on the largest integrin family which contains the β1 chain. The blood-cell-specific β2 integrin subfamily has been extensively reviewed by others [4]. We will try to dissect the different cell-biological functions that β1 integrins mediate when leukocytes emerge from the blood vessels, locate within tissues, become activated and re-enter the blood circulation.

Extravasation from the blood circulation

One of the best-established concepts in leukocyte biology is the extravasation paradigm. When hematopoietic cells leave the blood stream they go through a sequential adhesion cascade to overcome both the high shear forces within the blood vessel and the tight seal of the endothelial cells (see Figure 1). Transient selectin–carbohydrate interactions cause hematopoietic cells to begin to roll along an activated endothelium. While rolling, the cells sense chemokines that are immobilized on heparan sulfate residues on the luminal side of the endothelial cells. The ligated chemokine receptors then transmit signals into the leukocyte that lead to the rapid activation of integrins (inside-out signaling — see Box 1), which results in the integrins adhering firmly to their counter receptors on the endothelial cell. Although adhesion during extravasation is an essential step during leukocyte trafficking, it has little in common with cell migration in the true sense. It is rather a cell adhesion event of the hematopoietic cell to the two-dimensional surface of the endothelial lumen.

The crucial β1 integrin family member involved in extravasation is α4β1, which binds to the endothelial Ig

Box 1 Regulation of β1 integrin activation

On circulating leukocytes, integrins are locked in the low-affinity state. Only upon 'inside out' signaling (triggered, for example, by chemokines, growth factors or T cell receptor activation) integrins adopt a high-affinity conformation (termed integrin activation) facilitating ligand binding and subsequent cell adhesion. Ligand binding in turn induces integrin 'outside in' signaling that (among many other effects) further consolidates cell binding by clustering the integrins and thereby increasing binding avidity. Cytoplasmic key players mediating 'inside-out' signaling are the small GTPases of the Rap family and talin.

Rap1: Several recent *in vitro* studies have proven that Rap1, the best-characterized member of the five Rap proteins, is essential for β1 integrin activation on leukocytes. Studies with cell lines revealed that activated Rap1 increases β1 integrin-mediated adhesion and migration on VCAM-1 via α4β1 and on fibronectin via α4β1 and α5β1 [57,58]. The same was shown for primary thymocytes of transgenic mice expressing the constitutively active Rap1-mutant Rap1V12 [59], whereas T and B cells derived from Rap1-deficient mice show impaired adhesion on fibronectin [60]. Rap1 is recruited to the plasma membrane by PKD1, where it is activated upon integration into a complex containing the β1 integrin cytoplasmic tail [61,62]. For Rap1-mediated inside-out signaling, the two Rap1 binding effectors RIAM and RAPL are essential. Accordingly, T cells and dendritic cells from RAPL-deficient mice show impaired adhesion to β1 integrin ligands and reduced transmigration through endothelial monolayers [63]. Overexpression of RIAM enhances Rap1-mediated T cell adhesion to fibronectin. Through its interaction with profilin and ENA/Vasp proteins, RIAM probably links Rap1–GTP to the actin cytoskeleton [64].

Talin: Talin is a large rod-like molecule that binds via its globular head domain to the membrane proximal NPXY motif of β integrins in a phosphorylation-regulated manner. Talin acts as a physical link between integrins and the actin cytoskeleton and its binding to integrin β chains is regarded as the final common step in integrin activation [65]. Two recent studies assessed the *in vivo* role of the β1 integrin NPXY motifs by employing mouse genetic models. They revealed that the intact conformation of the NPXY motifs are essential, as substitution of the tyrosines by alanine abolishes β1 integrin function and leads to a β1 integrin-null phenotype [66,67]. Accordingly, chimeric mice with alanine substitutions, similar to a β1 integrin-null chimera, fail to develop hematopoietic cells, probably as a result of impaired talin binding [67]. Both studies, however, challenged the former view that tyrosine phosphorylation is essential for affinity regulation of β1 integrins, as replacement of both cytoplasmic tyrosines with phenylalanine did not result in an obvious phenotype, indicating that tyrosine phosphorylation is dispensable for the physiological β1 integrin function *in vivo*.

superfamily cell surface receptor VCAM-1 (vascular cell adhesion molecule 1). This interaction is conserved in many different physiological settings where extravasation occurs. In the steady state, lymphocyte recirculation via high endothelial venules [5], T cell precursor entry into the thymus [6] and T cell and stem cell homing into the bone marrow [7–9] are regulated via this pathway. During inflammation, lymphocytes and monocytes use α4β1 to immigrate into the skin, lung, peritoneum and liver [10,11,12•]. For several cell types it has also been shown that VCAM-1-α4β1 binding can mediate not only firm adhesion but also rolling along the endothelium [12•]. In a somewhat controversial deviation from the paradigm, there is evidence that extravasation of lymphocytes into the central nervous system during autoimmune inflammation is possible in the absence of previous rolling [13,14•]. Here the cells can be rather abruptly captured by VCAM-1 exposed on the endothelial lumen.

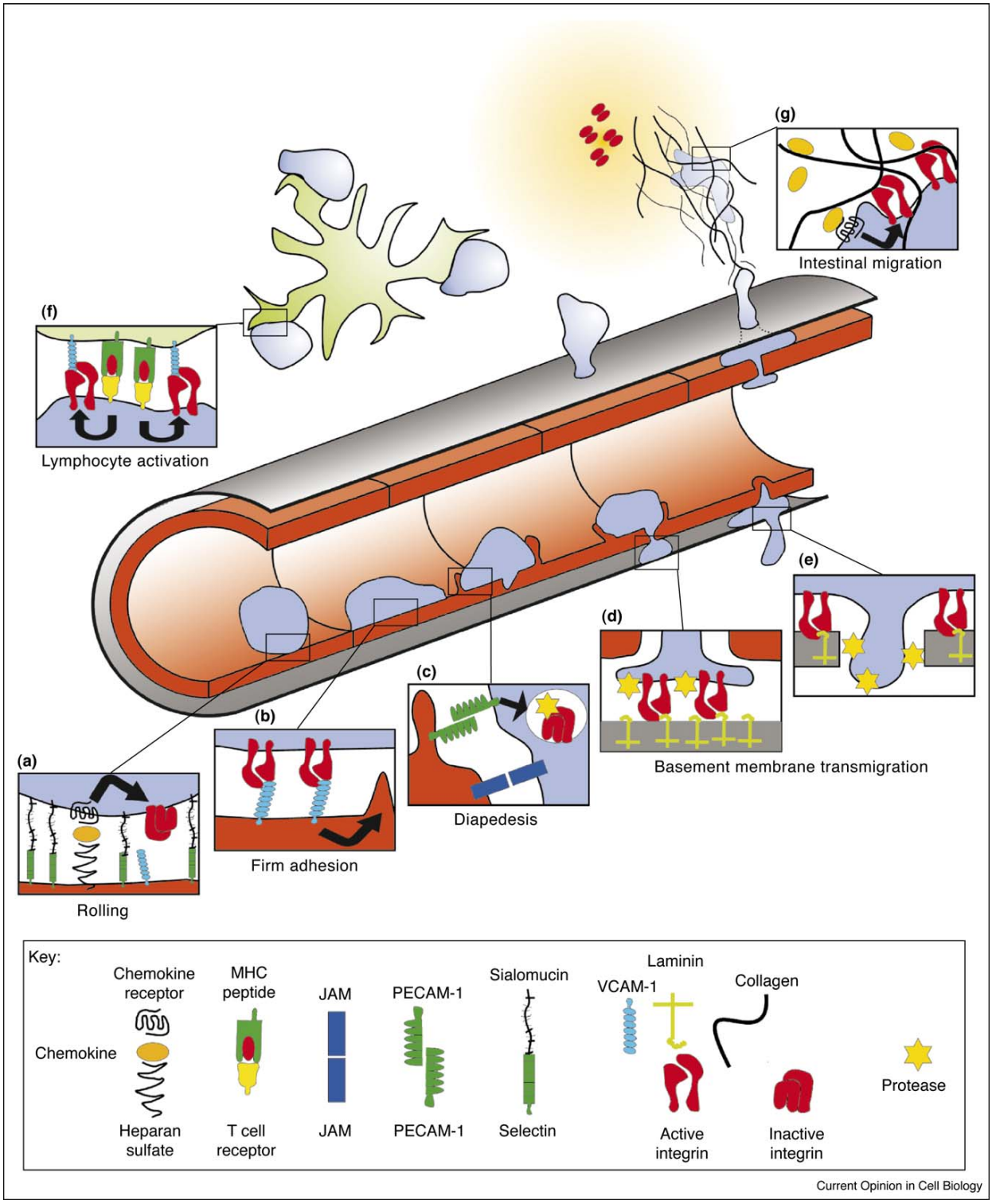
In most cases the function of α4β1 is partially redundant with that of β2 integrins and α4β7, which bind the endothelial counter-receptors intercellular adhesion molecule (ICAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), respectively. An impressive example of this redundancy is lymphocyte recirculation into lymph nodes, which is only partially affected by β2 and β7 elimination [5] and unimpaired in the absence of β1 integrin [15]. Combined blockade, however, results in almost 100% reduction of lymphocyte recirculation [5]. Many details concerning the overlapping functions of these integrins remain to be clarified using genetic models in which the separate families are targeted simultaneously.

Important exceptions to this redundancy are T cell trafficking into the CNS, which is largely inhibitable by α4β1 blockade [16], stem cell homing into the bone marrow, which is completely defective in the absence of β1 integrin [7], and the migration of hematopoietic progenitor cells from the fetal blood into the fetal liver during early development. In early hematopoietic development, we could show that genetic deletion of the β1 integrin gene in progenitor cells leads to their accumulation in the fetal blood and hence the inability to populate hematopoietic tissues [7,17]. Although it is likely that α4 is of major importance in this setting, other α chains may be involved. A possible candidate is the largely neglected integrin α9β1, which is highly expressed on granulocytes and binds to VCAM-1 and the ECM proteins tenascin, osteopontin and fibronectin. α9β1 has been shown to mediate transendothelial migration *in vitro* via interaction with VCAM-1 [18]. Interestingly, α4 and α9 chain are closely related and together form a sub-family that binds to the cytoplasmic adaptor paxillin [19].

Migration through the endothelium

Tight adhesion to the endothelium is followed by a cascade of migration events that probably successively trigger each other. First the cells have to pass through the endothelial monolayer. There is no *in vivo* evidence that this process — called 'diapedesis' — is directly dependent on β1 integrins. It is rather mediated by cell adhesion molecules of the JAM family, CD99 and PECAM-1 [1]. Nevertheless, ligation of endothelial VCAM-1 via α4β1 seems to be a prerequisite for diapedesis, as it triggers a signal within the endothelial cell that is transmitted via the cytoplasmic tail of VCAM [20]. The endothelial cell then reacts by actively extending protrusions to capture and guide the extravasating cell through intercellular junctions or by transcytosing it directly

Figure 1



Roles of β1 integrins during extravasation, interstitial migration and lymphocyte activation. **(a)** During selectin-mediated rolling, the extravasating cell senses chemokines that are immobilized on the surface of the endothelium, leading to the inside-out activation of integrin α4β1. **(b)** The α4β1–VCAM-1 interaction mediates firm adhesion and triggers a reverse signal via VCAM-1 that induces the extension of endothelial protrusions,

through the endothelial cell body — called emperipolesis [20,21].

Transmigration through the basement membrane

All the events following diapedesis are far less characterized, which is mainly due to their experimental inaccessibility and the lack of established *in vitro* models to study molecular interactions. Directly after passing through the endothelium, the transmigrating cells face a seemingly impermeable barrier of ECM: the endothelial basement membrane (BM). BMs are tightly interconnected and thin (~50–100 nm) sheets of specialized ECM components of the laminin and collagen IV family [22]. It is possible that β1 integrins play an active role during this passage and *in vitro* studies have demonstrated that leukocytes can actively bind BM components [23]. If BM transmigration is selectively blocked *in vivo* one would expect that extravasating leukocytes become trapped between the endothelial cell layer and the underlying BM. Indeed this phenomenon was observed when extravasation of granulocytes triggered by interleukin (IL)-1 was studied in mice in which platelet endothelial cell adhesion molecule-1 (PECAM-1) was functionally inactivated by blocking antibodies or genetic deletion [24]. The homophilic interaction between PECAM-1 on granulocyte and endothelium induced the up-regulation of the laminin binding α6β1 integrin on the granulocyte surface, which in turn was necessary for BM transmigration [25]. Although this sequence of events was well demonstrated in this specific experimental setting, it is not a general phenomenon, as granulocyte extravasation in response to tumor necrosis factor (TNF)α occurred independently of PECAM-1 and α6β1 [26]. Another recently reported example of the possible involvement of a BM binding integrin during extravasation is the reduced homing of hematopoietic stem cells into the bone marrow after antibody blockade of α6β1 [27].

A physiologically distinct situation where leukocytes cross a BM occurs during the emigration of Langerhans cells from the epidermis. Their penetration through the epidermal BM (which is biochemically distinct from blood vessel BMs [28]) can also be inhibited by antibody blockade of α6 integrin [29]. Although in this case it remains to be shown which β chain (β1 or β4) pairs with α6, laminin is the likely ligand.

Apart from these fragmentary data about molecular players, the progression of physiological events that leads to BM transmigration is completely enigmatic. Proteolytic digestion via proteases, especially by the matrix metalloproteinase (MMP) family, has been suggested in several *ex vivo* models [30,31]. In this context it is interesting that ligation of integrins can lead to the induction of MMPs on leukocytes [31,32]. It remains to be shown if integrins merely signal the presence of a BM to induce proteolytic cascades or other events leading to the BM's local disassembly, or if integrin-mediated adhesion is also a physical requirement for the translocation of the cell body through the BM.

Migration through the interstitium

The diverse extracellular environments that leukocytes face upon passaging through the BM range from the loosely packed and fibrillar-collagen-dominated connective tissue of the mesenchymal interstitium to the cell-rich environment of secondary lymphatic organs. At this stage, true directed migration takes over and it is assumed that leukocytes navigate along gradients of chemotactic agents towards their destinations. Despite numerous *in vitro* studies using artificial settings such as transwell filters coated with ECM components, it is still controversial if this directed migration depends on integrins at all *in vivo*. The most direct experimental evidence for integrin involvement is provided by a series of intravital microscopy studies revealing that migration of granulocytes through the mesenteric interstitium can be partially inhibited by blocking antibodies against the collagen-binding β1 and α2 integrin chains [33–35]. However, measured reductions in speed of only ~30% raise the question of whether the remaining migratory activity is mediated by compensating (β2 or αv) integrins or whether it is completely integrin-independent. *In vitro* experimental approaches using three-dimensional gels of the fibrillar collagens I and III, which mimic the interstitial ECM, can be utilized as migration matrices and studied by video microscopy. Even in this artificial and very defined setting, the results obtained with integrin-blocking antibodies are controversial. By combined antibody blockade of αv, β2 and β1 integrins, it was shown that random T cell migration in the gel can occur in an 'ameboid' fashion in the complete absence of integrin-mediated binding [36] and proteolytic activity [37]. However, others demonstrated that in the presence of chemotactic agents, T cells utilize β1 (α1, α2, α6) integrins for locomotion within collagen gels [38]. Although the issue of interstitial migration remains to be clarified using genetic approaches in combination with intravital microscopy, it is evident that it is essential to define not only the nature of the ECM ligand but also the spatial configuration of the extracellular environment in order to establish

(Figure Legend 1 continued) establishing a 'docking structure'. **(c)** Diapedesis is mediated via PECAM-1, JAMs, CD99 and β2 integrins (not shown). Signals from the endothelium induce surface expression of α6β1 and proteases on the transmigrating cell. **(d,e)** In some cases the laminin-binding integrin α6β1 and cell surface proteases mediate passage of the basement membrane. **(f)** α4β1 localizes to synapses between follicular dendritic cells and B cells and dendritic cells and T cells where it promotes lymphocyte activation. **(g)** Interstitial migration along chemotactic gradients is possibly mediated by the collagen-binding α2β1 integrin.

integrin dependency. In an elegant study, Malawista *et al.* [39] showed that in the spatially constrained environment of a narrow space between two closely adjacent glass surfaces, granulocytes can switch to biophysical mechanisms of translocation (squeezing or 'chimneying') that are independent of integrin binding. By contrast, on two dimensional surfaces granulocytes are completely dependent on integrins to generate traction forces [39]. These findings impressively demonstrate the importance of using 3-dimensional model systems to study interstitial blood cell migration.

In the light of these diverse and partially controversial data, it seems possible that within the 3-dimensional environment of the interstitium the quickly migrating blood cells employ adhesive mechanisms that are fundamentally different from the integrin-dependent migration strategies used by mesenchymal cells.

Retention and positioning within the tissue

While it still remains to be clarified to what extent integrins are involved in interstitial leukocyte migration, there is solid evidence that integrin-mediated binding can define the position of hematopoietic cells by immobilizing and retaining them in their niches. Owing to the poor knowledge about the spatial configuration and molecular composition of these niches, it is not known if retention simply reflects integrin-mediated cell binding or if more complex processes are triggered via integrin signaling that ultimately lead to retention. Two prominent examples of integrin-mediated retention are illustrated by studies involving marginal zone B cells and hematopoietic progenitor cells.

Several studies revealed that different precursors can be released from the bone marrow by antibody blockade or genetic inactivation of the α4 integrin [40,41]. Similar results were obtained with mice lacking the α4β1 ligand VCAM-1 [42]. It has been proposed that in the bone marrow the stroma-derived chemokine CXCL12 triggers a sustained signal that keeps the integrin in the active state and therefore immobilizes the cells to VCAM-1 and fibronectin on stroma cells [43].

Marginal zone B cells are part of the first line defense system against circulating soluble antigens. As such, they are located in a defined ring area around the white pulp follicles of the spleen, where they capture blood-borne antigens. Only upon activation by microbial stimuli or antigen do they downregulate integrin avidity, detach from the marginal zone and follow a chemotactic gradient of the chemokine CXCL13 that guides them into the follicle, where they produce immunoglobulins. The retention of marginal zone B cells is redundantly mediated by α4β1 and αLβ2 integrin, and blockade or genetic ablation of these integrins causes the cells to dislocate from the marginal zone [44]. In marginal zone B cells, integrin affinity was shown to be regulated via the GTPase RhoA and the exchange factor lsc, as in the absence of lsc these cells are unable to leave their niche following stimulation owing to an insufficient down-modulation of integrin avidity [45•].

An interesting series of studies that suggests a role for integrin-mediated retention during pathological processes has been performed by de Fougerolles and co-workers. Using antibody blockade and genetic inactivation, they demonstrated that the collagen receptors α1β1 and α2β1 are critically involved in the course of cutaneous hypersensitivity, experimental arthritis and colitis by localizing T cells within the interstitium [46–48]. Furthermore, in two models of murine virus infection, the number of virus-specific CD8 memory T cells in the lung of infected animals could be reduced by anti-α1β1 administration without affecting T cell recruitment during primary infection [49,50•]. Therefore it is likely that α1β1 is needed for the long term retention of CD8 memory T cells in the lung.

Cell–cell interactions and activation of lymphocytes

The initiating step in all T-cell-dependent immune responses is the formation of an immunological synapse — the contact between an antigen-presenting cell (B cell or dendritic cell) and a T cell. A main functional constituent of the synapse is a sealing zone (the peripheral supramolecular activation cluster or pSMAC), which is defined and maintained by the interaction of LFA1 on the T cell with ICAM 1 on the antigen presenting cell.

Although early *in vitro* studies already suggested that β1 integrins have potent co-stimulatory functions, conditional knockout mice lacking β1 integrins on hematopoietic cells showed a relatively weak immunological phenotype: T-cell-dependent immune responses were grossly unaffected with the surprising exception that IgM production was severely decreased [15]. However, recent data suggest that members of the β1 integrin family might play a more subtle regulatory role during immune synapse formation. Mittelbrunn *et al.* showed that α4β1, like αLβ2, localizes within the pSMAC of synapses between T cells and dendritic cells/B cells. Furthermore, they could demonstrate that this interaction is important for shifting of the T cells towards a Th1-type cellular immune response [51••]. Another recent study showed that B cells utilize α4β1 to bind VCAM-1 co-expressed with antigen on the surface of fibroblasts *in vitro*, which might reflect B cell interaction with follicular dendritic cells in the lymph node [52,53•]. Moreover, it could be shown that this interaction synergizes with the B cell receptor signal and triggers B cell activation. It will be important to test if integrins assist T/B cell receptor signaling only indirectly, by establishing and maintaining

the cell–cell contact, or if active cross talk occurs between the signaling pathways triggered by both receptors.

Members of the β1 integrin subfamily as anti-inflammatory drug targets

Pharmacological interference with leukocyte extravasation is an attractive strategy for anti-inflammatory therapies that was sparked off by the discovery of the extravasation paradigm in the late 1980s. Table 1 lists some selected diseases where blockade of β1 integrins showed beneficial effects, together with a proposed mechanism of action. Although the intended therapeutic effect of most of these therapeutic approaches involves the inhibition of firm adhesion to the endothelium, it is not clear whether other processes, for example lymphocyte activation, could be affected as well. A prominent example of this uncertainty is autoimmune inflammation of the central nervous system. It is well established that the binding of lymphocytes to inflamed brain blood vessels during experimental autoimmune encephalomyelitis in rodents is inhibited by antibodies against α4β1 and that these antibodies prevent the development of the disease [16]. This therapeutic principle was used in a clinical trial to treat patients suffering from the equivalent human disease, multiple sclerosis [54]. Despite very promising results, approximately one out of thousand patients acquired a deadly opportunistic viral infection of the CNS during chronic treatment [55]. These could have been caused either by impaired trafficking of non-pathogenic lymphocytes that are essential for normal CNS immunosurveillance or by a more general immunosuppression. Indeed it has been shown in rodent EAE that an anti-α4 antibody which does not inhibit lymphocyte homing *in vivo* still ameliorates EAE [56]. This argues in favor of an additional role for α4β1 apart from mediating extravasation. In this context the recent data suggesting a role for α4β1 in T cell activation are of special interest.

Conclusions

Advances in the field of intravital imaging make it now possible to track the dynamic behavior of cells in most tissues of living animals. In combination with genetic models where integrins are specifically deleted on defined blood cell lineages, this approach will allow the pinpointing of many of the cell biological roles of β1 integrins on hematopoietic cells. This knowledge will be decisive to predict side effects when pharmacological approaches are developed in which integrins are targeted in a non-cell-type-specific manner. Investigating cytoplasmic players involved in the activation of the β1 integrins will further teach us to what extent the signaling pathways are cell-type- and α-chain-specific and will eventually reveal new drug targets to inhibit extravasation in a more cell- and tissue-type-specific manner.

Table 1

Model system	Involved integrin dimer and mode of inhibition	Effects of integrin inhibition	Proposed mode of action	References
EAE, multiple sclerosis (Lewis rat, mouse, human)	α4β1 Anti-α4 mAb	Reduced clinical signs of disease, reduced inflammatory infiltrate	Blockade of firm adhesion to endothelium and thereby extravasation	[16,54,56]
Morbus Crohn (human)	α4 integrins Humanized anti-α4 mAb	Reduced clinical signs and lowered C-reactive protein levels	Not addressed, probably extravasation blockade	[68]
Arthritis (mouse)	α4β1 S18407: synthetic α4β1 inhibitor	Reduced clinical signs, reduced inflammatory infiltrate and mediators; bacterial clearance not affected.	Interference with neutrophil activation, cellular trafficking not severely affected	[69]
Hepatitis (mouse)	α1β1, α2β1 Anti-α1/α2 mAb, α1 deficiency	Reduced clinical signs, reduced inflammatory infiltrate	Unclear; either migration in tissue or activation of cells	[46]
	α4β1 Anti-α4 mAb, anti-VCAM-1 mAb	Reduced clinical signs of disease	Interference with α4β- mediated rolling, adhesion in sinusoids	[12•]
Peritonitis (mouse)	α4 integrins Y991A mutation in α4, blocks paxillin binding	Defective recruitment of lymphocytes and monocytes to the peritoneum	Probably extravasation blockade	[70]
Influenza (mouse)	α1β1 α1 deficiency; anti-α1 mAb	No inhibition of the recruitment to the lung during primary infection; reduced number of memory CD^{8+} T cells in the tissue and compromised secondary immunity	Inhibition of long term retention of CD8 memory T cells in the lung	[50•]
Colitis (mouse)	α1β1 α1 deficiency; anti-α1 mAb	Reduced clinical symptoms, reduced i nflammatory infiltrate, decreased IFN-γ and TNF-α production	Reduced extravasation, migration or retention of monocytes; reduced cytokine production	[48]

CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; TNBS, 2, 4, 6-trinitrobenzene sulfonic acid.

Acknowledgements

We thank Kyle Legate and Carsten Grashoff for critical reading of the manuscript. Our work on integrins is supported by DFG and the Max Planck Society.

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Paper II

Adult murine hematopoiesis can proceed without β1 and β7 integrins

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The function of α4β1 and α4β7 integrins in hematopoiesis is controversial. While some experimental evidence suggests a crucial role for these integrins in retention and expansion of progenitor cells and lymphopoiesis, others report a less important role in hematopoiesis. Using mice with a deletion of the β1 and the β7 integrin genes restricted to the hematopoietic system we show here that α4β1 and α4β7 integrins are not essential for differentiation of lymphocytes or myelocytes. However, β1β7 mutant mice displayed a transient increase of colony-forming unit (CFU-C) progenitors in the bone marrow and, after phenylhydrazine-induced anemia, a decreased number of splenic erythroid colony-forming units in culture (CFUe's). Array gene expression analysis of $CD4^+CD8^+$ double-positive (DP) and $CD4^-CD8^-$ double-negative (DN) thymocytes and $CD19^+$ and $CD4^+$ splenocytes did not provide any evidence for a compensatory mechanism explaining the mild phenotype. These data show that α4β1 and α4β7 are not required for blood cell differentiation, although in their absence alterations in numbers and distribution of progenitor cells were observed. (Blood. 2006;108:1857-1864)

Introduction

The development and maintenance of hematopoietic stem cells (HSCs), which can self-renew and differentiate into all hematopoietic blood cell lineages, is thought to depend on their interactions with the microenvironment. Integrins expressed on HSCs are capable of mediating several of those interactions as they can bind extracellular matrix components such as fibronectin and laminin, but also cellular receptors like VCAM-1 expressed on bone marrow (BM) stroma cells. Integrins are a family of heterodimeric cell-surface receptors consisting of an α and a β subunit.[1] Integrins provide mechanical support by connecting the extracellular matrix (ECM) with the cytoskeleton, but are also capable of transducing chemical signals upon ligand binding. This signaling results in cytoskeleton reorganization and changes in gene expression affecting proliferation, differentiation, and survival of cells.[2] Molecules inside the cell, on the other hand, can modulate the affinity and avidity of integrins, called inside-out signaling, which is, for example, crucial for the extravasation of leukocytes.[3]

In vitro and in vivo experiments suggests an important role of α4β1 and α5β1 integrins for the adhesion of HSCs and hematopoietic progenitor cells (HPCs) to fibronectin in the bone marrow matrix.[4,5] Integrin α4β1 was additionally shown to mediate binding to VCAM-1, which is expressed on BM stroma cells.[6] Injection of fibronectin fragments and blocking antibodies against α4β1 and VCAM-1 led to a release of HSCs/HPCs into the blood, supporting the proposed importance of these interactions in vivo.[5,7] Conditional deletion of the VCAM-1 gene resulted in an early exit of B-cell precursors into the blood.[8] Finally, it was shown that α4β1–mediated attachment of HPCs to fibronectin promotes proliferation and survival,[9,10] suggesting a crucial role for self-renewal and survival of HSCs.

In vivo studies with β1 integrin–deficient somatic chimeric mice, which are generated by injecting β1-null embryonic stem (ES) cells into wild-type host blastocysts, demonstrated that β1 integrin is not required for the formation of HSCs, but is essential for their migration to the fetal liver.[11] Additionally, β1 integrin–deficient HSCs failed to engraft lethally irradiated mice.[12] Altogether, these data pointed to a key role of α4β1 integrin in hematopoiesis. This notion was corroborated by the analysis of α4-null somatic chimeric mice, which have almost no mature B cells, T cells, or erythroblasts derived from α4-null ES cells.[13,14] In vitro experiments with cells derived from the α4-null chimeric mice suggested that both erythroid and B-cell precursors are less able to transmigrate through the stroma, which may result in reduced cell proliferation.[14] Also, the number of α4-deficient myeloid cells was reduced compared with control chimera. Since β7 integrin constitutive null mice displayed normal hematopoiesis,[15] it was suggested that α4β1 integrin might be the pivotal integrin during hematopoiesis, as α4 can dimerize only with β1 and β7 integrins. Therefore, it was unexpected when β1 mutant BM chimeras showed no defects in blood cell development.[16] The simplest explanation at that time was that α4β1 and α4β7 integrin might have redundant functions in blood cell development and that only the absence of both receptors leads to the described hematopoietic defects. However, further experiments showed that inducible deletion of the α4 integrin gene has only subtle effects on

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Submitted October 20, 2005; accepted May 7, 2006. Prepublished online as *Blood* First Edition Paper, May 30, 2006; DOI 10.1182/blood-2005-10-007658.

Supported by the Max Planck Society and the German Research Council (DFG BR 2201/1; DFG FOR471/2 DFG SFB571).

The online version of this article contains a data supplement.

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hematopoiesis.[17] These mutants showed only a partial reduction of the B220$^+$ B-cell and CD4$^+$ T-cell populations in BM. Monocytes (Mac-1$^+$) and erythroblasts (Ter119$^+$) were reported to occur in normal amounts in the BM. In this study, however, the α4 integrin gene was not only deleted in hematopoietic cells but also in many nonhematopoietic cells such as hepatocytes, endothelial cells, and so on, which could contribute to the phenotype. An alternative explanation for these contrasting results could be that fetal hematopoiesis is more dependent on α4 integrin than adult hematopoiesis.

To better understand the role of α4β1 and α4β7 integrin in adult hematopoiesis, we generated and analyzed mice with a blood cell–restricted knockout of β1 and a constitutive knockout of β7 integrin. As a consequence α4β7, α4β1 and also other β1 integrins expressed on blood cells are lost. In contrast to the α4-null somatic chimeras[13,14] or the α4 conditional knockout mice[17] used previously, we can exclude any effects due to deletion of α4 on nonhematopoietic cells, which might influence hematopoiesis through altered production of cytokines and growths factors or different cell-cell interactions. This model was used to study HSC maintenance, HPC distribution and differentiation, and the migration of differentiated cells in the absence of β1 and β7 integrins in adult mice. We demonstrate now that even in the absence of both α4β1 and α4β7 integrins, hematopoiesis is normal.

Materials and methods

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)[16] were mated with mice with a neomycin-disrupted β1 integrin gene (+/−),[18] mice lacking a functional β7 integrin gene (β7$^{-/-}$),[13] and with mice carrying the Mx transgene (+Mx).[19] β1β7 mutant BM chimeras were generated by BM transplantation as described previously.[20] Recipient Ly-5.1$^+$ mice were lethally irradiated and received BM cells from Ly-5.2$^+$ β1$^{fl/+}$β7$^{-/-}$+Mx or β1$^{fl/fl}$β7$^{-/-}$ mice (β7 mutant BM chimeras) or from β1$^{fl/fl}$β7$^{-/-}$+Mx mice (β1β7 mutant BM chimeras). Four weeks after the transfer, deletion of the β1 gene was induced by polyIC injections as described previously.[20]

Animal treatment

Mice were maintained and bred under pathogen-free conditions. All animal experiments were approved by the local ethics committee. Blood samples were obtained from the retro-orbital plexus under anesthesia. Acute hemolysis was assessed after phenylhydrazine (PHZ; Sigma, Steinheim, Germany) treatment as described.[16]

Türk staining

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

Flow cytometry

Single-cell suspensions were prepared and analyzed as described.[16] Erythrocytes in blood samples were lysed by incubation in ACK-lysis buffer for 5 minutes at room temperature prior to staining.[21]

Deletion of the β1 integrin gene on BM stroma cells was assessed by measuring the activity of the β-galactosidase reporter.[16] Five days after a single injection of 250 μg polyIC, BM cells were plated on tissue-culture plates as described.[22] After 24 hours, nonadherent cells were removed and adherent cells detached by trypsin/EDTA. Nonhematopoietic BM stroma cells were characterized as Ly-5.2$^-$Ter119$^-$adherent cells, which consist of mesenchymal stem cells, fibroblasts, endothelial progenitor cells, and endothelial cells.[23] Hematopoietic cells, on the other hand, were identified as Ly-5.2$^+$ or Ter119$^+$ nonadherent cells. Cells were stained for β-galactosidase activity as described,[24] with minor changes. Briefly, 4×10^6 cells were suspended in 20 μL phosphate-buffered saline (PBS) added to 20 μL of 2 mM fluorescein-di-(beta-D-galactopyranoside) (FDG; Sigma). Cells were incubated at 37°C for 75 seconds and subsequently 200 μL ice-cold PBS was added. Cells were incubated for 3 hours on ice and analyzed by flow cytometry as described.[16]

For the analysis of platelets, 5 μL antibody solution containing FITC-conjugated anti–β1 integrin (Ha2/5; 1:10 diluted; BD Pharmingen, San Diego, CA) and PE-conjugated anti–GPIb-IX (p0p1) (kindly donated by Dr B. Nieswandt, University of Würzburg, Germany; 1:10 diluted) was added to 1 μL whole blood. After a 15-minute incubation at room temperature in the dark, 100 μL PBS was added and samples were analyzed by fluorescence-activated cell sorting (FACS).

Colony formation assay

Pre-B and CFU-C colony formation assays were performed as described previously.[16] CFUe assays were carried out following the instructions of the manufacturer (Stem Cell Technologies, Vancouver, BC, Canada).

Separation of splenocytes by MACS

Leukocyte subpopulations were isolated from single-cell suspensions of splenocytes by positive selection using FITC-conjugated antibodies against B220 (B cells), CD4 (CD4 T cells), or CD8 (CD8 T cells) and anti-FITC MACS beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the sort was checked by FACS analysis.

Southern blot analysis

Southern blot analysis was carried out as described.[16] Membranes were exposed to x-ray films and the resulting bands quantified using Bio-PROFIL Bio-1D V97.03 software (Vilber Lourmat, Marne-la-Vallée, France).

DNA microarray hybridization and analysis

Total RNA was isolated from FACS-sorted populations of thymocytes (DN, CD4$^-$CD8$^-$; DP, CD4$^+$, CD8$^+$) and splenocytes (CD19$^+$ B cells; CD4$^+$ T cells). For biotin-labeled target synthesis reactions were performed using standard protocols supplied by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, 5 μg total RNA was converted to double stranded (ds) DNA using 100 pmol of a T7T23V primer (Eurogentec, Seraing, Belgium) containing a T7 promotor. The cDNA was then used directly in an in vitro transcription reaction in the presence of biotinylated nucleotides.

The concentration of biotin-labeled cRNA was determined by UV absorbance. In all cases, 12.5 μg of each biotinylated cRNA preparation was fragmented and placed in a hybridization cocktail containing 4 biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix MOE430A for 16 hours. After hybridization, the GeneChips were washed, stained with SA-PE, and read using an Affymetrix GeneChip fluidic station and scanner. Gene expression levels were determined by means of Affymetrix's Microarray Suite 5.0 (MAS 5.0).

Results

β1 and β7 integrins are coexpressed in many hematopoietic cells, including HSCs

In order to replace each other functionally, α4β1 and α4β7 must be expressed in the same cells. While β1 integrin is expressed on all hematopoietic cells besides erythrocytes,[12,16] the expression of β7 integrin is more restricted (Figure S1, available on the *Blood*

website; see the Supplemental Figure link at the top of the online article). In BM, β7 integrin was found on lin$^-$c-kit$^+$Sca1 high cells (ie, bona fide stem cells), most mature B cells (B220 high), on subpopulations of mature and immature granulocytes (Gr-1 high; Gr-1 medium), and on few erythroid cells (Ter119$^+$) and immature B cells (B220 low). β7 integrin was furthermore found on subsets of DN, CD4SP, and CD8SP thymocytes, whereas it was virtually absent on DP thymocytes. In spleen and lymph nodes, β7 integrin was present on most B cells (B220$^+$), T cells (CD4$^+$, CD8$^+$), and granulocytes (Gr-1$^+$). In lymph nodes, about 50% of the erythroid cells (Ter119$^+$) expressed β7 integrin, whereas only a few percent of the erythroid cells in the spleen had β7 on their surface.

Normal maintenance of HSCs in the combined absence of β1 and β7 integrins

To directly assess possible redundant functions of α4β1 and α4β7, mice were generated lacking both receptors in the hematopoietic system. 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.[15] Thus, mice that were deficient for β7 integrin and carried an inducible β1-null gene ($\beta1^{fl/-}\beta7^{-/-}$Mx-cre$^+$ or $\beta1^{fl/fl}\beta7^{-/-}$Mx-cre$^+$) were obtained. Mice lacking β7, but constitutively expressing β1 ($\beta1^{fl/+}\beta7^{-/-}$Mx-cre$^+$ or $\beta1^{fl/fl}\beta7^{-/-}$) were used as controls. To restrict the deletion to the hematopoietic system, BM from these mice was transplanted into lethally irradiated recipient mice (Figure 1A). The ablation of the conditional β1 gene was induced by 3 intraperitoneal injections of polyIC after reconstitution of the hematopoietic system (4 weeks after irradiation). Mice were analyzed 2, 6, and 10 to 12 months after the polyIC treatment.

At all time points analyzed, no expression of β7 integrin was detectable in any tissue by FACS (data not shown). To analyze the time course of the β1 integrin gene ablation, we monitored the loss of β1 integrin expression on short-lived platelets. Two days after the first polyIC injection, β1-deficient platelets were already detectable in the blood of β1β7 mutant BM chimeras (Figure 1B). The relative amount of β1-deficient platelets increased continuously to reach 93% after 14 days and 97% after 21 days and later. In control BM chimeras, on the other hand, virtually all platelets expressed β1 integrin at all time points analyzed. 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 integrins. Southern blot analysis of BM, spleen, and thymus of 2- and 10-month-old β1β7 mutant and control BM chimeras confirmed the efficient β1 gene deletion in all these tissues (Figure 1C and data not shown). Since only HSCs can sustain hematopoiesis for more than 3 months, these data indicate that β1β7-deficient HSCs are maintained in vivo.

To investigate the development of different hematopoietic lineages that derive from HSCs, we first checked the cellularity of

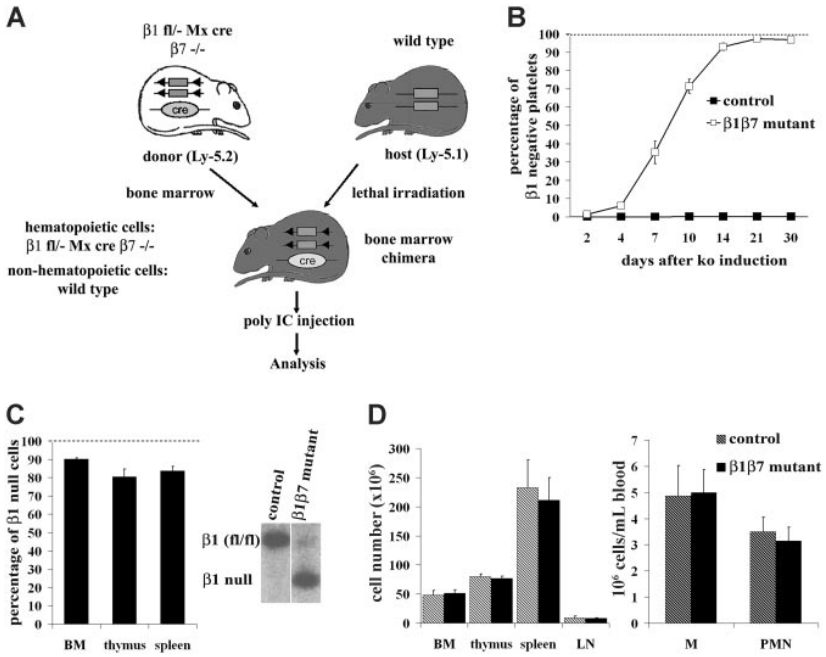

Figure 1. Efficient deletion of β1 integrin in the hematopoietic system does not affect cellularity of lymphoid organs. (A) Bone marrow of mice with a conditional knockout of β1 integrin, an Mx-cre transgene, and a constitutive knockout of β7 integrin ($\beta1^{fl/-}\beta7^{-/-}$Mx cre) was transplanted into lethally irradiated wild-type host mice. In bone marrow chimeras, donor and host cells could be distinguished by expression of Ly-5.1/Ly-5.2 surface marker. After repopulation of the hematopoietic system (4 weeks), polyIC was injected to induce expression of the cre recombinase in the donor cells and deletion of the β1 integrin gene. (B) Percentages of β1 integrin–negative platelets isolated from control and β1β7 mutant BM chimeric mice are shown at indicated time points after the first of 3 polyIC injections (day 0). Error bars show the standard deviation (n [control BM chimera]/[β1β7 mutant BM chimera]: 9/6). (C) 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 [control BM chimera]/[β1β7 mutant BM chimera]: 3/3). (D) Left panel: Single-cell suspensions were made from BM (2 femurs), thymus, spleen, and lymph nodes (2 inguinal, 2 axial, 1 para-aortic) of control and β1β7 mutant BM chimeric mice 2 months after the gene deletion. Cells were counted using a hemacytometer. The bar graph shows the absolute cell number in the respective tissues. Error bars show the standard deviation (n [control BM chimera]/[β1β7 mutant BM chimera]: 4/4). Right panel: Whole blood was collected retro-orbitally from control 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 hemacytometer. The bar graph shows the concentration of mononuclear (M) and polymorphonuclear (PMN) cells in the blood. Error bars show the standard deviation (n [control BM chimera]/[β1β7 mutant BM chimera]: 4/4).

different lymphoid organs. At 2 months (Figure 1D, left) and 10 to 12 months (data not shown) after induction of the gene deletion there were no differences observed in the cellularity of BM, thymus, or spleen of control and β1β7 mutant BM chimeras, providing no evidence for defective hematopoiesis in the absence of β1 and β7 integrins. Differential blood counts revealed similar numbers of mononuclear and polymorphonuclear cells in the peripheral blood (PB) of control and β1β7 mutant BM chimeras 6 months after polyIC treatment (Figure 1D, right).

Normal B-cell development in the absence of β1 and β7 integrins

Since previous studies suggested that normal B-cell development was dependent on α4 integrin,[13,14,17] but not on α4β1[16] or α4β7[15] alone, we investigated whether β1 and β7 integrins have a redundant function in B-cell development. In pre-B colony assays, control and β1β7 mutant BM gave rise to colonies that were derived each from a single pre-B-cell pecursor. FACS analysis of randomly picked colonies confirmed that 36 of 39 colonies (92.3%) of β1β7 mutant BM did not express β1 integrins, whereas all tested colonies derived from control BM expressed β1 integrins. No host-derived colonies expressing Ly-5.1 were detected. To further monitor B-cell development, single-cell suspensions from BM, spleen, and lymph nodes (LNs) were analyzed using B-cell–specific markers: B220 (pre-proB and later), CD19 (proB and later), IgM (immature B), and IgD (all mature B). The relative amount of cells positive for the respective markers was unaltered in β1β7 mutant BM chimeras compared with control BM chimeras 2 and 12 months after the knockout induction (Table 1 and data not shown).

FACS analysis of immature B cells (B220 medium) proved the loss of β1 integrin (Figure 2A). Mature B cells (B220 high) express only low amounts of β1 integrin, which makes it difficult to distinguish normal from β1-deficient mature B cells by FACS (Figure 2A). Therefore, the knockout efficiency in B220$^+$ B cells purified from spleen was determined by Southern blot analysis (Figure 2B). B220$^+$ B cells were enriched by MACS beads to a purity of more than 95% (Figure 2B, left). Southern blot of genomic DNA isolated from these cells revealed a deletion efficiency of the β1 integrin gene of 93.5% ± 8.3% (n = 5). These data indicate that in the absence of β1 and β7 integrins, B cells can fully mature. Furthermore, since spleen, LN, and BM contained normal numbers of B cells, migration of immature B cells to spleen and of mature B cells to LN and BM is apparently not impaired by the combined loss of β1 and β7 integrins.

Table 1. B-cell population sizes

	Population size	
	Control	β1β7 mutant
BM		
B220^{med}	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		
B220^{hi}	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		
B220^{hi}	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

For all populations, n = 4. Single-cell suspensions from BM, spleen, and LN of control and β1β7 mutant BM chimeras 2 months after polyIC treatment were prepared; stained with antibodies against B220, CD19, IgM, IgD (med indicates medium; hi, high); and β1 integrin and analyzed by FACS. The averages of the population sizes in the respective tissues are shown with standard deviations.

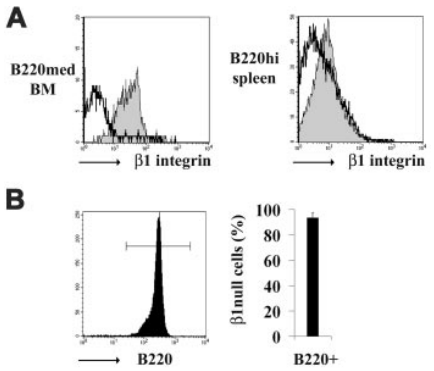

Figure 2. Normal B-cell population sizes in the absence of β1 and β7 integrins. (A) Representative histogram overlays show the β1 integrin expression on immature (B220med) and mature (B220hi) B cells of BM and spleen, respectively, of control (filled) and β1β7 mutant BM chimeras (line). (B) Single-cell suspensions from spleen of control 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. Left panel: FACS analysis of the B220$^+$-enriched fraction indicated higher than 95% purity (representative histogram is shown). Right panel: DNA was prepared from MACS-enriched B220$^+$ splenocytes, analyzed by Southern blot, and densitometrically 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 [control BM chimera]/[β1β7 mutant BM chimera]: 5/5).

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

Since in α4-null somatic chimeric mice α4-null T-cell precursors were described to be unable to migrate to the thymus for further differentiation, thymocyte development was analyzed in β1β7 mutant BM chimeric mice using the T-cell markers CD4 and CD8. No significant difference was found in the population sizes of CD4$^-$CD8$^-$ (DN) thymocytes, which contain the early thymic immigrants indicating that thymic colonisation was not altered in β1β7 mutant BM chimeric mice which lack both α4β1 and α4β7 integrins. Furthermore, the relative amounts of CD4$^+$CD8$^+$ (DP), CD4$^+$ (CD4SP), and CD8$^+$ (CD8SP) cells in the thymus were normal in β1β7 mutant BM chimeric mice (Figure 3A and Table 2). Staining of thymocytes for β1 integrin and subsequent FACS analysis proved the absence of β1 integrin from DP T cells (Figure 3B). Normal numbers of CD4 and CD8 T cells in spleen, LN, and BM of β1β7 mutant BM chimeras 2 and 12 months after induction of the β1 gene deletion suggested normal migration of these cells to secondary lymphoid organs and to the BM (data not shown). Since mature CD4$^+$ and CD8$^+$ T cells express only low levels of β1 integrin, the deletion efficiency in these populations was tested on the genomic level. Southern blot from CD4$^+$ and CD8$^+$ T cells, enriched from the spleen, revealed that 78.5% ± 5% (n = 3) of CD4$^+$ T cells and 83.4% ± 10.8% (n = 4) of CD8$^+$ T cells lacked a functional β1 integrin gene (Figure 3C). These data show that β1 and β7 integrins are neither essential for the migration of T-cell precursors to the thymus nor for T-cell maturation within the thymus.

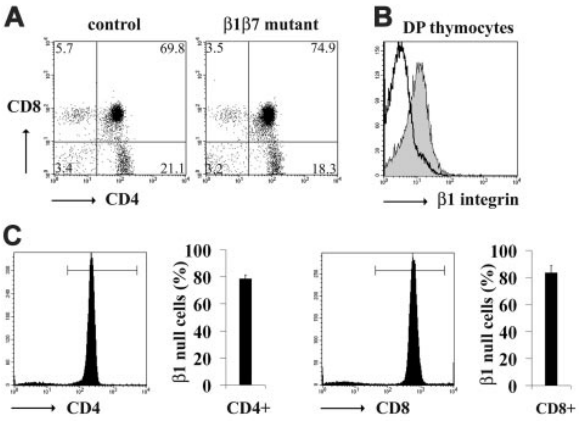

Figure 3. β1 and β7 integrins are not essential for normal T-cell development. Single-cell suspensions from thymus of control and β1β7 mutant BM chimeras 2 months after polyIC injection were prepared, stained with antibodies against CD4, CD8, and β1 integrin, and analyzed by FACS. (A) The dot blots show a representative staining of DN, DP, CD4SP, and CD8SP thymocytes for both control and β1β7 mutant BM chimeras. (B) A representative histogram displays β1 integrin expression on DP T cells of control (filled) and β1β7 mutant (line) mice. (C) Single-cell suspensions from spleen of control and β1β7 mutant BM chimeras 6 months after polyIC treatment were prepared, stained with CD4-FITC or CD8-FITC antibody, and subsequently sorted using anti-FITC MACS beads. FACS analysis of the $CD4^+$- or $CD8^+$-enriched fraction indicated higher than 95% purity (representative histogram is shown). DNA was prepared from MACS-enriched $CD4^+$ or $CD8^+$ splenocytes and analyzed by Southern blot and densitometrically evaluated. The bar graphs show the relative amount of $CD4^+$ or $CD8^+$ cells deficient for a functional β1 integrin gene. Error bar shows the standard deviation (n [control BM chimera]/[β1β7 mutant BM chimera]: $CD4^+$, 3/3; $CD8^+$, 4/4).

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

To analyze myeloid development, we first studied the capacity of myeloid progenitors in control and β1β7 mutant BM chimeras lacking α4β1 and α4β7 integrins to form colonies in vitro (CFU-C). All CFU-C colonies analyzed from BM (n = 37), spleen (n = 38), and PB (n = 36) from control mice were positive for β1 integrin. 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. These results show first, that in the absence of β1 and β7 integrin granulocyte/monocyte precursors have the potential to form colonies in vitro and second, that the efficiency of the β1 integrin gene deletion is very high in the myeloid lineage. Both control and mutant BM cells also formed erythroid colonies (CFUe's) in vitro. Of 42 colonies tested from mutant BM, none showed a functional β1 integrin gene as tested by genomic PCR, whereas in 24 of 24 colonies from control BM a functional β1 gene was detected.

Monitoring the development of monocytes, granulocytes, and erythroblasts in β1β7 mutant BM chimeric mice 2 and 12 months after the β1 integrin gene deletion in vivo revealed no significant differences in the numbers of granulocytes, monocytes, and erythroblasts, indicating no developmental defects in the absence of both α4β1 and α4β7 integrins (Table 3 and data not shown). The β1 gene deletion on these cells was confirmed by staining for β1 integrin and subsequent FACS analysis (Figure 4). These data strongly suggest that HSCs and HPCs continuously provide myeloid and erythroid cells in the absence of α4β1 and α4β7 integrins.

Table 2. Thymic T-cell population sizes

Thymus subpopulation	Population size	
	Control	β1β7 mutant
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

For both populations, n = 4. The averages and standard deviation of the population sizes are shown in the table for the respective subpopulations.

Table 3. Myeloid and erythroid cell populations in the BM

BM subpopulation	Population size	
	Control	β1β7 mutant
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

For both populations, n = 4. Single-cell suspensions from BM of control and β1β7 mutant BM chimeras 2 months after polyIC treatment were prepared; stained with antibodies against Gr-1, Mac-1, and Ter119 in combination with antibodies against β1 integrin; and analyzed in FACS. The table shows the relative size of granulocyte (Gr-1), granulocyte/monocyte (Mac-1), and erythroblast (Ter119) subpopulations and standard deviation.

Emigration of granulocyte/monocyte progenitors from the BM to the blood and spleen

Induced deletion of α4 integrin in hematopoietic and many nonhematopoietic cells resulted in a slow increase of CFU-Cs in the BM, an overproportional release into the PB, and an accumulation of CFU-Cs in spleen, suggesting a role for α4 in the retention of progenitor cells in the BM.[17] To test this in β1β7 mutant BM chimeras, we determined the frequency of CFU-C progenitors in BM, PB, and spleen 2 and 10 months after the β1 integrin gene deletion (Figure 5A). At 2 months, the number of precursor cells was significantly elevated in the BM of β1β7 mutant mice as compared with controls. We also observed an increase of progenitors in PB roughly proportional to the progenitor increase in the BM, but significantly less than reported for α4 conditional knockout mice 8 weeks after induced gene deletion, thus not indicating a severe defect in progenitor retention in the BM. Furthermore, these alterations were transient, since they were observed 2 months but not 10 months after knockout induction, when β1β7 mutant and control mice had similar CFU-Cs, both in BM and PB (Figure 5A). Unlike the conditional α4 integrin knockout mice 2 weeks and 6 months after gene deletion,[17] β1β7 mutant BM chimeras did not accumulate precursor cells over time in the spleen, as tested 2 and 10 months after the knockout induction (Figure 5A). To the contrary, CFU-Cs were significantly decreased in 10-month-old mutant chimera.

FACS analysis of BM cells of nonchimeric ($β1^{fl/fl}$Mx-cre^+) mice 3 days after a single polyIC injection revealed that the β1 integrin gene is not only deleted on most hematopoietic cells

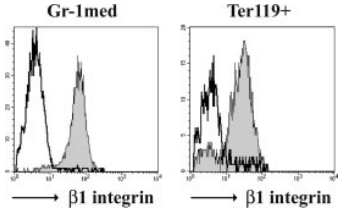

Figure 4. β1 and β7 integrins are not essential for myeloid and erythroid development. The β1 integrin expression of immature granulocytes (Gr-1med) and erythroblasts ($Ter119^+$) of control (filled) and β1β7 mutant BM chimeras (line) is shown in representative histogram overlays.

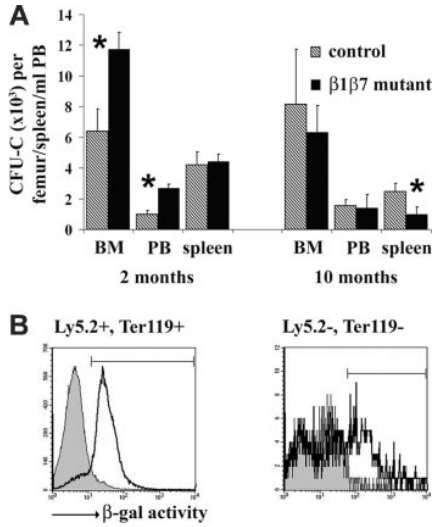

Figure 5. No overproportional release of CFU-Cs from the BM into the PB. (A) Single-cell suspensions were made from BM and spleen, and erythrocyte-depleted blood cells (PB) from control and β1β7 mutant BM chimeras were prepared at indicated times after the gene deletion. BM cells (180 000), splenocytes (3 600 000), and PB (250 μL) were seeded into MethoCult GF M3534 medium and counted 7 days later. Total numbers of colonies per femur, spleen, and mL PB are shown. Error bars show the standard deviation, star indicates significant difference ($P < .05$) (n (control BM chimera)/(β1β7 mutant BM chimera): 2 months 5/5, 10 months 3/3). (B) Single-cell suspensions from BM of nonchimeric control ($\beta1^{fl/fl}$Mx-cre$^-$) and $\beta1^{fl/fl}$Mx-cre$^+$ mutant mice were plated on tissue-culture plastic dishes. After 24 hours, nonadherent cells were removed and adherent cells detached. Adherent and nonadherent cells were then stained for Ly-5.2 and Ter119 and tested for β-galactosidase activity by an FDG assay as described in "Materials and methods." Since loss of β1 integrin results in expression of the β-galactosidase reporter,[12] high β-galactosidase activity indicates deletion of the β1 gene. Representative histogram overlays show the β-galactosidase activity on hematopoietic (Ly-5.2$^+$ or Ter119$^+$) and nonhematopoietic (Ly-5.2$^-$, Ter119$^-$) cells of control (filled line) and mutant mice (line). The marked region on the overlay indicates cells with high β-galactosidase activity. PolyIC injection induced an efficient deletion of the β1 integrin gene on hematopoietic cells; about 86% of the (Ly-5.2$^+$ or Ter119$^+$) cells of the mutant mice showed high green fluorescence, compared with only 6% of the corresponding cells of the control mice. Also, among the nonhematopoietic BM cells (Ly-5.2$^-$, Ter119$^-$) the percentage of cells with high β-galactosidase activity increased from less than 5% in control to more than 42% in mutant, clearly indicating the presence of β1 integrin–deficient nonhematopoietic cells in the BM of mutant mice.

(Figure 5B, Ly-5.2$^+$, Ter119$^+$), but also on many nonhematopoietic BM stroma cells, defined as (Ly-5.2$^-$, Ter119$^-$) plastic adherent cells (Figure 5B). To assess whether loss of β1 and β7 integrin on nonhematopoietic cells might contribute to the progenitor release, the frequency of progenitor cells was determined in the PB of nonchimeric β1β7 mutant mice 4 weeks after the knockout induction. We found that the progenitor content in PB increased approximately 8-fold in β1β7 mutant mice (data not shown), comparable to the more than 10-fold increase of the CFU-Cs in α4 conditional knockout mice 4 weeks after gene deletion,[17] indicating that loss of α4 integrin on nonhematopoietic cells might contribute to the release of CFU-C progenitors from BM to PB.

Expansion of erythrocyte precursors after hemolytic anemia

After phenylhydrazine (PHZ) induced lysis of erythrocytes in vivo, erythroid precursor cells expand in order to compensate for the loss of erythrocytes. In addition, hemolytic anemia promotes extramedullary erythropoiesis leading to proliferation of progenitors in the spleen.[25] Since in α4 conditional knockout mice the ability of erythroblasts to expand in response to a PHZ-induced hemolytic anemia was reduced,[17] we investigated whether combined loss of β1 and β7 integrins shows a similar effect. For better comparison with the nonchimeric α4 conditional knockout mice we used nonchimeric β1β7 mutant mice.

Two days after PHZ treatment the amount of erythrocytes dropped in both control and β1β7 mutant mice by more than 55% in BM (n = 3) and was not significantly different between both groups. Similarly, also the number of erythroblasts in the BM as assessed by Ter119 staining was reduced after the PHZ treatment but comparable between β1β7 mutant mice and controls (Figure 6A). Since α4 conditional knockout mice were reported to have fewer erythroid progenitor cells in the BM after hemolytic stress, we tested at the same time point (ie, 2 days after PHZ treatment) the relative amounts of cells of different erythroid developmental stages by Ter119-CD71 staining and subsequent FACS analysis separating different maturation stages of BM erythroblasts[18] (Figure 6B). Neither in BM nor in spleen was a significant difference detected between β1β7 mutant and control mice at any of these stages, providing no evidence for an impaired recovery from hemolytic anemia in the absence of β1 and β7 integrin (Figure 6C and data not shown). Analysis of CFUe's confirmed a normal frequency of erythroid progenitors in BM, but surprisingly revealed a significant reduction of CFUe's in the spleen of β1β7 mutant mice compared with controls. Since the spleen is the most prominent place for hematopoiesis after PHZ treatment these data support a role for α4β1 and α4β7 integrin in the recovery of the erythropoietic system after hemolytic anaemia.

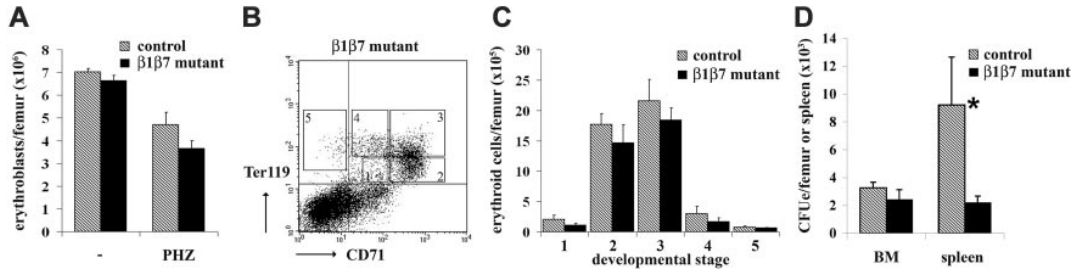

Figure 6. Reduced number of splenic CFUe's after hemolytic stress. (A) Single-cell suspensions from BM of control and β1β7 mutant mice (no BM transplantation) untreated and treated at day 1 and 2 with PHZ were prepared at day 4, stained with Ter119 antibody, and subsequently analyzed by FACS. The total amount of Ter119$^+$ erythroblasts per femur is shown. Error bars show the standard deviation (n (controls)/(β1β7 mutants): 4/4). (B) BM single-cell suspensions from PHZ treated β1β7 mutant BM chimeras were prepared, stained with antibodies against α4, β1 integrin, CD71, and Ter119 and subsequently analyzed by FACS. (Ter119-CD71 staining distinguishes 5 different developmental stages.) (C) The amount of erythroid BM cells of PHZ-treated β1β7 mutant mice for each of the 5 developmental stages as distinguished by Ter119 and CD71 is shown. Error bars show the standard error (n (controls)/(β1β7 mutants): 4/4). (D) Single-cell suspensions from BM of control and β1β7 mutant mice (no BM transplantation) treated at day 1 and 2 with PHZ were prepared at day 4 and tested for CFUe's. Total numbers of colonies per femur and spleen are shown. Error bar shows the standard error (n (controls)/(β1β7 mutants): 3/3).

No apparent compensatory change in gene expression in the combined absence of β1 and β7 integrin in different leukocyte subsets

To investigate whether hematopoietic subpopulations of β1β7 mutant mice show major alterations in gene expression, we tested mRNA levels of different hematopoietic subsets (DN, DP, B cells, $CD4^+$ T cells) by array analysis. RNA was prepared from DP and DN cells from the thymus, and $CD19^+$ (B cells) and $CD4^+$ cells from the spleen, obtained from 5 pooled mutant and control mice, respectively, and tested on affymetrix chips. All mutant mice had an efficient knockout of β1 integrin indicated by a loss of surface β1 integrin on more than 97% of the platelets.

We then analyzed the data by searching for genes that are up- or down-regulated in mutant mice in all 4 different populations investigated, which would suggest a crucial compensatory response. However, only 3 genes encoding heat shock proteins (heat shock protein 1α, heat shock protein 1β, heat shock protein 105) were found with increased expression in the absence of β1 and β7 integrin. No genes were found with reduced expression in all subpopulations derived from mutant mice.

We then screened the genes up- or down-regulated in the individual hematopoietic subpopulations (thymus: DN, DP; spleen: B cells, $CD4^+$ T cells) for integrins (β3-β6, α2-α10, αX, αD, αM, αL, αE), selectins (P-, L-, E-), CD44, and for the α4β1 and α4β7 integrin ligands VCAM-1 and MAdCAM-1. All these genes showed normal expression in β1β7 mutant cells compared with control cells.

Discussion

It has been reported that several classes of integrins play an important role to attach hematopoietic stem cells and progenitor cells as well as their differentiated lineages to the extracellular matrix and to other cells.[1] α4 integrins, for example, have been suggested to be crucial for the retention of hematopoietic stem cells in the bone marrow, for the homing of lymphocytes to Peyer patches and for the migration of T cells during inflammation.[13,15,26] In addition, development of the hematopoietic system, characterized by the formation of the different blood cell lineages and their distribution within hematopoietic organs, was reported to be α4 integrin–dependent, although the gradual contribution of α4 integrins differed significantly depending on the experimental approach.[7,17] To study the role of α4 integrins in hematopoiesis, mouse models were applied in which the α4 integrin gene was deleted on hematopoietic as well as on nonhematopoietic cells. Loss of the α4 integrins on the latter cell population might affect hematopoietic development. To overcome this problem and to assess by an alternative approach the function of α4 integrins in hematopoiesis, we decided to generate and analyze mice, which lack β1 and β7 integrins, and hence both α4β1 and α4β7 integrins, exclusively in the hematopoietic system. Unexpectedly, we could not find an essential function for α4β1 and α4β7 integrins in blood cell development or in progenitor retention in the bone marrow. Detailed analysis of lymphoid and myeloid development by testing the size of different blood cell subsets in lymphoid organs at different time points and investigating the formation of pre-B and CFU-C colonies did not reveal any impairment in the hematopoiesis of β1β7 mutant mice.

The only abnormality we observed in untreated mutant BM chimeras was an increase in the amount of CFU-C precursors in the bone marrow coupled with a proportional increase in the number of CFU-C progenitor cells in the peripheral blood. This impairment indicates a role of α4 integrins in the maintenance of HPCs. Whether the increase in BM CFU-Cs is due to elevated proliferation, decreased cell death, or changed migratory behavior of progenitor cells is currently unclear. The increase, however, was only transient, since it was detected 2 months after knockout induction, but not 8 months later, pointing to compensatory mechanisms that kick off in the β1β7 mutant mice leading to a reduction of progenitor numbers in BM and PB back to normal levels. Alternatively, it is possible that transplantation-dependent effects affect β1β7 mutant and control mice with different efficiencies, thus contributing to the reduction of CFU-Cs. While in α4 integrin conditional knockout mice HPCs accumulate in spleen,[17] this was not the case in β1β7 mutant BM chimeras. To the contrary, relative to PB the number of HPCs in spleen was decreased in young and old β1β7 mutant BM chimeras, which might indicate a migration defect of β1β7 mutant HPCs to the spleen. Interestingly, no increased CFU-Cs were observed in β1-null BM chimera.

Induction of hemolytic anemia revealed a reduced number of splenic CFUe's in β1β7 BM chimera, indicating a role for α4β1 and α4β7 integrin in erythroid recovery after stress. Also, α4 mutant mice showed an impaired recovery after PHZ-induced hemolysis, although in that case the frequency of CFUe's was reduced in BM and not in spleen.[17]

Different explanations might be possible to reconcile the published data with the findings of our investigations. First, in α4-null somatic chimera α4 integrin is lost already before the development of hematopoietic stem cells, whereas in our system α4β1 and α4β7 are lost in adult animals 1 month after bone marrow transplantation. It is possible, therefore, that the absence of these integrins during the entire embryogenesis impairs development of the hematopoietic system and causes defects that persist to adulthood, whereas the loss of α4 in adult animals has no severe consequences for hematopoiesis. However, recent data by Gribi et al[27] showed that transplantation of fetal α4-null hematopoietic stem cells derived from the aorta-gonad-mesonephros (AGM) region of α4-deficient embryos into adult microenvironment results in long-term generation of mature B and T lymphocytes and myeloid cells. These data indicate that neither fetal nor adult HSCs require α4 integrins for hematopoietic development.

Second, the defects observed in α4 integrin conditional knockout mice might be related to the fact that in this model α4β1 and α4β7 integrin are lost also on nonhematopoietic cells, whereas deletion of the β1 and β7 integrin in our BM chimeras was restricted to the hematopoietic system. Interference with the functions of α4 integrins in nonhematopoietic tissues such as BM stroma cells or endothelial cells might result in the production of cytokines, which inhibit lymphopoiesis or favor the mobilization of HPCs. We tested this possibility and found that nonchimeric β1β7 mutant mice, which lack α4β1 and α4β7 integrin with a similar tissue distribution as the α4 conditional knockout mice, do show an overproportional release of HPCs into the peripheral blood. Furthermore, we could demonstrate that induction of Cre in these mice disrupts the β1 integrin gene in BM stromal cells. Altogether, these data underline the importance of a strict tissue-restricted gene deletion in order to avoid unwanted cross-talk between different tissues.

In summary, we demonstrate that α4β1 and α4β7 integrins are not crucial for the differentiation of lymphoid and myeloid cells in

adult mice. In fact, even the additional loss of all other β1 integrin receptors besides α4β1, as occurring in our β1β7 mutant mice, did not prevent hematopoietic development. β1 and β7 integrins affect maintenance and distribution of CFU-C progenitors, though differently than previously suggested for α4 integrins.[17] Our results do not exclude effects of α4β1 and α4β7 integrins on fetal hematopoiesis, during immune response or in inflammation.

Acknowledgments

We thank Dr Bernhard Nieswandt for generously providing antibodies, Dr Robert Geffers for generating array data, Dr Lothar Gröbe for flow cytometry assisstance, and Dr Alexander Medvinsky for discussion and sharing unpublished data.

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Paper III

RhoH is important for positive thymocyte selection and T-cell receptor signaling

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RhoH is a small GTPase expressed only in the hematopoietic system. With the use of mice with targeted disruption of the *RhoH* gene, we demonstrated that RhoH is crucial for thymocyte maturation during DN3 to DN4 transition and during positive selection. Furthermore, the differentiation and expansion of DN3 and DN4 thymocytes in vitro were severely impaired. These defects corresponded to defective TCR signaling. Although RhoH is not required for TCR-induced activation of ZAP70 and ZAP70-mediated activation of p38, it is crucial for the tyrosine phosphorylation of LAT, PLCγ1, and Vav1 and for the activation of Erk and calcium influx. These data suggest that RhoH is important for pre–TCR and TCR signaling because it allows the efficient interaction of ZAP70 with the LAT signalosome, thus regulating thymocyte development. (Blood. 2007;109:2346-2355)

Introduction

RhoH is a member of the Rho GTPase family, expressed only in the hematopoietic system. Rho GTPases are small GTPases that regulate cytoskeletal organization, proliferation, survival, and cell polarization.[1] They are present in an active, GTP-bound form and an inactive, GDP-bound form. Only in the GTP-bound conformation can they interact with a wide range of effector molecules, including serine-threonine kinases, lipid kinases, and cytoskeletal proteins.

RhoH was shown to be expressed in hematopoietic progenitor cells (HPCs), lymphocytes, and neutrophils.[2,3] Because RhoH has no functional intrinsic GTPase activity, it is thought to be constitutively active and controlled only at the transcriptional level. Indeed, RhoH expression is regulated in lymphocytes.[2] In T cells, activation of the T-cell receptor reduced the RhoH message within a few hours, whereas PMA treatment decreased RhoH mRNA levels in Jurkat cells within 60 minutes.[2] In diffuse large B-cell lymphoma, *RhoH* is frequently mutated in the noncoding region, which might affect mRNA stability or translation efficiency.[4,5] Functionally, RhoH was proposed to be a negative regulator of Rho GTPases and integrins. RhoH inhibits Rac1-mediated activation of p38 MAPK and NFκB in Jurkat cells but has no effect on JNK or Erk or on the activation of Rac1 in these cells.[2] In hematopoietic stem cell (HSC)/HPC preparations, RhoH negatively regulates the proliferation, survival, and migration and reduces the SCF-induced activation of Rac1.[3] Finally, RhoH expression maintains lymphocytes in a nonadhesive state by decreasing integrin-mediated attachment.[6]

These observations suggested an important role of RhoH in the maintenance of HSCs, in leukocyte migration, and possibly in the development of B-cell lymphoma. To test these hypotheses in vivo, we generated mice that lacked a functional *RhoH* gene and analyzed the development of different hematopoietic lineages. RhoH-null mice did not develop lymphoma and had no obvious defects in HSC maintenance, but they showed impaired T-cell differentiation attributed to defective T-cell receptor (TCR) signaling.

Materials and methods

Mice

RhoH-deficient mice were generated using procedures described previously.[7] As a targeting vector, a 6.9-kb *Eco*RI-*Eco*RI genomic DNA fragment was used in which a 0.9-kb *Hin*dIII-*Aat*II fragment encoding the first 42 amino acids of the RhoH protein was replaced by a neomycin resistance expression cassette. Wild-type and heterozygous knockout mice were used as controls, with indistinguishable results in all assays. If not stated otherwise, mice were kept as 129Sv/C57BL6 outbreds. β2-integrin[8]–deficient mice and RhoH-null mice were intercrossed to obtain RhoH-β2-null double-knockouts. RhoH mutant mice backcrossed for 6 generations to C57BL6 were mated with OT-II transgenic mice[9] to obtain RhoH-null/OT-II mice. All mice were kept in a barrier facility in accordance with the German policies on animal welfare.

Flow cytometry

Single-cell suspensions were prepared by gentle disaggregation of the dissected organs through 70-μm cell strainers. Cells were stained with antibodies (all BD PharMingen [San Diego, CA] unless otherwise specified) conjugated to FITC, PE, APC, or biotin in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and analyzed on a FACSCalibur with CellQuest software. Biotinylated antibodies were detected by streptavidin-Cy-5 (Jackson ImmunoResearch, West Grove, PA) or streptavidin-Cy-Chrom. Dead cells were excluded by forward-scatter and side-scatter profiles and by 1 μg/mL propidium iodide counterstaining. The following

Submitted April 25, 2006; accepted November 2, 2006. Prepublished online as *Blood* First Edition Paper, November 21, 2006; DOI 10.1182/blood-2006-04-019034.

The online version of the article contains a data supplement.

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antibodies were used: anti-B220 (RA3-6B2), anti-IgM (R6-60.2), anti-IgD (11-26c.2a), anti–Gr-1 (RB6-8C5), anti-Mac1 (M1-70), anti-Ter119, anti–NK1.1 (PK136), anti-CD4 (H129.19) (GK1.5; eBioscience, San Diego, CA), anti-CD8 (53-6.7), anti-CD5 (53-7.3), anti-TCRβ (H57-597), anti-CD69 (H1.2F3), anti-Vα2 (B20.1), anti-TCRγδ (GL3), and anti-CD62L (MEL-14; eBioscience). For the characterization of double-negative (DN; Lin^- $CD4^-CD8^-$) thymocytes, cells were gated for lineage-negative (Lin^-) (B220, CD4, CD8, NK1.1, Mac1, Gr-1, Ter119) cells and analyzed for the expression of CD25 (7D4) and CD44 (IM7).

For proliferation assays, mice were given intraperitoneal injections of 1 mg BrdU and were killed 2 hours later. BrdU incorporation of thymocytes was determined using the BrdU Flow kit. AnnexinV staining was carried out in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) using Annexin V–Alexa 488 (kindly provided by Dr E. Pöschl, Erlangen, Germany).

T-cell development in vitro

In vitro T-cell development on OP9-DL1 stromal cells was carried out as described previously.[10] OP9-DL1 cells were maintained in α-MEM medium (Invitrogen, Carlsbad, CA) containing 20% FCS and penicillin/streptomycin. Control and $RhoH^{-/-}$ DN thymocytes enriched through MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) were sorted for DN3 and DN4 populations with FACSAria. DN3 (5×10^4) and DN4 (2.5×10^4) thymocytes were plated onto a subconfluent OP9-DL1 monolayer in a 24-well plate and cultured in the presence of 5 ng/mL Flt3 ligand (Peprotech, Rocky Hill, NU) and 1 ng/mL IL-7 (Peprotech) for 8 days. The medium with cytokines was replaced after 4 days. Developmental progression of cultured cells was assessed by flow cytometry after 4 and 8 days. Cells were harvested by forceful pipetting and filtered through a 70-μM cell strainer to remove OP9-DL1 cells.

TCR signaling

Freshly isolated thymocytes (0.5×10^7 to 1×10^7) were incubated with 5 μg/mL biotinylated anti-CD3ε (145-2C11) and anti-CD4 or anti-CD3ε alone for 20 minutes on ice and washed. Cross-linking of the bound antibodies was carried out with the addition of prewarmed streptavidin (5-10 μg/mL; Sigma, St Louis, MO) and incubation of the samples for 5 minutes at 37°C. After stimulation, 5×10^6 cells were directly fixed in an equal volume of 4% paraformaldehyde in PBS for 10 minutes at 37°C, washed with PBS, and permeabilized in ice-cold 90% methanol for 30 minutes on ice. Cells were then washed twice in PBS, incubated with fluorescence-labeled anti-CD4, anti-CD8, and anti–phospho-ZAP70 (Y319)/Syk (Y352), anti–phospho-Lck (Y505), anti–phospho-Erk1/2 (T202/Y204), or anti–phospho-p38 MAPK (T180/Y182) for 1 hour at room temperature, and analyzed by FACS.

Double-positive (DP) thymocytes (0.5×10^7 to 1×10^7) sorted by FACSVantage were stimulated as described, washed with cold PBS, and lysed in 100 μL 50 mM Tris pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 2 mM $MgCl_2$, 1 mM Na_3VO_4, and 100 mM NaF containing a protease inhibitor cocktail (Complete Mini, EDTA free; Roche, Basel, Switzerland) for 20 minutes on ice. Lysates were cleared by centrifugation at 20 800 *g* for 15 minutes at 4°C and immunoprecipitated with anti-LAT (Upstate Biotechnology, Lake Placid, NY) or anti-Vav1 (C-14; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies bound to protein A agarose (Sigma-Aldrich, St Louis, MO). Phosphorylation of the immunoprecipitated proteins was detected by Western blotting using anti–phosphotyrosine PY-7E1 (Zymed, San Francisco, CA) antibody. Total levels of the immunoprecipitated proteins were determined by reprobing the blots with anti-LAT (11B.12; Upstate Biotechnology) or anti-Vav1 (D-7; Santa Cruz Biotechnology) antibodies. Aliquots (20 μL) of cell lysates were analyzed by Western blotting using the following antibodies: anti–phospho-ZAP70(T319) (Cell Signaling, Beverly, MA), anti-ZAP70 (99F2; Cell Signaling), anti–phospho-Vav2(T172) (Santa Cruz Biotechnology), anti-Vav2 (H-200; Santa Cruz), anti–phospho-LAT(T191; Cell Signaling), anti-LAT (11B.12; Upstate Biotechnology), anti–phospho-PLCγ1(T783) (Cell Signaling), anti-PLCγ1 (Cell Signaling), anti–phospho-p44/p42 MAPK (T202/Y204) (New England BioLabs, Ipswich, MA), anti–p44/p42 MAPK (New England BioLabs). Pull-downs for active Rac1 and Rac2 were performed as previously described[11] with 0.5×10^7 to 1×10^7 DP sorted thymocytes stimulated as indicated by cross-linking of biotinylated CD3 and CD4 antibodies with streptavidin for 30 seconds or 1 minute and 5 minutes at 37°C. Total lysates and precipitates were analyzed by Western blotting with the use of antibodies against Rac1 (Transduction Laboratories, Lexington, KY) and Rac2 (Upstate Biotechnology).

For the detection of phosphorylated Vav2 and active Rac2, DP thymocytes were sorted by positive selection using anti-CD8-FITC and MACS anti-FITC microbeads (Miltenyi). The purity of obtained DP thymocytes was 93% to 97%, as determined by flow cytometry.

For measurement of calcium influx, 2×10^6 thymocytes were loaded with 2.5 μM Fluo-4 (Molecular Probes, Eugene, OR) for 25 minutes at 37°C and washed twice with RPMI 1640, 25 mM Hepes, without phenol red (Gibco, Grand Island, NY). Cells were stained on ice with 5 μg/mL anti–CD8α-APC (53-6.7; eBioscience), anti–CD4-PE-Cy5.5 (RM4-5; Caltag, Burlingame, CA), and biotinylated anti-CD3ε. Cells were warmed to 37°C and analyzed for 20 seconds to establish baseline calcium levels. Then CD3ε was cross-linked by the addition of 10 μg/mL streptavidin. Flow cytometric analysis was performed with FACSCalibur using FlowJo software (TreeStar, Ashland, OR).

Adhesion assays

Adhesion assays to ICAM-1, VCAM-1, and the endothelioma cell lines bEnd5 and bEndI1.1[12] (kindly provided by Dr Britta Engelhardt, University of Bern, Switzerland) were carried out as described previously[12,13]; 2×10^4 endothelioma cells were used per well.

Statistical analysis

All mean values are shown with standard deviation. Student *t* test was performed to assess the significance of observed differences.

Results

Generation of RhoH-deficient mice

To generate mice with targeted disruption of the *RhoH* gene, we replaced the genomic sequence encoding the translation start and the switch1 region, which is crucial for the interaction with GEFs or GAPs[14] effectors, by a neomycin expression cassette using homologous recombination (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Mutation of the *RhoH* gene was confirmed by Southern blot analysis and genomic PCR (Figure S1B-D). Northern blot analysis indicated the expression of a long transcript in RhoH mutant mice that contained the sequence of the inverted neomycin resistance gene, with at least 6 Kozak box ATGs followed by an in-frame stop codon and the truncated RhoH coding region (Figure S1E). These open-reading frames should prevent any expression of a truncated RhoH molecule. RhoH mRNA levels in heterozygous mutant mice were approximately 50% those of homozygous wild-type mice.

Because of the lack of functional antibodies, confirmation of the loss of RhoH on protein level was not possible. Homozygous mutant mice were born at Mendelian ratio (+/+, 23.2%; +/−, 50.8%; −/−, 26.0%; n = 214), indicating no embryonic lethality. RhoH-null mice were fertile and were of normal weight and life span.

Hematopoiesis

RhoH is expressed in HPCs, lymphoid cells, and myeloid cells.[2,3] We analyzed the development of different blood lineages in mutant and control mice. In 2-month-old mice, BM cellularity was not

Table 1. Absolute sizes of myeloid, erythroid, and lymphoid populations in hematopoietic organs

Hematopoietic organ	Control, × 10^6 cells		RhoH$^{-/-}$, × 10^6 cells		
	Mean	SD	Mean	SD	*P*
Bone marrow					
Mac1$^+$ Gr-1$^+$	14.849	2.357	14.889	1.501	—
Mac1$^+$ Gr-1$^-$	1.679	0.594	1.360	0.466	—
Ter119$^+$	7.131	4.622	6.733	4.560	—
NK1.1$^+$	0.151	0.100	0.355	0.092	< .01
CD4$^+$	0.218	0.123	0.101	0.021	< .05
CD8$^+$	0.225	0.159	0.054	0.007	< .05
B220$^+$IgM$^-$	2.377	1.110	2.620	0.745	—
B220$^+$IgM$^+$	1.602	0.645	2.084	0.591	—
B220$^+$IgM$^+$IgD$^+$	0.740	0.183	0.971	0.422	—
Spleen					
Mac1$^+$ Gr-1$^+$	10.192	3.666	9.721	4.169	—
Mac1$^+$ Gr-1$^-$	5.879	1.351	8.971	2.366	< .05
Ter119$^+$	24.934	5.423	26.361	8.124	—
NK1.1$^+$	2.791	1.337	7.816	2.617	< .01
CD4$^+$	64.082	7.282	23.741	5.426	< .001
CD8$^+$	27.418	3.342	7.500	0.743	< .001
IgD$^+$	97.934	12.041	89.384	7.611	—
Lymph nodes					
CD4$^+$	4.163	3.649	1.855	1.105	—
CD8$^+$	1.603	1.506	0.281	0.226	—
IgM$^+$IgD$^+$	1.077	0.646	0.959	0.434	—

Data shown are the averages (means and SD) of the absolute sizes of the cell populations carrying indicated surface markers in single-cell suspensions of bone marrow, spleen, and lymph nodes of 2-month-old control and RhoH-null mice. n [control]/[RhoH$^{-/-}$]: 5/5.

— indicates not significant ($P > .05$).

significantly different between RhoH-null and control mice, but splenocyte counts were lower in RhoH-deficient mice (Figure S2). Lymph node cellularity varied but tended to be lower in mutant mice (Figure S2). Population sizes of granulocytes (Mac1$^+$ Gr-1$^+$), monocytes (Mac1$^+$ Gr-1$^-$), erythroblasts (Ter119$^+$), and different stages of B cells (proB + preB [B220$^+$IgM$^-$], immature [B220$^+$IgM$^+$], mature [B220$^+$IgM$^+$IgD$^+$]) in BM were similar in mutant and control mice (Table 1), indicating that RhoH is not essential for the differentiation of myeloid, erythroid, or B cells. Splenic granulocyte counts were unperturbed, whereas splenic monocyte counts were slightly increased in RhoH-deficient mice. B-cell counts (IgD$^+$) in spleen and lymph nodes were similar in mutant and control mice (Table 1). In contrast, T-cell counts were strongly decreased in BM, spleen, and lymph nodes. CD8$^+$ T-cell counts were reduced by approximately 75%, and CD4$^+$ T-cell counts were reduced by 50% (Table 1). Heterozygous mice showed a normal phenotype and were included in the control group. These data suggest a defect in the production, survival, or migration of T cells in the absence of RhoH.

NK1.1$^+$ cell counts, including those of NK and NKT cells, were elevated in BM and spleen (Table 1). Six-month-old mutant mice still had normal numbers of myeloid, erythroid, and B cells in the BM, indicating that RhoH is not crucial for the maintenance of HSCs (Table S1). Splenic granulocyte, monocyte, and B-cell populations were similar in 6-month-old control and RhoH-deficient mice, whereas the number of splenic Ter119$^+$ erythroblasts was greater in mutant mice. CD4$^+$ and CD8$^+$ T cells were reduced in BM, spleen, and lymph nodes. In contrast to counts in 2-month-old mutant mice, NK1.1$^+$ cell counts were not significantly changed in BM and spleen (Table S1).

Defective T-cell development in vivo

To test whether the production of T cells is impaired in the absence of RhoH, we analyzed thymocytes of 2-month-old mice. Thymus cellularity of RhoH-null mice was approximately 60% lower than in control animals (Figure S2). In mutant mice, the absolute number of DN (CD4$^-$CD8$^-$) thymocytes was increased more than twofold, whereas the number of DP (CD4$^+$CD8$^+$) thymocytes was reduced by 60%, indicating an incomplete developmental block between the DN and the DP stages (Figure 1A, lower panel). Absolute amounts of CD4SP and CD8SP thymocytes were reduced by 80% and 85%, respectively, in RhoH-deficient mice, implying an additional defect during the development from DP to SP cells, where positive selection takes place (Figure 1A).

To define the block during DN to DP development in more detail, the population of DN cells was further divided into DN1 (CD25$^-$CD44$^+$), DN2 (CD25$^+$CD44$^+$), DN3 (CD25$^+$CD44$^-$), and DN4 (CD25$^-$CD44$^-$) cells. The number of DN1 cells was not significantly different between control and mutant mice, suggesting normal migration of T-cell precursors from the BM to the thymus (Figure 1B). The very small population of DN2 cells was slightly, but not significantly, increased in the absence of RhoH. The total number of DN3 cells, however, was elevated nearly 3-fold in the absence of RhoH. DN4 cell counts were not significantly different between control and mutant mice, whereas DP cell counts were reduced in the absence of RhoH. In addition, 6-month-old mutant mice showed a decrease in CD4SP and CD8SP cells and an increase in DN3 thymocytes (Figure S3A-B). These differences, however, were less pronounced than

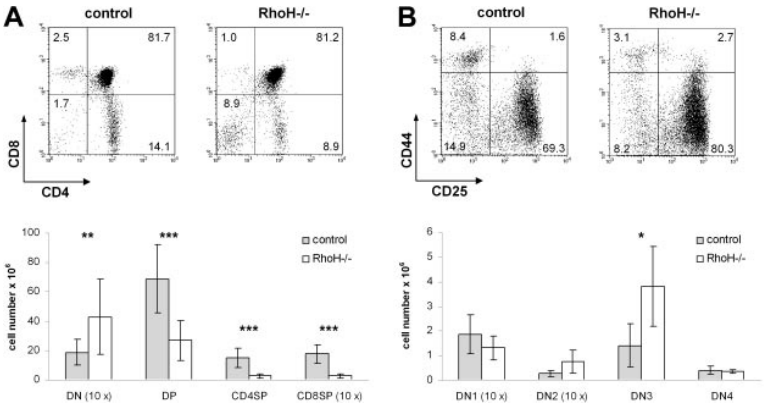


Figure 1. Impaired thymocyte development in the absence of RhoH. (A) Thymocytes of 2-month-old mice were analyzed for the expression of CD4 and CD8 by FACS. Bar graph presents the absolute cell number of each population. **$P < .01$; ***:$P < .001$. Error bars show the standard deviation (n [control]/[RhoH$^{-/-}$]: 11/11). (B) Thymocytes of 2-month-old mice were gated for lineage-negative (B220, CD4, CD8, NK1.1, Mac1, Gr-1, Ter119) cells and analyzed for the expression of CD25 and CD44. DN1, CD25$^-$CD44$^+$; DN2, CD25$^+$CD44$^+$; DN3, CD25$^+$CD44$^-$; DN4, CD25$^-$CD44$^-$. Bar graph presents the absolute cell number of each population. *$P < .05$. Error bars show the standard deviation (n [control]/[RhoH$^{-/-}$]: 4/4).

in 2-month-old mice. DP cell numbers were only slightly decreased in mutant mice (Figure 3A-B).

This T-cell phenotype was indistinguishable in 129Sv/C57BL6 outbred and backcrossed C57BL6 mice (data not shown). Total amounts of thymocytes with TCRγδ were normal in the absence of RhoH (Figure S4A), but the very small population of DN3 cells expressing TCRγδ was increased (Figure S4B). These data indicate that RhoH is only required for the development of T cells carrying αβ TCR. γδ T-cell counts were slightly increased in spleen and significantly increased in lymph nodes (Figure S4A).

Heterozygous mice showed a normal T-cell phenotype and were included in the control group. Quantitative RT-PCR revealed that RhoH is expressed at all stages of thymocyte development, with relative peaks at the DN3 and DP stages (Figure S5). These data indicate defects in the DN3 to DN4 transition and in the differentiation of DN4 to DP cells in RhoH-deficient mice.

Aberrant thymocyte proliferation and survival

To characterize whether impaired thymocyte development was caused by defective proliferation or increased apoptosis, we assessed cell proliferation by measuring the incorporation of BrdU and apoptosis by determining the percentage of AnnexinV binding to thymocyte subpopulations.

The percentage of BrdU-incorporating, proliferating DN1 and DN2 thymocytes was not significantly different between control and mutant mice (Figure 2A). However, DN3 and DN4 cells showed significantly decreased proliferation in the absence of RhoH (Figure 2A). Proliferation of DP cells was unchanged, whereas CD4SP and CD8SP cells showed increased proliferation (Figure 2A).

In DN1, DN2, and DN3 cells, apoptosis was not significantly different between RhoH-null and control mice (Figure 2B). In contrast, RhoH-deficient DN4 cells displayed increased apoptosis (Figure 2B). In addition, DP, CD4SP, and CD8SP thymocytes showed a higher percentage of AnnexinV$^+$ apoptotic cells, possibly indicating that fewer cells were positively selected (Figure 2B).

Defective TCR signaling suggested by altered expression of maturation markers

During thymocyte development, the expression of CD5, TCRβ, and CD69 is tightly regulated by pre–TCR and TCR signaling. At the DN stage, pre–TCR signaling induces the expression of CD5.[15] On DP cells, CD5 expression is maintained because of low-affinity TCR–major histocompatibility complex interactions. Finally, CD5 is up-regulated during DP to SP transition in response to TCR signaling by positive- or negative-selecting ligands.

In all thymocyte populations tested (DN, DP, CD4SP, CD8SP, and γδ T cells), the percentage of cells with low CD5 expression was significantly increased in RhoH-deficient mice (Figures 2C, S4C). In addition, RhoH-null DP cells expressed significantly lower levels of CD5 than control cells. In spleen, the percentage of CD5^{low} T cells was increased in the absence of RhoH (Figure S6A). Among the CD4$^+$ splenocytes, the amount of CD5^{low} cells increased from 1.5% to 24.6%, and among CD8$^+$ cells it increased from 3.7% to 18.5%. These data suggest defects in pre–TCR and TCR signaling.

The expression of TCRβ and CD69 becomes up-regulated during positive selection in response to TCR signaling.[16,17] In RhoH-null mice, the number of more mature, TCRβ^{high}, and CD69^{high} thymocytes was significantly decreased among DP, CD4SP, and CD8SP cells, suggesting impaired positive selection and decreased TCR signaling (Figure 2D-E). No difference was found in the number of CD69^{high} CD4$^+$ and CD8$^+$ cells in the spleen (Figure S6B). Interestingly, peripheral RhoH-null T cells in the spleen and lymph nodes showed a significantly increased amount of cells with cell surface characteristics of activated effector (CD62L^{low}CD44^{high}) T cells, and more CD8$^+$ T cells demonstrated the memory (CD62L^{high}CD44^{high}) phenotype (Figure S7A-B).

Defective thymocyte development in vitro

To directly assess the differentiation potential of DN3 and DN4 thymocytes in the absence of RhoH, thymocyte populations were sorted and differentiated in vitro on OP9-DL1 cells. RhoH-deficient DN3 cells showed significantly less ability than controls to differentiate into DN4 and DP cells after 4 days and 8 days in culture (Figures 3A, S8A). In addition, the total cell number was severely lower in the absence of RhoH, indicating an impaired expansion potential (Figures 3A, S8A). In addition, RhoH-null DN4 cells showed a strongly reduced ability to develop into DP cells compared with controls and did not expand as well as the controls (Figures 3B, S8B).

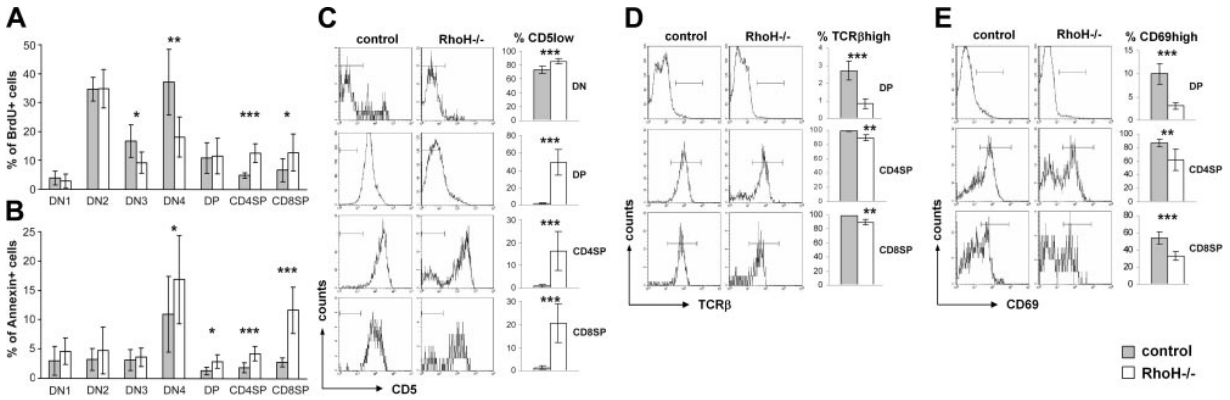


Figure 2. Impaired proliferation and survival and decreased expression of maturation markers in RhoH-null thymocytes. (A-B) Thymocytes of 2-month-old mice were analyzed for proliferating BrdU-incorporating cells and apoptotic AnnexinV$^+$ cells. Different thymocyte populations were distinguished (see Figure 1A-B). $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; BrdU, DN1-DN4, n [control]/[RhoH$^{-/-}$]: 6/10; DP, CD4SP, CD8SP, n [control]/[RhoH$^{-/-}$]: 8/10; Annexin V, DN1 to DN4, n [control]/[RhoH$^{-/-}$]: 10/10; DP, CD4SP, CD8SP, n [control]/[RhoH$^{-/-}$]: 8/9. (C-E) Thymocytes of 2-month-old mice were analyzed for the expression of CD4, CD8, and CD5 (C; n [control]/[RhoH$^{-/-}$]: 9/9) or TCRβ (D; n [control]/[RhoH$^{-/-}$]: 4/4) or CD69 (E; n [control]/[RhoH$^{-/-}$]: 6/6) by FACS. Percentages of cells marked in histograms are shown in graph. $^{**}P < .01$; $^{***}P < .001$. Error bars show standard deviation.

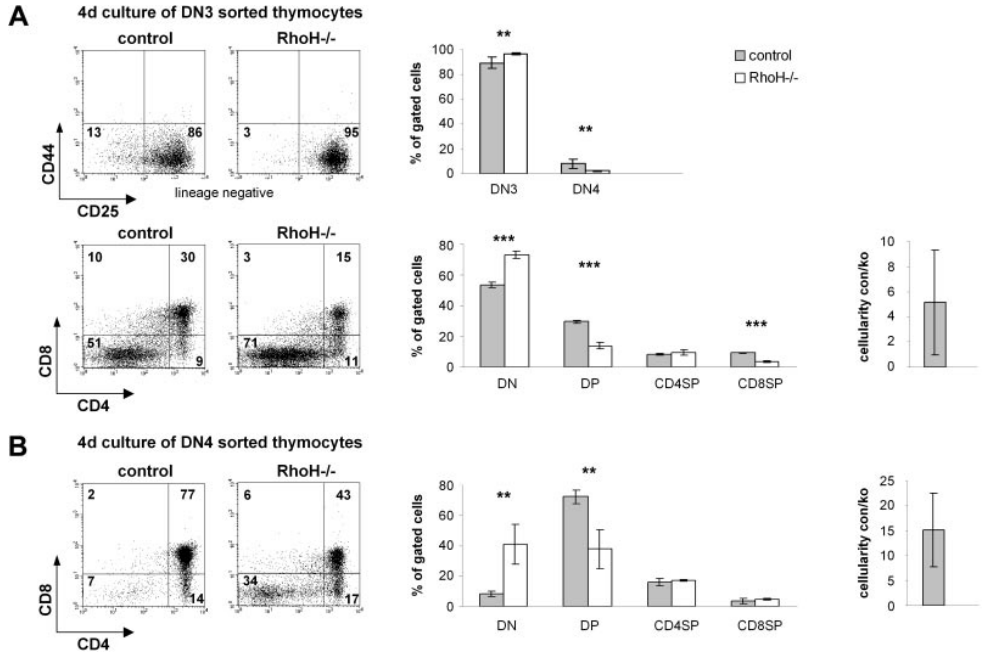


Figure 3. Impaired differentiation of RhoH-null DN3 and DN4 cells in vitro. (A) Sorted DN3 cells were cultured for 4 days on OP9-DL1 cells in the presence of IL-7 and Flt3 ligand. Differentiation from DN3 to DN4 was tested by FACS analysis of lineage-negative cells for the expression of CD44 and CD25. RhoH-null cells showed a significantly higher percentage of DN3 and a lower percentage of DN4. FACS staining for CD4 and CD8 revealed significantly increased levels of DN and reduced levels of DP in the absence of RhoH. Furthermore, cellularity of the RhoH-null cultures was approximately 5-fold decreased, indicating defective expansion in the absence of RhoH. $^{**}P < .01$; $^{***}P < .001$. Error bars show the standard deviation (n [control]/[RhoH$^{-/-}$]: 2/3). (B) Sorted DN4 cells were cultured for 4 days on OP9-DL1 cells in the presence of IL-7 and Flt3 ligand. FACS staining for CD4 and CD8 revealed significantly increased levels of DN and reduced levels of DP in the absence of RhoH. Furthermore, cellularity of the RhoH-null cultures was approximately15-fold decreased, indicating defective expansion in the absence of RhoH. $^{**}P < .01$. Error bars show the standard deviation (n [control]/RhoH$^{-/-}$]: 3/4.

These data demonstrate that in the absence of RhoH, the differentiation and expansion potential of DN3 and DN4 are decreased, confirming the in vivo data. Furthermore, they prove that this is a cell-autonomous defect of RhoH-null thymocytes.

Defect in positive selection

To test the positive selection of thymocytes into the CD4 lineage, we intercrossed RhoH-deficient mice backcrossed to C57BL6 with OT-II transgenic mice, which express an ovalbumin-specific TCR restricted to MHC class I-A^b as present in the RhoH-null mice. FACS analysis for the transgenic TCRα chain Vα2 indicated that nearly all DP and CD4SP thymocytes expressed the ovalbumin-specific TCR in control and mutant mice, though within the RhoH-deficient CD4SP cells a population expressing lower Vα2 levels could be detected (Figure 4C). Among the DN cells, which up-regulate Vα2 expression during β-selection, RhoH-null mice showed a reduced amount of Va2$^+$ cells compared with controls (Figure 4C).

Expression of ovalbumin TCR in control mice led to a reduced DP thymocyte count and an increased CD4SP cell count (Figures 1A, 4A). Furthermore, the percentage of DN3 cells was decreased, and the DN4 population was increased (Figures 1B, 4B). In contrast, RhoH deficiency severely impaired the generation of CD4SP cells in mice expressing the ovalbumin TCR (Figures 1A, 4A). No increase in DN4 population was observed (Figures 1B, 4B). In addition, in the presence of the ovalbumin TCR, the percentage of CD5^{low} cells was higher in RhoH-null mice than in controls (Figure 4D), and the relative amount of CD69^{high} cells was lower (Figure 4E).

These data indicate an impaired positive selection of DP cells into the CD4 lineage in the absence of RhoH and conceivably a defective β-selection, which controls the DN3 to DN4 transition.

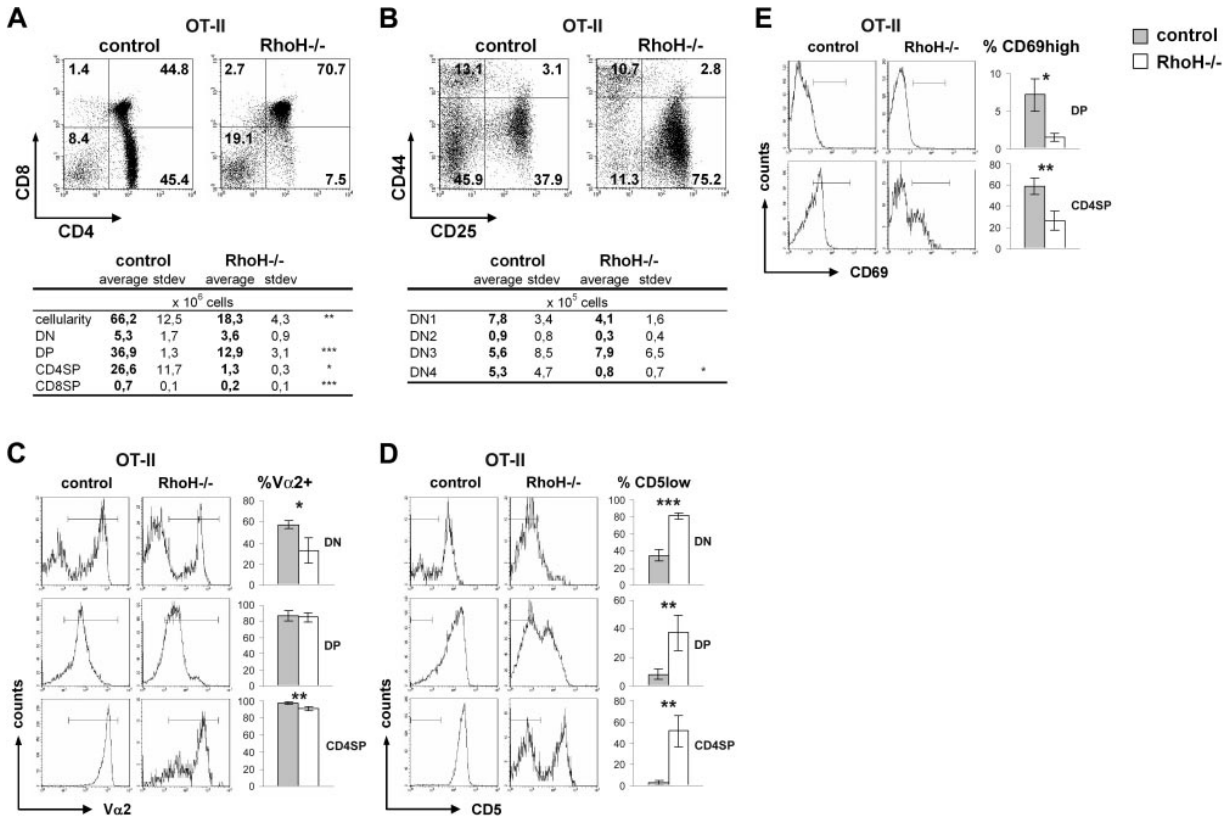
Defect in TCR signaling

In the absence of RhoH, the defects observed in thymocyte development and positive selection were consistent with impaired TCR signaling. To test this possibility directly, we analyzed TCR signaling in vitro. We induced TCR signaling in FACS-enriched preparations of DP cells by cross-linking biotinylated antibodies against CD3 or CD3 and CD4 with streptavidin and investigated the activation of different steps of the TCR signaling cascade. In addition, we measured the phosphorylation of different signaling molecules by intracellular FACS staining of stimulated thymocyte preparations.

TCR cross-linking triggers the activation of the tyrosine kinases lck and ZAP70. ZAP70-mediated phosphorylation of the scaffold protein LAT and associated molecules such as SLP-76 and PLCγ1, which together form the LAT signalosome, is then crucial to initiate downstream events such as calcium influx or Erk activation.

In the absence of RhoH, TCR-induced autophosphorylation of ZAP70 in DP cells was not altered (Figures 5A, S9A). Also, in CD4SP cells, TCR-dependent activation of ZAP70 did not require RhoH (Figure 6A). Furthermore, the phosphorylation of lck at Y505 was not altered in the absence of RhoH in DP and CD4SP cells (Figure 6B). Lck is negatively regulated by the phosphorylation of Y505, though this inhibition is overruled by the activating autophosphorylation of Y394.[18] These data indicate that early events in TCR signaling are not strongly affected by the loss of RhoH. In CD4$^+$ T cells, ZAP70 activates p38 MAPK independently of LAT.[19] In RhoH-null CD4SP thymocytes, p38 was weakly, but significantly, activated as in controls (Figure 6D), suggesting that this downstream pathway of ZAP70 is normally intact in the absence of RhoH.

However, ZAP70-mediated total tyrosine phosphorylation of LAT and tyrosine phosphorylation of LAT at Y195, which is



	control average	stdev	RhoH-/- average	stdev	
x 10^6 cells					
cellularity	**66,2**	12,5	**18,3**	4,3	**
DN	**5,3**	1,7	**3,6**	0,9	
DP	**36,9**	1,3	**12,9**	3,1	***
CD4SP	**26,6**	11,7	**1,3**	0,3	*
CD8SP	**0,7**	0,1	**0,2**	0,1	***

	control average	stdev	RhoH-/- average	stdev	
x 10^5 cells					
DN1	**7,8**	3,4	**4,1**	1,6	
DN2	**0,9**	0,8	**0,3**	0,4	
DN3	**5,6**	8,5	**7,9**	6,5	
DN4	**5,3**	4,7	**0,8**	0,7	*

Figure 4. Defective positive selection in the absence of RhoH. (A) Thymocytes of 4- to 7-week-old OT-II mice transgenic for an ovalbumin-specific TCR, either expressing or not expressing RhoH, were analyzed for the expression of CD4 and CD8 by FACS (upper panel). Quantification of absolute numbers of thymocyte subpopulations (lower panel). $*P < .05$; $**P < .01$; $***P < .001$. Error bars indicate the standard deviation (n [control]/RhoH$^{-/-}$]: 3/4). (B) Thymocytes of 4- to 7-week-old OT-II mice transgenic for an ovalbumin-specific TCR, either expressing or not expressing RhoH, were gated for lineage-negative (B220, CD4, CD8, NK1.1, Mac1, Gr-1, Ter119) cells and analyzed for the expression of CD25 and CD44. DN1, CD25$^-$CD44$^+$; DN2, CD25$^+$CD44$^+$; DN3, CD25$^+$CD44$^-$; DN4, CD25$^-$CD44$^-$; upper panel). Quantification of absolute numbers of thymocyte subpopulations (lower panel; $*P < .05$. Error bars indicate the standard deviation (n [control]/RhoH$^{-/-}$]: 3/4). (C) Decreased amount of Vα2$^+$ DN thymocytes in 4- to 7-week-old OT-II mice transgenic for an ovalbumin-specific TCR in the absence of RhoH. $*P < .05$; $**P < .01$. Error bars indicate the standard deviation (n [control]/RhoH$^{-/-}$]: 4/4). (D) Increased amount of CD5^{low} thymocytes in 4- to 7-week-old OT-II mice transgenic for an ovalbumin-specific TCR in the absence of RhoH. $**P < .01$; $***P < .001$; Error bars indicate the standard deviation (n [control]/RhoH$^{-/-}$]: 4/4). (E) Decreased amount of CD69^{high} thymocytes in 4- to 7-week-old OT-II mice transgenic for an ovalbumin-specific TCR in the absence of RhoH. $*P < .05$; $**P < .01$. Error bars indicate the standard deviation (n [control]/RhoH$^{-/-}$]: 4/4).

important for the interaction of LAT with Gads and SLP-76 and which indirectly supports the interaction of LAT with PLCγ1,[20,21] were reduced in RhoH-null DP cells (Figures 5B-C, S9B-C). Phosphorylation of PLCγ1 at Y783, which is important for the activation of PLCγ1,[22] was reduced in DP cells of RhoH-null mice (Figures 5D, S9D). In addition, the TCR-dependent phosphorylation of Vav1, which is required for the GEF activity of Vav1,[23] was decreased in DP thymocytes in the absence of RhoH (Figures 5E, S9E). Interestingly, the phosphorylation of Vav2 after TCR ligation was similar in RhoH-null and control mice (Figures 5F, S9F).

Despite the decreased TCR-induced activation of Vav1, Rac1 and Rac2 activity after TCR activation were normal in RhoH-deficient DP cells (Figures 5G-H, S9G-H). However, the basal level of Rac1 activity appeared to be higher in RhoH-null DP cells than in controls, suggesting compensatory up-regulation of Rac1 activity in the absence of RhoH or inhibition of basal Rac1 activity by RhoH (Figures 5G, S9G). Basal levels of active Rac2 were not significantly different between RhoH-null and control mice (Figures 5H, S9H).

We then tested TCR-dependent activation of Erk and calcium influx, each of which requires PLCγ1 activation. In the absence of RhoH, TCR-induced Erk phosphorylation was severely reduced in DP, CD4SP, and CD8SP cells (Figures 5I, 6C, S9I). To assess TCR-induced calcium influx, thymocytes and splenocytes were loaded with the calcium-sensitive dye Fluo-4 and were incubated with biotinylated antibodies against CD3. After the induction of TCR signaling with streptavidin, the level of intracellular calcium was determined by FACS. Costaining for CD4 and CD8 allowed us to distinguish DN, DP, CD4SP, and CD8SP cells. No strong calcium influx was detected in DN control and mutant cells (Figure 6E). In DP, CD4SP, and CD8SP cells, however, RhoH-deficient cells exhibited a significantly decreased percentage of cells responding to stimulation and a decreased mean fluorescence of all cells, indicating a partially impaired TCR-dependent calcium influx (Figure 6E). In addition, RhoH-deficient CD4$^+$ and CD8$^+$ splenocytes showed a reduced stimulation of calcium influx after CD3 cross-linking, suggesting that RhoH is also important for TCR signaling in peripheral T cells (Figure 6F).

These data show that RhoH is required for TCR signaling downstream of ZAP70 in a cell-autonomous manner in thymocytes and in mature T cells.

RhoH is not crucial for the regulation of β2 integrin–mediated adhesion on thymocytes

Previously, it was suggested that RhoH is required to maintain integrin LFA-1 (αLβ2) in a nonadhesive state on lymphocytes.[6] To test whether this function could contribute to the defect observed in

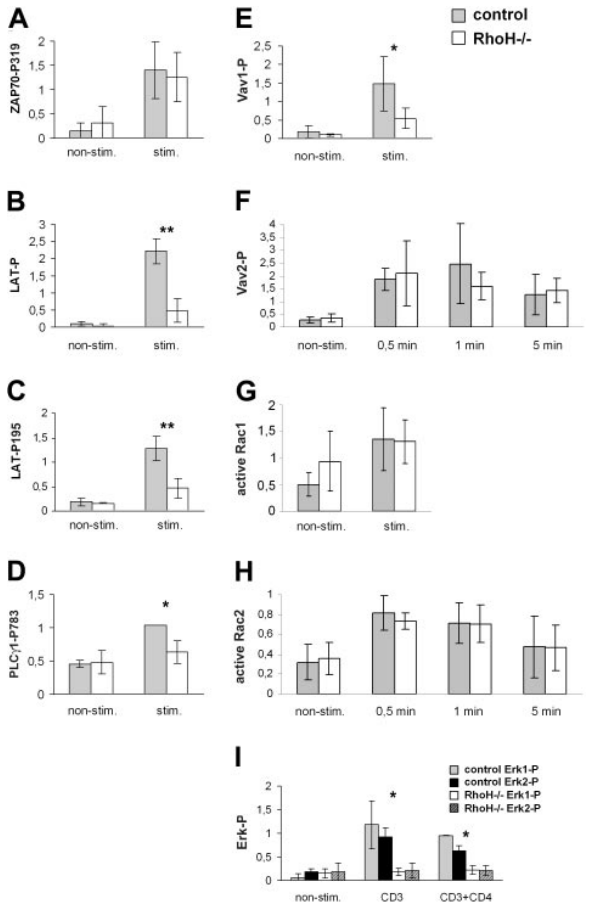


Figure 5. Impaired TCR signaling in RhoH-null DP thymocytes. DP thymocytes of 4- to 8-week-old mutant mice were sorted by FACS or MACS microbeads. TCR signaling was induced as indicated by cross-linking of biotinylated CD3 and CD4 antibodies with streptavidin for 5 minutes (A-E, I), 30 seconds (G), or indicated times (F, H) at 37°C. Total lysates were analyzed by Western blot for ZAP70-P319 (A), LAT-P195 (C), PLCγ1-P783 (D), Vav2-P (F), and Erk-P (I). Immunoprecipitations (IPs) of LAT (B) and Vav1 (E) were blotted with antiphosphotyrosine antibodies and reprobed with LAT (B) or Vav1 (E). Amounts of active Rac1 and Rac2 were determined by pull-down assays (G-H). Bar graphs represent quantifications of 5 (A), 3 (B-D,I), 4 (E), 5 (F), 10 (G), or 4 (H) independent experiments. *$P < .05$; **$P < .01$. All values are normalized to equal total amounts of the corresponding protein determined by Western blot. Representative examples of the Western blots are shown in Figure S9. Error bars show the standard deviation.

thymocyte development, we measured the adhesion of RhoH-null thymocytes to the immobilized LFA-1 ligand ICAM-1. Manganese (Mn^{2+}; 2 mM) or PMA (100 ng/mL) activated RhoH-null thymocytes bound to ICAM-1 with an efficiency equal to that of control cells (Figure 7A). At lower concentrations of Mn^{2+} (1 mM) or PMA (20 ng/mL), the combination of Mn^{2+} and PMA or, in the presence of Mg^{2+}, the binding to ICAM-1 was similar in RhoH-null and control thymocytes (Figure S10). This demonstrated that LFA-1–mediated adhesion of thymocytes is normally regulatable in the absence of RhoH, at least under the conditions tested. Adhesion to the α4β1 integrin substrate VCAM-1, carried out as an additional control, was indistinguishable between control and mutant thymocytes, even when stimulated with Mg^{2+}, Mn^{2+}, or PMA (Figures 7A, S10).

In a more physiological setting, we investigated the adhesion of T-cell blasts to the endothelial cell line bEnd5, which up-regulates the expression of ICAM-1 and VCAM-1 on treatment with TNF. Both RhoH-null and control T cells showed TNF-stimulated binding to these endothelial cells, which was partially inhibited by antibodies against LFA-1 and α4-integrin, respectively (Figure 7B). TNF-induced binding to the ICAM-1–deficient cell line bEndI1.1 was low for control and RhoH-null T cells and was completely inhibited by antibodies against α4-integrin but was insensitive to LFA-1 antibody inhibition (Figure 7B).

To investigate, by an alternative approach, whether RhoH-dependent modulation of β2-integrin function was involved in the defective thymocyte development in RhoH-null mice, we crossed RhoH-deficient mice with mice lacking β2-integrin and analyzed T-cell development in the absence of both RhoH and β2-integrin. If loss of RhoH indeed constitutively up-regulated αLβ2 activity and this contributed to the thymocyte phenotype, ablation of the β2-integrin gene would logically have rescued the defect. However, we found that population sizes of DN, DP, CD4SP, and CD8SP thymocytes were identical in RhoH-null and RhoH-β2-integrin double-knockout mice (Figure 7C). Furthermore, the increase of $CD5^{low}$ cells in different RhoH-null thymocyte populations was unaffected by the additional loss of β2-integrin (Figure 7D). These data indicate that the impaired thymocyte development of the RhoH-null mice is independent of β2-integrin function.

Discussion

Previous work has shown that RhoH is a negative regulator of Rac1-dependent signaling, HPC proliferation and survival, and LFA-1–mediated adhesion.[2,3,6] We demonstrated that during thymocyte development, RhoH is a positive regulator of thymocyte differentiation and TCR signaling and that it is crucial for β-selection and positive selection. Furthermore, we report that RhoH also contributes to the TCR signaling of mature T cells.

In the absence of RhoH, thymocyte development was partially blocked at the DN3 to DN4 transition. β-Selection takes place at this transition, which ensures that only thymocytes that have generated a functional TCRβ chain can differentiate to DP cells. A second partial block occurred at the transition from DP to CD4SP and CD8SP cells, where positive selection allows only MHC-restricted, self-tolerant thymocytes to develop further. In line with defects in β-selection and positive selection, the proliferation of RhoH-null DN3 and DN4 cells was decreased, whereas apoptosis of DN4, DP, CD4SP, and CD8SP cells was significantly increased. Furthermore, RhoH-null DN3 and DN4 cells showed a clearly reduced ability to differentiate and expand in vitro. Previously, it was documented that decreased RhoH expression increased the proliferation and survival of HPCs.[3,24] Our data suggest that RhoH regulates proliferation and cell survival independently and in a cell type– and differentiation stage–specific manner.

While β-selection is dependent on pre–TCR signaling, positive selection is thought to require weak TCR signaling.[25] CD5 expression correlates with pre–TCR and TCR signaling strength.[15] Reduced expression of CD5 on DP cells and an increased number of $CD5^{low}$ cells among DN, CD4SP, and CD8SP thymocytes of RhoH-null mice suggests defective pre–TCR signaling in DN and impaired TCR signaling in DP, CD4SP, and CD8SP cells. Furthermore, reduced numbers of $TCR\beta^{high}$ and $CD69^{high}$ cells among DP, CD4SP, and CD8SP thymocytes indicated defective positive selection. Analysis of RhoH-null mice expressing an ovalbumin-specific, MHC class II–restricted TCR confirmed that RhoH is important for positive selection because no significant increase in positively selected CD4SP thymocytes was observed, in contrast to controls. Among DN cells, RhoH-null/OT-II mice did not display

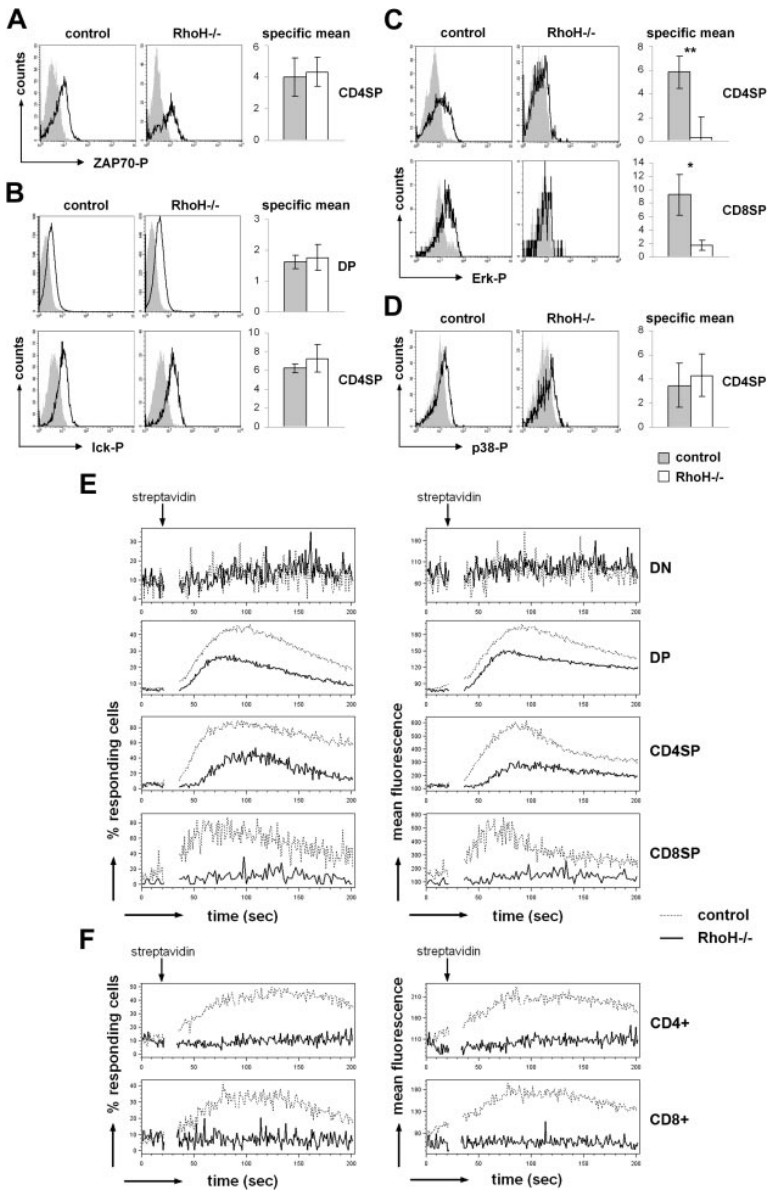


Figure 6. Defective TCR signaling and calcium influx in RhoH-null thymocytes. Thymocytes of 2-month-old mice were stimulated with biotinylated antibodies CD3 (C) or CD3 and CD4 (A-B, D) and streptavidin for 5 minutes at 37°C. Phosphorylation of ZAP70(Y319)/Syk(Y352) (A), lck(Y505) (B), Erk1/2(T202/Y204) (C), and p38 MAPK (T180/Y182) (D) was measured by FACS. Different thymocyte populations were distinguished (filled, nonstimulated; line, stimulated). Differences between the mean of stimulated and nonstimulated (specific mean) cells are shown. $*P < .05$; $**P < .01$. n [control]/RhoH$^{-/-}$]: 5/5 (A-B, D). n [control]/RhoH$^{-/-}$]: 4/5 (C). Thymocytes (E) or splenocytes (F) of 2-month-old mice were loaded with Fluo-4 and stained on ice for CD4, CD8, and CD3. After warming to 37°C, baseline Fluo-4 fluorescence was determined, and TCR signaling was induced by cross-linking CD3 with streptavidin. Presented is the percentage of cells above a threshold fluorescence (responding cells; left panel) and the mean fluorescence of all cells (right). Shown are representative results of 5 (E) or 2 (F) independent experiments. Error bars indicate the standard deviation.

the obvious reduction of DN3 or the increase of DN4 thymocytes observed in controls, suggesting impaired β-selection. Interestingly, an increased proportion of peripheral T cells displayed surface characteristics of activated and memory cells in the absence of RhoH. We will test in the future whether this activation might be attributed to an increased amount of autoreactive T cells escaping negative selection that are activated in the periphery by high amounts of self-antigen. Such a phenotype has been observed in mice with a mutation in the *ZAP70* gene; these mice also have partially impaired TCR signaling.[26]

Our data indicated that RhoH is important for pre–TCR and TCR signaling by facilitating the phosphorylation of the LAT signalosome by ZAP70. TCR activation by cross-linking of CD3 leads to activation of the lck tyrosine kinase, which phosphorylates the CD3 complex. Docking of ZAP70 to the phosphorylated ITAMs of the TCR and phosphorylation by lck stimulates its tyrosine kinase activity and results in ZAP70-dependent phosphorylation of the membrane-anchored LAT protein and of LAT-associated molecules, forming together the "LAT signalosome." Normal autophosphorylation of ZAP70 at Y319 in RhoH-null thymocytes after cross-linking of CD3 suggests that TCR signaling is not impaired at the level of ZAP70 activation. This notion is strengthened by the normal TCR-dependent activation of p38 in CD4SP cells because in T cells this activation is mediated by ZAP70 independently of LAT.[19] However, the phosphorylation of LAT and the LAT-associated proteins PLCγ1 and Vav1 and the activation of Erk and calcium influx, which are downstream of PLCγ1, were dramatically reduced in RhoH-deficient thymocytes. RhoH, therefore, seems to specifically interfere with the LAT branch of ZAP70 signaling. The impaired TCR-dependent calcium influx in peripheral CD4$^+$ or CD8$^+$ T cells indicates that RhoH is also important for TCR signaling in mature T cells and that loss of RhoH is not compensated during development.

When comparing the RhoH-null phenotype with other mouse mutants with defects in thymocyte development, striking similarities with Vav1-deficient mice become obvious[27-29] and make it

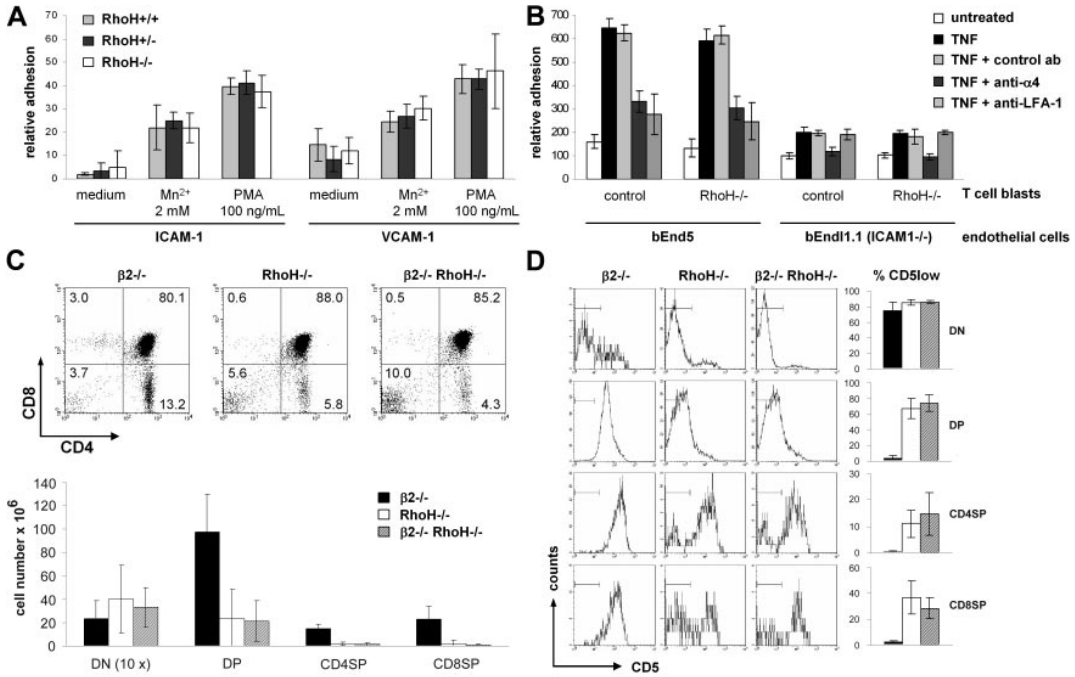


Figure 7. RhoH does not affect thymocyte development through regulation of αLβ2 integrin-mediated adhesion. (A) Relative adhesion of thymocytes to the immobilized αLβ2 ligand ICAM-1 and to the α4β1 ligand VCAM-1. Integrins were activated by treatment with 2 mM Mn^{2+} or 100 ng/mL PMA as indicated. Adhesion of wild-type (RhoH $^{+/+}$), heterozygous (RhoH$^{+/-}$), and homozygous RhoH-null thymocytes (RhoH $^{-/-}$) was indistinguishable at all conditions tested. Error bars show the standard deviation (n [RhoH$^{+/+}$]/[RhoH$^{+/-}$]/[RhoH$^{-/-}$]: 1/2/3. (B) Relative adhesion of T-cell blasts to the endothelial cell line bEnd5 and the ICAM-1–deficient endothelial cell line bEndl1.1 (ICAM1$^{-/-}$). Adhesion was stimulated by treating the endothelial cells with TNF. Antibodies against α4-integrin and LFA-1 were used to determine the specific contribution of these adhesion receptors to the attachment. An unrelated antibody was added as a control for nonspecific effects. Wild-type (RhoH$^{+/+}$), heterozygous (RhoH$^{+/-}$), and homozygous (RhoH$^{-/-}$) RhoH-null T cells showed indistinguishable adhesion at all conditions tested. Error bars show the standard deviation (n [RhoH$^{+/+}$]/[RhoH$^{+/-}$]/[RhoH$^{-/-}$]: 2/2/4. (C)Thymocytes of 6- to 10-week-old β2-integrin-null, RhoH-null, and β2-integrin-RhoH double-knockout mice were analyzed for the expression of CD4 and CD8 by FACS. Bar graph presents the absolute cell numbers of each population. Error bars indicate the standard deviation (n [β2$^{-/-}$]/[RhoH$^{-/-}$]/[β2$^{-/-}$RhoH$^{-/-}$]: 3/3/3. (D) Expression of CD5 and amount of CD5^{low} thymocytes in 6- to 10-week-old β2-integrin-null, RhoH-null, and β2-integrin-RhoH double-knockout mice. Error bars indicate the standard deviation (n [β2$^{-/-}$]/[RhoH$^{-/-}$]/[β2$^{-/-}$RhoH$^{-/-}$]: 3/3/3.

tempting to speculate that RhoH function is closely linked to Vav1. Given that Vav1 is a GEF binding to Rho GTPases, the simplest link would be a direct interaction of Vav1 with RhoH. This could lead to the recruitment of a constitutively active RhoH to the TCR complex, enabling further protein–protein interactions through RhoH. As a second possibility, Vav1 might activate RhoH by catalyzing the exchange of GDP to GTP. This would imply that, in thymocytes, RhoH activity is regulated by GEFs and GAPs and that RhoH is not constitutively active, as presently assumed.[2] Like RhoH, the small GTPase Rap1 does not have detectable intrinsic GTPase activity because of the mutation of a catalytic glutamine.[30] However, Rap1 can hydrolyze GTP with the help of Rap1GAP, which provides a catalytic asparagine.[31] It remains to be tested whether such a GAP exists for RhoH. Interestingly, we did not observe any defect in hematopoiesis in heterozygous RhoH-mutant mice, though mRNA levels of RhoH were reduced by half.

Our data indicate that RhoH is upstream of Vav1 because TCR-dependent activation of Vav1, as determined by tyrosine phosphorylation, is reduced in RhoH-null mice, whereas total levels of Vav1 are unchanged. A conceivable scenario might be that though RhoH is weakly associated in the resting state with Vav1 or other members of the LAT signalosome, this interaction becomes strengthened on TCR stimulation, resulting in the stabilization of the entire LAT signalosome complex. Thus, RhoH can be involved in a positive feedback loop downstream of Vav1, which increases the tyrosine phosphorylation of LAT, PLCγ1, and Vav1 itself. However, thus far we have been unsuccessful in coimmunoprecipitation of recombinant RhoH with Vav1, ZAP70, or LAT from lysates of resting and stimulated thymocytes or Jurkat cells transfected with tagged RhoH, suggesting that such interactions, if existent, are weak or transient (data not shown).

Several functions have previously been assigned to RhoH. First, RhoH was described as a negative regulator of p38 MAPK in Jurkat and 293 cells.[2] In thymocytes lacking RhoH, we did not detect increased activation of p38 MAPK, suggesting a cell type–specific function of RhoH in this respect.

Second, RhoH was reported to decrease LFA-1–mediated adhesion in Jurkat cells and human peripheral blood lymphocytes.[6] We showed here that LFA-1–mediated adhesion of thymocytes and T cells to ICAM-1 is not affected by the absence of RhoH. Our results do not rule out a more subtle role for RhoH in cell adhesion and cell–cell contact in vivo.

Finally, RhoH inhibited SCF-induced Rac1 activation in HPCs.[3] Indeed, basal Rac1 activity was increased in RhoH-null DP thymocytes, suggesting that RhoH is a negative regulator of Rac1 activity in the resting state. Increased Rac1 activity could be a compensatory change in response to the loss of RhoH. Other compensatory changes might not yet have been found, and future studies will address this search. It is unlikely that the increased basal activity of Rac1 in RhoH-null DP thymocytes is the reason for the impaired thymocyte development. Constitutive activation of Rac1 should rather result in "augmented" TCR signaling because constitutively active Rac1 can rescue the defective DN3 to DN4 transition in Vav1-null thymocytes and can increase the expression of TCRβ, CD5, and CD69 on DP and CD4SP cells, in contrast to

the phenotype of RhoH-deficient cells.[32,33] Conceivably, the different phenotypes are explained by the fact that the level of active Rac1 is lower in RhoH-deficient thymocytes than in thymocytes overexpressing the constitutively active mutant form (L61Rac1).[32,33]

In conclusion, our data suggest that RhoH is required for efficient β-selection and positive selection because it promotes the ZAP70-dependent phosphorylation of the LAT signalosome during pre–TCR and TCR signaling.

Acknowledgments

This work was supported by the German Research Council (DFG), the Friis Foundation, the Lundbeck Foundation, and the Max Planck Society.

We thank Dr Cinthia Farina for help with the calcium measurements, Bianka Ksienzyk for her support with cell sorting, Dr Michael Bösl for generation of chimeric mice, Dr Ernst Pöschl for Annexin V, Dr Jürgen Wehland for antibodies, Dr Juan Carlos Zuniga-Pfluecker and Dr Britta Engelhardt for cell lines, Dr Hans-Reimer Rodewald and Dr Klaus-Dieter Fischer for advice, Dr Werner Müller, Dr Kathryn Rodgers, and Tine Lefever for critically reading the manuscript, and Dr Reinhard Fässler for his comments and his continuous and generous support.

Authorship

Contribution: T.D. designed and performed the research, collected and analyzed the data, and wrote the paper. U.K. performed the research. G.B. collected the data. S.S contributed analytical tools. M.B. collected the data. J.E. collected the data. T.P. contributed vital new reagents. K.S.-K. contributed vital new reagents. M.S. contributed vital new reagents. M.L. performed the research. B.H. contributed vital new reagents. W.E.F.K. collected the data. P.T.S. contributed vital reagents. T.K. collected the data. M.S. collected the data. C.B. designed the research, analyzed the data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Paper IV

Extravasation of autoreactive T cells into the central nervous system is controlled by β1 integrins

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Key words: EAE, integrin β1, natalizumab

Short title: Integrin β1 and T cell extravasation

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Funding: This research has been funded by the Max Planck Society, the German Research Council (DFG SFB571), the National Multiple Sclerosis Society (USA) and the Swiss Multiple Sclerosis Society. CC is additionally funded by a fellowship of the French Multiple Sclerosis Society (ARSEP).

Inhibition of the α4 subunit of the integrin heterodimers α4β1 and α4β7 with the monoclonal antibody natalizumab is an effective treatment of multiple sclerosis (MS)[1]. Since α4 integrins control multiple functions on almost all hematopoietic cells it is not clear where and how the antibody executes its beneficial effect. One assumption is that the interaction of α4 integrins on autoreactive T cells with VCAM-1 on the activated blood brain barrier endothelium is blocked, preventing their extravasation into the central nervous system (CNS). However, there are conflicting data regarding which integrin heterodimer is responsible for the clinical effect[2,3], and whether more fundamental processes such as T cell activation and polarization are affected. Finally, it is unknown whether inhibition of cell types other than T cells contributes to the beneficial effect of α4 blockade. To address these questions, we studied MS-like experimental autoimmune encephalomyelitis (EAE) in mice with a β1 integrin gene deletion in either all hematopoietic cells or selectively in T cells. We found that β1 integrin-deficient autoreactive T cells were unable to firmly adhere to CNS endothelium *in vivo* while priming and expansion of T cells and CNS infiltration of myeloid cells remained unaffected. Our data suggest that α4β1 integrin is essential for T cell entry into the CNS.

Multiple sclerosis is considered an autoimmune disease where activated $CD4^+$ T cells reactive against myelin components enter the central nervous system, recruit additional inflammatory cells like macrophages and cause inflammation, edema and demyelination which set the stage for the clinical picture of this chronic disabling disease[4]. Thus, T cell trafficking into the CNS is a major hallmark of the immunopathogenesis of MS. Natalizumab (marketed as Tysabri), a humanized monoclonal antibody against the α4 integrin subunit has been approved for the treatment of relapsing-remitting MS under the assumption that it prevents extravasation of activated T cells into the CNS[5]. However, there is accumulating evidence that α4 integrins are also involved in immunological processes other than T cell extravasation including activation of myeloid cells[6], activation of naïve T and B lymphocytes[7], polarization of effector T cells into the T_H1 or T_H2 lineage[8], retention of memory T cells in their niches[9] and localization of hematopoietic stem cells[10]. While these findings largely stem from studies in rodents, the very rare but fatal side effects such as the development of progressive multifocal leukoencephalopathy (PML) in natalizumab-treated patients also pointed to the possibility that the antibody has a broader systemic immunosuppressive or immunmodulatory effect[11,12].

To investigate these issues we chose a genetic approach where we selectively deleted the gene encoding the β1 integrin subunit, thus restricting our findings to the α4β1 integrin heterodimer and excluding potential effects of α4β7 blockade. In order to directly compare the fate and the functional properties of wild type (wt) and β1 integrin-deficient T cells within the same animal we established an animal model in which Cre-mediated deletion of the β1 integrin gene occurred in approximately half of the T cells. This was achieved by generating bone marrow (BM) chimeric mice carrying floxed β1 integrin alleles and an inducible Mx1-Cre transgene[13] ($\beta1^{fl/fl}$/MxCre+) in the hematopoietic compartment. This genetic set-up restricts the ablation of the conditional β1 integrin gene exclusively to hematopoietic cells, which occurs in T cells to a rate of approximately 60% and in other hematopoietic cells to a rate of 95-98% (Fig. 1a). The presence of β1 integrin-positive T cells is thought to be due to absent Mx1-Cre transgene expression in thymic and peripheral T cells[14]. Active immunization of $\beta1^{fl/fl}$/MxCre+ bone marrow chimeras (called $\beta1^{-/-}$ BM chimeras) with the myelin oligodendrocyte glycoprotein (MOG)-peptide MOG_{35-55} led to the development of EAE with a similar devastating impact on weight and clinical scores as for control ($\beta1^{fl/fl}$/MxCre-) BM chimeras (Fig. 1b). Moreover, histology of diseased animals revealed no qualitative difference in leukocyte infiltration in the CNS (Fig. 1c). These findings suggest that (i) loss of β1 integrin expression on the myeloid and B cell lineage did not influence the development of the disease, and that (ii) a bisected pool of naïve T cells was sufficient to trigger disease induction.

To assess the contribution of β1 integrin-deficient inflammatory cells within the diseased tissue we isolated the infiltrating leukocytes from the CNS of animals at the peak of their disease and determined the cellular composition of the infiltrates. In line with the severity of the clinical course the total number of isolated leukocytes was similar between control and $\beta1^{-/-}$ BM chimeras (Fig. 2a). Furthermore, the population sizes of isolated $CD4^+$ and $CD8^+$ T cells, macrophages and granulocytes were comparable in control and $\beta1^{-/-}$ BM chimeras (Fig. 2b). We next took advantage of our internally controlled system and determined the contribution of β1-deficient cells to the inflammatory infiltrates. In $\beta1^{-/-}$ BM chimeric mice $CD45.1^{neg}Gr\text{-}1^{med}Mac\text{-}1^+$ infiltrating macrophages and $CD45.1^{neg}Gr\text{-}1^{high}Mac\text{-}1^+$ granulocytes were uniformly β1-deficient (Fig. 2c) indicating that inflammatory bystander cells are recruited into the CNS in a β1 integrin-independent manner. In contrary, $CD4^+$ and $CD8^+$ T cells isolated from the CNS of $\beta1^{-/-}$ BM chimeric mice were almost entirely β1 integrin-positive (Fig. 2c). Thus, a 60:40 ratio of β1 integrin-negative to β1 integrin-positive peripheral T cells in the

peripheral circulation shifted towards an almost pure population of β1-positive cells within the inflamed CNS. These data impressively demonstrate that T cells but not myeloid cells require β1 integrins to infiltrate the CNS.

To further corroborate our findings we employed the CD4-Cre transgene[15] to specifically ablate the β1 integrin gene on T cells. Mice carrying floxed β1 integrin alleles and the CD4-Cre transgene (named $\beta1^{fl/fl}$/CD4Cre+) displayed a 90% deletion efficiency on T lymphocytes (Fig. 3a). Upon active immunization these mice exhibited a significant delay in the onset of clinical EAE symptoms (Fig. 3b+c). Interestingly, these animals eventually developed clinical symptoms, which were accompanied by an influx of β1 integrin expressing T cells into the CNS that escaped CD4-Cre-mediated deletion (Supplementary Fig. 1). Taken together, these findings indicate that loss of β1 integrin expression on 90% of the peripheral T lymphocyte population delays the development of EAE, while the presence of only 10% of β1 integrin expressing T cells is sufficient to eventually trigger the disease.

In order to further clarify this issue, we next investigated whether lack of β1 integrin on T cells leads to their insufficient activation, to their impaired extravasation into the CNS, or both. To test T cell activation we intercrossed $\beta1^{fl/fl}$/MxCre+ mice with transgenic animals expressing MHC II-restricted T cell receptors (TCR) specific for the ovalbumin (Ova)-peptide $Ova_{323-339}$ (OT-II.2)[16]. Two to three months after polyIC injection $CD4^+$ T cells were purified and injected intravenously into wild-type recipients. LPS matured $Ova_{323-339}$-loaded bone marrow-derived dendritic cells were intravenously co-injected and the proliferative response was tracked by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. β1 integrin-deficient T cells showed comparable proliferation and division indices to control T cells (Fig. 3d+e). This indicates that β1 integrins were not required to induce T cell priming. Similarly, intracellular flow cytometry revealed similar cytokine responses between $\beta1^{fl/fl}$/MxCre+ and control cells (Supplementary Fig. 2). Together, these findings indicate that the lack of β1 integrin-deficient T cells in the EAE lesions is not due to impaired activation.

To investigate whether β1 integrins are required for T cell infiltration into the CNS we employed an *in vitro* as well as an *in vivo* approach that allowed assessing the interaction of β1 integrin-deficient T cells with inflamed CNS endothelium. We isolated T cells from polyIC-induced OT-II.2 transgenic $\beta1^{fl/fl}$/MxCre+ BM chimeras and triggered their proliferation by co-culturing them with $Ova_{323-339}$ loaded dendritic cells. For further analysis T cell blasts were sorted into β1 integrin-positive and -negative populations. Flow cytometry of the surface integrins showed a high expression of the α4, β1 and β7 subunit on control cells. On the knockout cells expression of the entire

β1 integrin subfamily was lost, while the αLβ2 integrin and the β7 subunit was slightly increased (Fig. 4a). First, we tested their static adhesion to the TNF-α stimulated brain endothelioma cell line bEnd5 and observed no difference in binding between β1-positive and -negative T cells at room temperature (Fig. 4 b). Since the adhesion was likely due to a predominant interaction between αLβ2 integrin and endothelial ICAM-1 we also performed adhesion assays on the ICAM-1$^{-/-}$ endothelioma cell line bEndI1.1, which significantly impaired adhesion of β1 integrin-deficient T cells (Fig. 4 b). The reduced adhesion of β1$^{-/-}$ T cells was even further impaired when the assays were performed at 4°C (Supplementary Fig. 3), which diminishes the contribution of αLβ2-mediated adhesion[17]. These findings suggest that T cells adhere to activated endothelial cells by binding VCAM-1 with α4β1 integrin and ICAM-1 likely with αLβ2 integrin. To test whether the αLβ2 integrin binding to ICAM-1 plays a compensatory role for T cell adhesion to inflamed brain endothelium, we performed intravital fluorescence videomicroscopy (IVM) of the spinal cord white matter microvasculature. Interestingly, adoptive T cell transfers into diseased wild-type animals revealed that adhesion of β1-deficient $Ova_{323-339}$-specific T cells to the endothelium was significantly diminished in comparison to control $Ova_{323-339}$-specific T cells (Fig. 4c). Since the events during initial cell contact (i.e. rolling and immediate capture) are slightly but not significantly impaired (Fig. 4d) the dramatic reduction of T cell adhesion is probably due to their inability to maintain a firm contact without β1 integrins. Performing the same experiment with β1 deficient MOG_{35-55}-specific T cells[18] produced similar results (Supplementary Fig. 4). Together, these findings impressively demonstrate that the interaction of α4β1 integrin with endothelial VCAM-1 is the critical molecular interaction for the stable adhesion of activated T cells to the brain vasculature.

Our results show that the genetic deletion of β1 integrin in the hematopoietic system does not impair the potency of antigen presenting cells, granulocytes and macrophages to contribute to the development of EAE. Furthermore we show that active EAE development is significantly delayed in mice lacking β1 specifically in the majority of their T lymphocytes. Taken together, these results suggest that the beneficial clinical effects of natalizumab are due to a direct effect on the T cell lineage. We further show that adhesion of T cells to inflamed endothelial cells is mediated by β1 integrins, which suggests that the anti α4 antibody is interfering with α4β1 and not α4β7 binding. Our data also demonstrate that β1 integrins play an essential role during extravasation but not during T cell priming or polarization. Hence it is likely, that the profound clinical efficacy of the antibody therapy is due to interference with the α4β1-integrin mediated extravasation of effector T cells into the CNS.

Methods

Animals. Mice carrying a floxed β1 integrin gene[19] (referred to as $\beta1^{fl/fl}$) were intercrossed with mice carrying a Mx1 promoter-driven Cre recombinase transgene[13] (referred to as MxCre+). To obtain T cell receptor (TCR) transgenic β1-deficient T cells $\beta1^{fl/fl}$/Mx1Cre+ mice were intercrossed with ovalbumin-specific TCR transgenic mice[16] (referred to as OT-II.2). To obtain mice with a deletion of the β1 integrin subunit restricted to the hematopoietic system of $\beta1^{fl/fl}$/MxCre+ irradiation bone marrow chimeras were made as described[20]. Mice with a Cre-mediated deletion of the T lymphocyte compartment were obtained by crossing $\beta1^{fl/fl}$ mice with mice carrying a CD4 promoter-driven Cre recombinase (referred to as $\beta1^{fl/fl}$/CD4Cre+)[15]. Mice were kept on a mixed 129Sv/C57BL/6 genetic background and bred in the animal facilities of the Max Planck Institute of Biochemistry. All animal studies were performed with the license of the government of Oberbayern. Female C57BL/6 mice (8-10 weeks) for IVM were purchased from Harlan (Netherlands). All IVM experiments were performed in accordance with the requirements of the local government in Bern, Switzerland (permission number 55/04 for EAE experiments and 104/04 for IVM).

Induction and evaluation of active EAE. Active EAE was induced by subcutaneous injection at the tail base of an emulsion of complete Freund´s adjuvant (CFA) and 200 µg $MOG_{35–55}$ peptide (Biotrend) in PBS (1:1 volume ratio). CFA was prepared by supplementation of incomplete Freund´s adjuvant (Sigma-Aldrich) with 5 mg/ml inactivated *Mycobacterium tuberculosis* (H37Ra; Difco). 400 ng Pertussis toxin (List Biological Laboratories) in PBS was injected intraperitoneal on days 0 and 2 after immunization. Clinical disease and weight were checked daily and scored as described[21]. Mice scored 1 (limp tail) to 2 (hind leg weakness) were used as recipients for IVM experiments.

Southern Blot analysis. Southern blotting to detect the Cre-mediated knockout efficiency was carried out as described[14,22].

Histological analysis. The spinal cord was rapidly dissected and in part frozen unfixed in optimal cutting temperature compound. Cryosections of 10 µm thickness were cut and used for immunofluorescence staining according to standard protocols. The following biotinylated monoclonal antibodies were used: Ly5.1 (A20) and Mac-1 (M1/70) (both from BD Pharmingen). After blocking unspecific binding with a Streptavidin/Biotin blocking kit (Vector Laboratories), Cy3-conjugated Streptavidin was used to detect the primary antibodies. A pan-laminin antibody (L9393, Sigma) was used to stain blood vessels. Images were taken with a DMIRE2 confocal microscope (Leica).

Flow cytometry. Mononuclear cells were isolated from the central nervous system (CNS) by Percoll density gradient centrifugation[23] of the dissected brain and spinal cord. Single-cell suspensions from hematopoietic organs and staining for FACS analysis were prepared as described[23]. The following fluorochrome or biotin-labelled monoclonal antibodies were used: CD4 fluorescein isothiocyanate (FITC), phycoerythrin (PE) and biotin (H129.19), CD8 biotin (53-6.7), Gr-1 PE (RB6-8C5), integrin α4 biotin (9C10), integrin α5 biotin (5H10-27), integrin α6 PE (GoH3), integrin αE PE (M290), integrin αV PE (RMV-7), integrin β1 FITC (Ha2/5), integrin β2 biotin (C71/16), integrin β7 PE (M293) and Mac-1 biotin (M1/70) (all from BD Pharmingen), integrin β1 PE (HMbeta1-1) (BioLegend), integrin αL biotin (M17/4) and Vα 2 TCR biotin (B20.1) (both from eBioscience). As controls the following isotype-matched antibodys were used: hamster IgM FITC (G235-1), rat IgG1 (R3-34) and rat IgG2a,κ PE (R35-95) (all from BD Pharmingen), hamster IgG PE (HTK888) (BioLegend) and rat IgG2a, κ biotin (eBiosciences). All biotinylated antibodies were detected with Cy5-conjugated streptavidin (Jackson ImmunoResearch). Dead cells were excluded from the analysis by staining with propidium-iodide (2.5 µg/ml, Sigma), residual host cells in the bone marrow chimeras were excluded by staining with CD45.1 CyChrome. Measurements were performed on a FACSCalibur (BD Biosciences) and analyzed using the FlowJo software (version 6.1.1, TreeStar).

Cell culture. Dendritic cells (DC) were generated from murine bone marrow cells as described[24]. Activated T cells for adhesion assays and IVM experiments were generated from $\beta1^{fl/fl}$/Cre+; OT-II.2 mice. Mice received a single intraperitoneal injection of 250 µg polyIC (Amersham Biosciences) in PBS to induce the knockout. Six to eight weeks after knockout induction the mice were sacrificed and single cell suspensions of splenocytes were obtained. After ACK-lysis of red blood cells according to standard protocols[22] splenocytes from one mouse were cocultured with 4×10^6 DCs loaded for 2 hours with 20 µg/ml $Ova_{323-339}$ peptide (in-house peptide synthesis service). After four days of co-culture the cell suspensions were split 1:2 into fresh medium containing 5 ng/ml IL-2 (R&D Systems). After another two days of co-culture dead cells were removed by Nycoprep 1.077 A (Axis-Shield) density gradient centrifugation. For depletion of β1 positive T cells the cell suspension was subsequently incubated with a biotinylated integrin β1 antibody (Ha2/5) (BD Pharmingen) followed by streptavidin-microbead separation according to the manufacturers protocol (Miltenyi Biotech). The efficiency of sorting was monitored by staining with fluorochrome-labelled CD4 and β1 antibodies and subsequent FACS analysis. On average 90,4% of the cells were $CD4^+$ and 92,2% of the β1-depleted cells were β1 negative. For unspecific stimulation single

cell suspensions of splenocytes were stimulated after ACK-lysis for 2 days with 50 ng/ml Phorbol-12-Myristate-13-Acetate (PMA; Calbiochem) and 500 ng/ml Ionomycin (Calbiochem).

Proliferation assays. Sorting of T cells, *in vivo* proliferation assays and FACS analysis of the proliferated cells were performed as described[23]. Proliferation parameters were analyzed using the proliferation platform of the FlowJo software (version 6.1.1, TreeStar). The division index indicates the average number of division that a dividing cell underwent, whereas the proliferation index indicates the average number of divisions all cells underwent. The percentage of initially present cells which divided is shown by the percent of dividing cells. For intracellular cytokine stainings splenocytes were stimulated after isolation for 4 hours with 50 ng/ml PMA, 500 ng/ml Ionomycin and 10 µg/ml Brefeldin A (Sigma). Intracellular cytokine staining was performed according to the manufacturers guidelines using the Leucoperm kit (AbD Serotec) and the following PE-labelled monoclonal antibodies: IL-2 (JES6-5H4), IL-4 (11B11), IL-17 (TC11-18H10.1), IFN-γ (XMG1.2) and TNF-α (MP6-XT22) (all from BD Pharmingen). As controls the following isotype-matched antibodys were used: rat IgG1 (R3-34) (BD Pharmingen) and rat IgG2b (eB149/10H5) (eBioscience).

Adhesion assays. Adhesion assays to the endothelioma cell lines bEnd5 and bEndI1.1 were carried out as described previously[25]. 2 x 10^4 endothelioma cells per well were plated. 1 x 10^5 cultured, MACS-sorted OT-II.2 transgenic T cell blasts were allowed to adhere per well either at room temperature or at 4 °C. All conditions were performed in duplicates. After fixation two pictures were acquired from each well with an Axiovert 200M microscope (Zeiss) and the number of adherent T cells was quantified using the MetaMorph software (version 6.3r6, Molecular Devices).

Intravital fluorescence videomicroscopy. Surgical preparation of the spinal cord window, intravital microscopy and quantitative analysis of the spinal cord white matter microcirculation were performed exactly as described[26] using a custom made Mikron IVM500 fluorescence microscope connected to a SIT camera (Dage-MTI). Epi-illumination techniques were used to visualize the spinal cord microvasculature after injection of 0.1 ml 1 % TRITC-conjugated dextran (MW = 155,000; Sigma-Aldrich). Real time observations were made using x4, x10 and x20 long-distance working objectives and microscopic images were recorded using a DSR-11 digital videocassette recorder (Sony) for later offline analysis, which was performed exactly as described before[26,27]. β1 integrin-deficient $CD4^+$ T cells and control $CD4^+$ T cells, isolated as described above, were fluorescently labelled with 125 nM Calcein-AM (Molecular Probes) prior to their infusion via the right carotid artery. For direct comparison of the interaction of β1 integrin-deficient and β1 integrin-positive T cells

within the same spinal cord white matter vascular bed, the injection of β1 integrin-deficient $CD4^+$ T cells (4×10^6 cells in 300 µl 0,9 % NaCl injected in aliquots of 100 µl) was followed by injection of control T cells (4×10^6 cells in 300 µl 0,9 % NaCl injected in aliquots of 100 µl) into the same mouse 1 hour later. This technique might produce artefacts due to the extended observation time necessary in this experimental setup. Thus, the interaction of β1 integrin-deficient and β1 integrin-positive $CD4^+$ T cells was additionally compared in individual mice injected with one T cell population only. The same results were obtained using both experimental approaches.

T cells passing through the spinal cord microvessels (∅ 20-60 µm) and T cells, which visibly initiated contact with the spinal cord microvascular endothelium and thus moved at a slower velocity than the main blood stream were counted during an observation period of 1 minute in frame by frame analysis of the videos using the CapImage software (version 8.3, Dr. Zeintl)[26]. The fraction of T cells initiating contact with the vascular wall was calculated for each microvessel as the percentage of interacting T cells among the total number of T cells passing through a given post-capillary venule during this 1 minute observation window. The rolling fractions and the capture fractions were counted accordingly. Permanently adherent T cells were identified as cells stuck to the vessel wall without moving or detaching from the endothelium within an observation period of ≥ 20 seconds and were counted 10 min, 30 and 60 minutes after T cell infusion (4×10^6 cells/mouse).

Statistical analysis. All statistical analysis was performed using the GraphPad Prism software (version 5.00, GraphPad Software). Data are presented as medians with interquartile ranges. Mann-Whitney U statistics were used for comparisons between different data sets. Asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.005). For analysis of adherent T cells in the IVM analysis mean values were calculated from the values in each animal and the two groups were compared using a Mann-Whitney U test.

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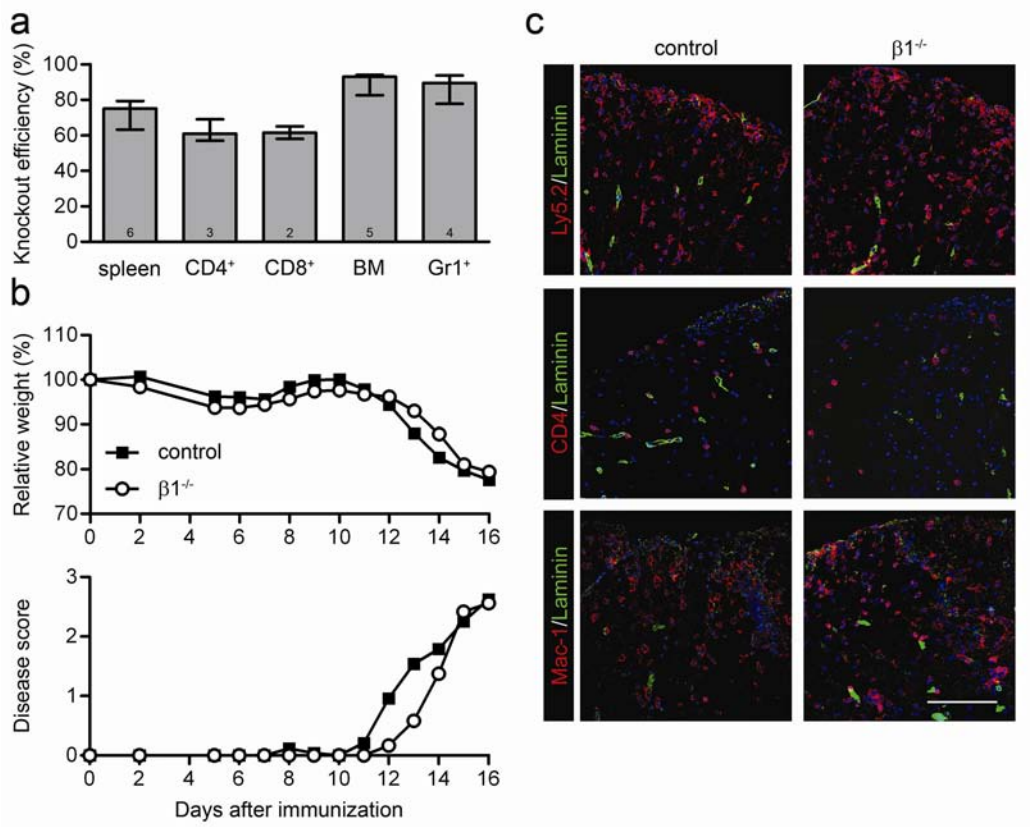


Figure 1: The clinical course of EAE is not altered in β1$^{-/-}$ BM chimeras. (a) Knockout efficiency for the indicated total and MACS-sorted cell populations was determined by southern blotting. The number of samples for each population is given in each bar. Bars represent medians and interquartile ranges. (b) The relative weight normalized to day 0 and the clinical disease score of control and β1$^{-/-}$ BM chimeras with active EAE are shown. Data points indicate the means of 13 mice from 3 independent experiments. Around day 16, mice were sacrificed for histological and flow cytometric analysis. (c) Immunostaining of the spinal cord white matter of control and β1$^{-/-}$ BM chimeras with ongoing active EAE (clinical score 3). Infiltrating leukocytes were stained with Ly5.2, CD4 or Mac-1 antibodies (red), blood vessels with a laminin antibody (green) and nuclei with DAPI (blue) (scale bar, 100 µm).

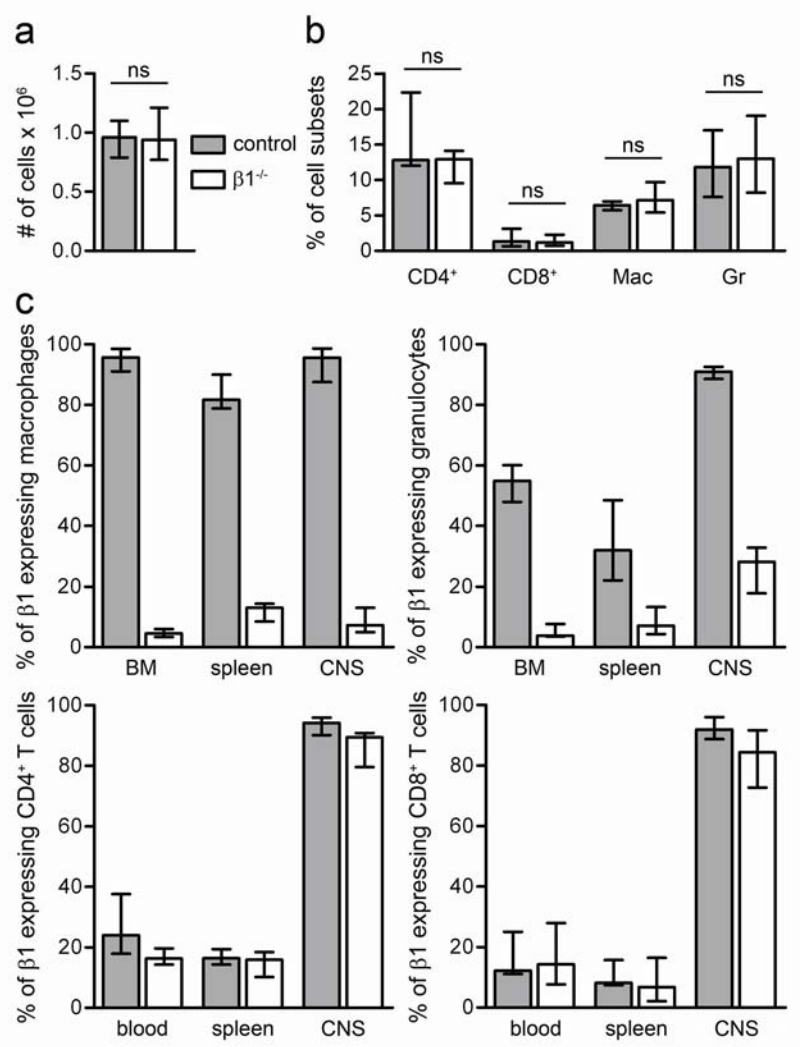


Figure 2: T lymphocytes depend on β1 integrins to enter the CNS. (a) Shown is the total number of leukocytes and microglia cells isolated by density gradient centrifugation from the brain and spinal cord of control and $\beta1^{-/-}$ BM chimeras with ongoing active EAE (average clinical score = 3, n = 5). Shown is the median number of isolated cells per CNS of one animal and the interquartile range. (b + c) The isolated leukocytes were analyzed by flow cytometry. In (b) the relative numbers of $CD4^+$ T cells ($CD4^+$), $CD8^+$ T cells ($CD8^+$), Macrophages (Mac) and Granulocytes (Gr) are shown (controls, n = 8, $\beta1^{-/-}$ n = 9). (c) The β1 expression of the four leukocyte subsets was analyzed by flow cytometry. Macrophages and granulocytes were distinguished by their different expression levels of Gr-1 and Mac-1. Microglia cells are mainly host cell derived and were excluded based on their cell surface expression of CD45.1. Each bar represents at least 5 mice. (b + c) Bars in all panels represent medians and interquartile ranges.

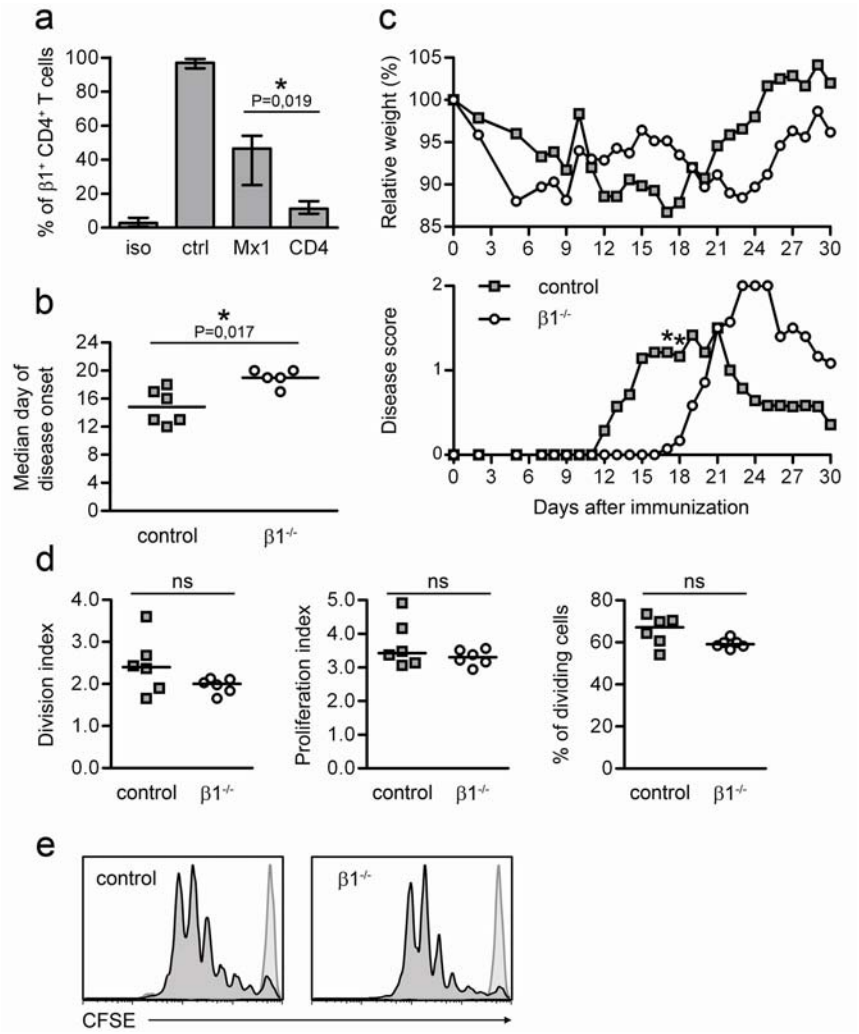


Figure 3: β1 integrin is important for T cell extravasation but not proliferation. (a) Splenocytes from control, $\beta1^{fl/fl}$/MxCre+ or $\beta1^{fl/fl}$/CD4Cre+ mice were stimulated unspecifically *in vitro*. Subsequently, isotype control staining and β1 integrin expression of $CD4^+$ T cells were analyzed by FACS. Graphs show the medians and interquartile ranges of at least 5 animals per group. (b) The median day of disease onset of control and $\beta1^{fl/fl}$/CD4Cre+ mice with active EAE is shown. (c) The relative weight normalized to day 0 and the clinical disease score of control and $\beta1^{fl/fl}$/CD4Cre+ mice with active EAE are shown. Data points indicate the means of 7 mice from 3 independent experiments. (d) Naïve CFSE-labelled $CD4^+$ OT-II.2 T cells were cotransferred with $Ova_{323-339}$ peptide loaded dendritic cells into wildtype mice. After three days cells were isolated from the spleen and analyzed for CFSE-dilution. The division and proliferation index and the percentage of dividing cells were calculated from the generation sizes (n = 6). (e) CFSE stainings from one representative control and $\beta1^{-/-}$ sample are shown. Light grey shaded histograms represent control animals that received dendritic cells without $Ova_{323-339}$ peptide.

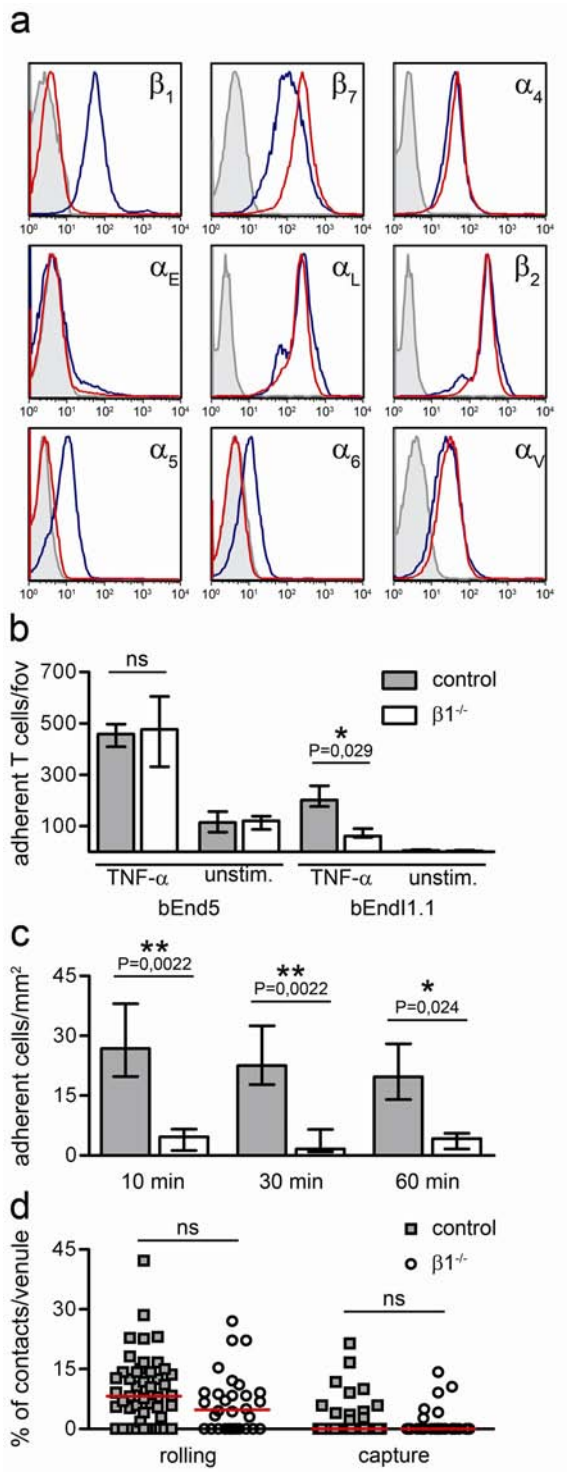
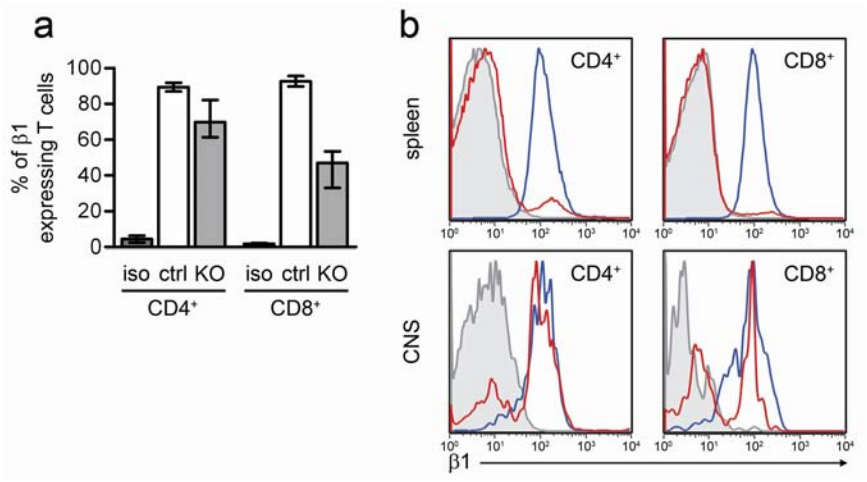
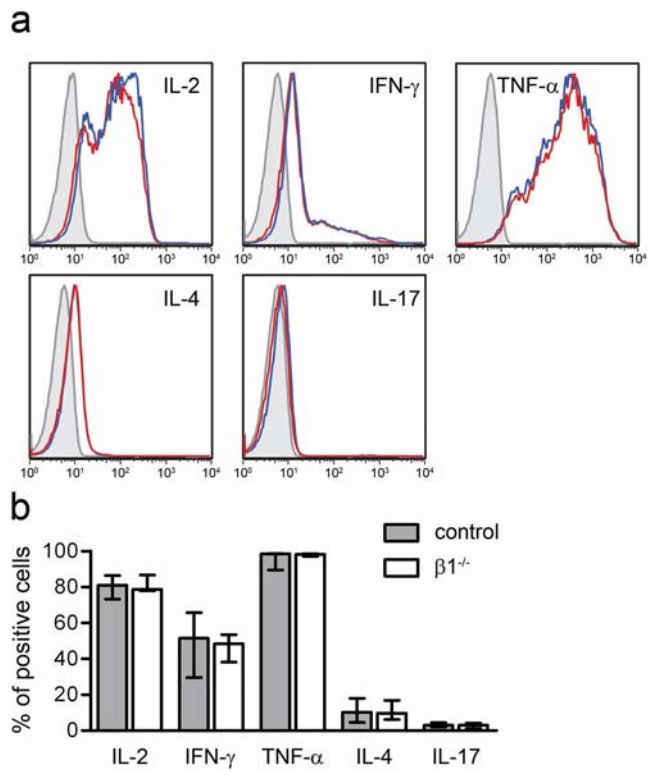


Figure 4: *In vivo* firm adhesion of β1$^{-/-}$ T lymphocytes in the spinal cord microvasculature is dramatically reduced. Proliferating CD4^{+} OT-II.2 transgenic control and β1$^{-/-}$ T lymphocytes were analyzed for their adhesive behaviour *in vivo* and *in vitro*. (a) Integrin expression of proliferating control and β1$^{-/-}$ T lymphocytes was analyzed by FACS. Blue and red histograms represent control and β1$^{-/-}$ T cells, respectively. Isotype control stainings are shown in shaded grey histograms. Histograms are representative of 3 independent experiments. (b) Adhesion of T cell

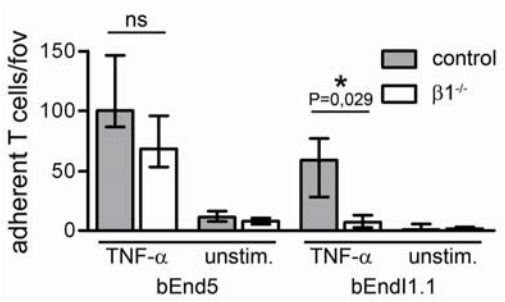
blasts to the endothelioma cell lines bEnd5 (wildtype) and bEndI1.1 (ICAM-$1^{-/-}$). Endothelioma cell lines were stimulated with TNF-α prior to the adhesion assay, which was performed at room temperature. Graphs show the medians and interquartile ranges (n = 4). (c) Firm adhesion of control and $\beta1^{-/-}$ T cell blasts to the spinal cord microvascular wall was analyzed by IVM of the spinal cord white matter microvasculature in wildtype mice with ongoing active EAE. Firm adhesion was analyzed 10 minutes, 30 minutes and 1 hour after infusion of T lymphocytes. Bars represent the medians and interquartile ranges (n = 6). (d) Initial contact events of T cell blasts with endothelial cells were analyzed by IVM. From 6 experiments with control and $\beta1^{-/-}$ T cells, 46 and 30 vessels were analyzed, respectively. Shown is the percentage of rolling or captured T cells among the total number of T cells passing through a given venule during a 1 minute observation period. Each dot represents one vessel, the red line indicates the median.



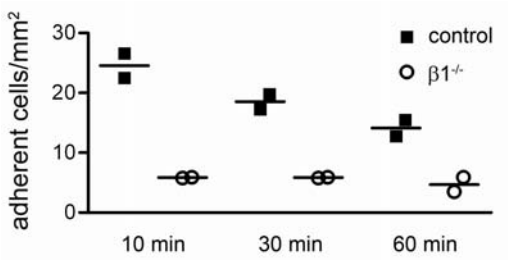
Supplementary Figure 1: $CD4^+$ T lymphocytes isolated from the CNS of $\beta1^{fl/fl}$/CD4Cre+ mice with active EAE are β1 positive. (a) Leukocytes and microglia cells were isolated by density gradient centrifugation from the brain and spinal cord of control (n = 2) and $\beta1^{fl/fl}$/CD4Cre+ (n = 3) mice with ongoing active EAE. The β1 expression of $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry. Bars represent medians and interquartile ranges. (b) Shown are representative histograms of unspecifically stimulated splenocytes and T lymphocytes isolated from the CNS of mice with active EAE gated for CD4 or CD8 expressing cells. Blue and red histograms represent control and $\beta1^{-/-}$ T cells, respectively. Isotype control stainings are shown in shaded grey histograms.



Supplementary Figure 2: The cytokine expression profile of β1 deficient T cells is not altered. The intracellular cytokine expression of control and $\beta1^{-/-}$ T lymphocytes was analyzed by FACS. Cells were isolated after 3 days of *in vivo* proliferation and additionally incubated for 4 hours with ionomycin, PMA and Brefeldin A. (a) Blue and red histograms represent control and $\beta1^{-/-}$ T cells, respectively. Isotype control stainings are shown in shaded grey histograms. Histograms are representative of 4 independent experiments. (b) Shown are the percentages of cytokine expressing T cells. Bars represent medians and interquartile ranges (n = 4).



Supplementary Figure 3: Static adhesion of T cells to endothelioma cell lines is mainly integrin αLβ2 dependent. Adhesion of T cell blasts to the endothelioma cell lines bEnd5 (wildtype) and bEndI1.1 (ICAM-$1^{-/-}$) were analyzed. Endothelioma cell lines were stimulated with TNF-α prior to the adhesion assay, which was performed at 4°C to reduce the contribution of the interaction between LFA-1 and ICAM-1. Graphs show the medians and interquartile ranges (n = 4).



Supplementary Figure 4: *In vivo* firm adhesion of $\beta1^{-/-}$ T lymphocytes to the spinal cord microvasculature is dramatically reduced. To obtain T cell blasts specific for the MOG_{35-55} peptide mice with β1-deficient T cells ($\beta1^{fl/fl}$/Mx1-Cre) were intercrossed with MOG-specific TCR transgenic mice (referred to as 2D2)[18]. Proliferating $CD4^+$ 2D2 transgenic control and $\beta1^{-/-}$ T lymphocytes were analyzed for their adhesive behaviour *in vivo*. Firm adhesion of control and $\beta1^{-/-}$ T cell blasts to the spinal cord microvascular wall was analyzed by intravital microscopy 10 minutes, 30 minutes and 1 hour after infusion. Each dot represents the mean of one experiment, lines indicate the medians (n = 2).

Supplementary Movie 1 (on the enclosed CD): *In vivo* adhesion of control T lymphocytes to the spinal cord microvasculature. Proliferating $CD4^+$ OT-II.2 transgenic control T lymphocytes were analyzed for their adhesive behaviour *in vivo*. The interaction of T cell blasts with endothelial cells was observed by IVM of the spinal cord white matter microvasculature in wildtype mice with ongoing active EAE. The movie shows first the injection of Calcein-AM labelled lymphocytes into the spinal cord white matter microvasculature of anesthetized mice. Afterwards scanning of the microvasculature for adherent T cells 10 minutes and 1 hour after infusion is shown.

Supplementary Movie 2 (on the enclosed CD): *In vivo* adhesion of $\beta1^{-/-}$ T lymphocytes to the spinal cord microvasculature. Proliferating $CD4^+$ OT-II.2 transgenic $\beta1^{-/-}$ T lymphocytes were analyzed for their adhesive behaviour *in vivo*. The movie shows first the visualization of the microvasculature by injection of TRITC-conjugated dextran and then injection of the Calcein-AM labelled lymphocytes. Afterwards scanning of the microvasculature for adherent T cells 10 minutes and 1 hour after infusion is shown. Supplementary movies 1 and 2 show the vascular bed of the same mouse into which first $\beta1^{-/-}$ and then control T cells were injected.

Paper V

3. Analysis of Integrin Functions in Blood

The leukocyte-specific $\beta 2$ and $\beta 7$ integrins as well as almost all members of the $\beta 1$ integrin subfamily play important roles in the hematopoietic system (Sixt *et al.*, 2006). The integrins are required for the homing of hematopoietic stem cells to the fetal liver and bone marrow (Hirsch *et al.*, 1996; Potocnik *et al.*, 2000), hematopoiesis (Bouvard *et al.*, 2001), extravasation of leukocytes at sites of inflammation (Mizgerd *et al.*, 1997; Yednock *et al.*, 1992), and the formation of lymphatic organs such as Peyer's patches, and so on. Consequently, the analysis of integrin function in the hematopoietic system will undoubtedly contribute to our understanding of how the various blood cells move from one site to another, how the cells communicate with each other, and how they exert their functional properties. A serious problem of genetic studies of blood cells can be early embryonic lethality, or the loss of gene expression in several non-hematopoietic cell types relevant for blood cells, such as endothelial cells or stromal cells. This problem can be elegantly circumvented with conditional and inducible gene deletions. Numerous, well-characterized *Cre* mouse lines are available that restrict the gene ablation in a constitutive or temporal manner to specific cell types or compartments of the hematopoietic system. This rich resource makes genetic studies of hematopoiesis very attractive and doable (Table 12.2).

The *Mx1-Cre* mouse strain represents an excellent option to temporally ablate genes in the hematopoietic system (Kuhn *et al.*, 1995). The *Mx1* promoter is activated by interferon-α or -β, whose expression in turn can readily be induced by the intraperitoneal injection of the synthetic double-stranded RNA polyinosinic-polycytidylic acid (pI-pC). Since the *Mx1* promoter is highly active in hematopoietic stem cells, pI-pC injections lead to a rapid and efficient deletion of *loxP*-flanked (also-called floxed) genes in all hematopoietic cells. Unfortunately, a large number of nonhematopoietic cells, such as hepatocytes and endothelial cells, among others, also express the interferon α/β receptors and hence also lose the floxed gene. To restrict the deletion to the hematopoietic compartment, the *loxP/Mx1-Cre* system must be combined with the generation of bone marrow chimeras (Fig. 12.3), which is a rather easy task. This task is usually done with C57BL/6 mice that express the Ly-5.2 and B6SJL that express the Ly-5.1 surface antigen on all leukocytes. The two mouse strains provide an elegant system to generate bone marrow chimeras between C57BL/6 donors (floxed gene/Mx1-Cre bone marrow) and lethally irradiated, wild-type B6SJL hosts, thereby providing a marker to easily exclude remaining cells of the irradiated host from the analysis.

Bone marrow chimeras cannot be generated when a gene mutation leads to embryonic lethality. In such a case, it may still be possible to isolate and transfer hematopoietic stem cells from the fetal liver cells or the adreno-gonadal-mesonephros region into lethally irradiated hosts (Gribi *et al.*, 2006).

Table 12.2 Cre lines for gene deletions in the hematopoietic system

Promoter	Expression pattern	Special notes/reference
Lck	T cells	Onset of deletion during the DN1 stage of thymocyte development (Lee *et al.*, 2001)
CD4	T cells	Onset of deletion during the double positive stage of thymocyte development (Lee *et al.*, 2001)
mb1	B cells	Onset of deletion in early pro-B cells, low frequency of deletion in T cells (Hobeika *et al.*, 2006)
CD19	B cells	Onset of deletion during the pre-B cell stage (Rickert *et al.*, 1997)
CD21	B cells	Onset of deletion in mature B cells, high deletion in the ovary (Kraus *et al.*, 2004)
hCD2	T cells and B cells	Onset of deletion before the DN4 stage of thymocyte development and in early pro-B cells, mosaic expression in testis (de Boer *et al.*, 2003)
Vav	All cells of hematopoietic system	Expression in ovaries and testis (de Boer *et al.*, 2003), endothelial cells, and testis (Georgiades *et al.*, 2002)
CD11c	Dendritic cells (DC)	Cre expression is induced by tamoxifen, only a few $CD11c^+$ DCs are expressing Cre (Probst *et al.*, 2003)
GATA1	erythroid cells and mast cells	Onset of deletion at the time of Ter119 expression, deletion also in megakaryocytes and eosinophils (Jasinski *et al.*, 2001)
LysM	granulocytes	Partial deletion in macrophages and splenic $CD11c^+$ DCs (Clausen *et al.*, 1999)
CD11b	peritoneal macrophages, mature osteoclasts	Partial deletion in macrophages and granulocytes, deletion in B and T cell subsets (Ferron and Vacher, 2005)
Pf4	megakaryocytes	Complete and specific deletion in the megakaryocytic lineage (Tiedt *et al.*, 2006)

3.1. Generation of bone marrow chimeras

3.1.1. Materials

Borgal: 7.5% antibiotic solution for small animals (commercially available from Hoechst).

pI-pC: Poly(I)-poly(C) (Amersham Biosciences); the stock solution with a final concentration of 2 mg/ml is prepared with PBS according to manufacturer's instructions and stored at –20°.

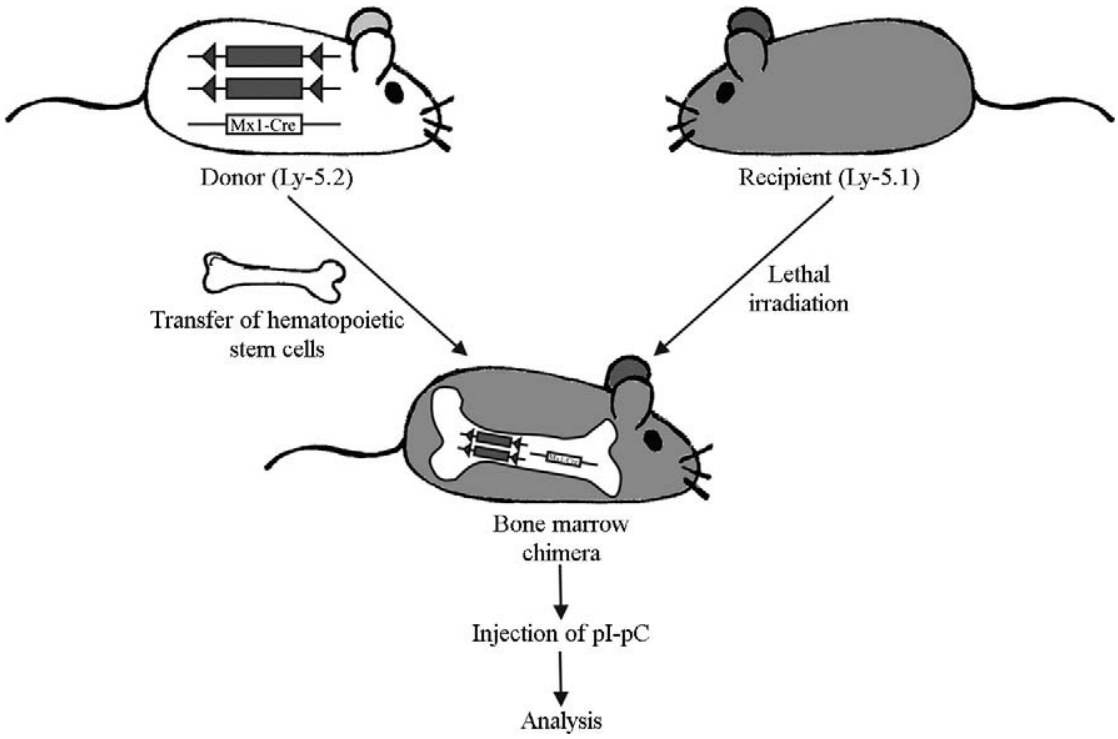


Figure 12.3 Generation of bone marrow chimeras using the *Mx1*-Cre/loxP system. Bone marrow from a Ly-5.2–positive donor mouse carrying the target gene flanked by *loxP sites*, and the *Mx1-Cre* transgene is transferred into an irradiated, Ly-5.1–positive wild-type recipient mouse. Four weeks after bone marrow transfer the hematopoietic system of the recipient mouse is fully reconstituted and the knockout can be induced by repeated pI-pC injections.

3.1.2. Procedure

1. The ideal recipients are 8- to 12-week-old B6SJL mice that are sex-matched with the donor mice (to prevent an immune response against donor cells). The recipient mice are γ-irradiated at 1000 rad with an X-ray machine.
2. Single-cell suspensions of bone marrow cells are prepared (see Section 3.2), resuspended in PBS, and adjusted to 5×10^6 cells/ml PBS.
3. The recipient mice are immobilized in custom-made conical metal chambers (Fig. 12.4) and the tails of the recipient mice are warmed for 30 sec in a 50° water bath (to increase blood flow and vessel size). Around 200 μl of the bone marrow cell suspension (1×10^6 cells/mouse) are injected with a 30G needle into the lateral tail vein.
4. The first 2 weeks after the transplantation, 1 ml of Borgal solution is added to 500 ml of drinking water; this will prevent infections in the irradiated, immunocompromised mice.
5. Four weeks after the bone marrow transplantation, the hematopoietic system of the recipient mouse is fully reconstituted with donor cells (this can be easily checked by determining the number of Ly-5.1–positive

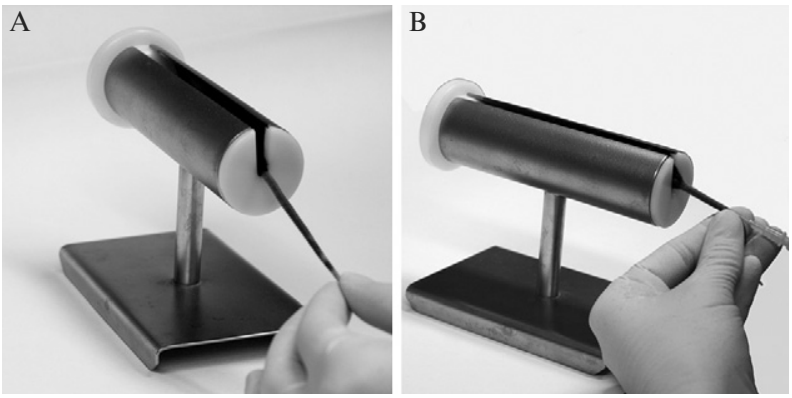


Figure 12.4 **Injection of bone marrow cells into a recipient mouse. (A) The mouse is immobilized in a conical metal chamber. (B) Bone marrow is injected into the lateral tail vein of the prewarmed tail with a 30G needle.**

cells in the peripheral blood using the flow cytometer). To induce the *Mx1-Cre*–mediated gene ablation, the pI-pC stock solution is diluted to 0.5 mg/ml with PBS, and 500 μl is then injected intraperitoneally. The pI-pC injection is repeated 2 and 4 days later.

6. It is necessary to wait at least 4 weeks before attempting *in vivo* analysis of the hematopoietic system to exclude effects caused by the pI-pC–triggered interferon production.

3.2. Standard flow cytometric analysis of cell surface receptors

Analysis of the cellular composition of hematopoietic tissues/compartments is done by determining the expression of cell surface receptors unique for distinct subsets of blood cells with the fluorescence-activated cell sorter (FACS).

3.2.1. Materials

Isoflurane: Isoba, a ready-to-use solution is commercially available (Essex Tierarznei)

Heparin: 20 U/ml heparin (Sigma) in TBS (50 m*M* Tris, 10 m*M* NaCl, pH 7.5).

ACK buffer: Ammonium chloride potassium phosphate buffer: 150 m*M* NH_4Cl, 1 m*M* $KHCO_3$, 0.1 m*M* EDTA, pH 7.3.

FACS-PBS: PBS supplemented with 1% BSA.

70-μm cell strainer: sterile 70-μm nylon cell strainer (BD Falcon).

Propidium-iodide: Propidium-iodide (Sigma) is prepared as a stock solution of 50 μg/ml in PBS, which can be stored at 4° in the dark.

3.2.2. Isolation of peripheral blood leukocytes

Procedure

1. The mouse is anesthetized by putting it into a beaker that contains a tissue drenched with 1 to 2 ml of isoflurane. When movements cease, the mouse should be immediately removed from the beaker. Extended exposure to isoflurane stops the heart beat and makes blood collection impossible. Around 50 μl of blood are collected with a heparin-soaked glass capillary from the retro-orbital venous plexus. The blood is resuspended in 500 μl PBS and centrifuged for 5 min at 400×*g*. The supernatant is carefully removed and discarded.
2. The cell pellet is resuspended in 500-μl ACK buffer, incubated for 5 min at RT, and centrifuged again. The supernatant, which should appear reddish due to the lysis of the erythrocytes, should be carefully removed and discarded. The cell pellet is resuspended in 100 μl of PBS. The amount of cells should be sufficient for two to three flow cytometry stainings.

3.2.3. Isolation of bone marrow cells

Procedure

1. The femurs of a mouse are carefully dissected and the muscles are removed by rubbing each femur with a Kleenex tissue. If cells should be sterile (e.g., for generation of dendritic cells), the femurs must stay intact during the removal of the muscle tissue and are incubated after muscle removal for 2 min in 70% ethanol and then rinsed in PBS.
2. Both ends of the femur are abscised with a sharp scalpel and the bone is subsequently flushed from both ends with 10-ml ice-cold PBS using a 10-ml syringe and a 23G needle. The isolated cell clumps are broken up into a single cell suspension by vigorous pipetting and then transferred through a 70-μm cell strainer into a 50-ml tube.

3.2.4. Isolation of cells from lymphatic tissues

Procedure

1. The organs of interests (such as thymus, spleen, lymph nodes, or Peyer's patches) are isolated, freed from surrounding connective tissue as much as possible, rinsed with PBS to remove blood and put into a cell strainer, which is placed in a Petri dish together with 10 ml of ice-cold PBS. To keep the cell strainer at 4°, the Petri dish is kept on ice.
2. Each organ is homogenized by gently squeezing it with the piston of a plastic syringe through the cell strainer. The cell suspension is transferred from the Petri dish into a 50-ml tube by filtering it again through the cell strainer. To obtain all remaining cells floating in the Petri dish, the Petri dish is rinsed again with 10 ml of ice-cold PBS and the suspension is combined with the other cells in the 50-ml tube.

3.2.5. Immunofluorescence staining of suspended hematopoietic cells

Procedure

1. The cells are pelleted by centrifugation at 300×*g*, resuspended in an appropriate volume (2 to 10 ml) of ice-cold PBS, and counted.
2. If a centrifuge capable of centrifuging 96-well plates is available, the staining is most conveniently performed in 96-well round-bottom plates. For each staining, 1×10^6 cells or 100 μl of peripheral blood suspension are pipetted into each well. The plate is centrifuged for 5 min at 300×*g*. The supernatant is removed and the pellet resuspended in 50 μl of FACS-PBS with the first antibody.
3. After a 30-min incubation at 4° in the dark, 200 μl FACS-PBS are added to each well. Subsequently, the cells are pelleted by centrifugation (300×*g*). Upon removal of the supernatant, the cell pellet is resuspended in 50-μl of FACS-PBS containing the secondary antibody.
4. The cell suspension is incubated for 15 min at 4° in the dark, and then diluted by adding 200 μl of FACS-PBS and finally centrifuged again at 300×*g* and RT. The supernatant is removed and the cell pellet resuspended in 200 μl FACS-PBS. Immediately before measuring, the fluorescent signal with the flow cytometer 10 μl of propidium-iodide (50 μg/ml) is added to each sample, which allows identification of dead cells. After brief vortexing, the fluorescence is measured with a cytometer.

Table 12.3 lists a number of surface markers that are commonly used singly or in combination to analyze the hematopoietic system. Please note that many of the markers are also useful for immunostaining of tissue sections of lymphatic organs, thereby also allowing assessment of the spatial distribution of blood cell types.

3.3. Flow cytometric lacZ staining of hematopoietic cells

The bacterial *lacZ* gene is an elegant means to examine the expression of a gene of interest. If knockout constructs are engineered to replace the disrupted gene with a *lacZ* gene, *Cre*-driven excisions can easily be monitored histochemically. Expression of the *lacZ* gene results in the production of β-galactosidase (β-gal), which can be readily detected by a number of chromogenic or fluorogenic β-gal substrates (Rotman *et al.*, 1963). This allows visualization of the gene deletion by histochemically staining tissue sections (see Section 4.4) or whole-mount embryos. Importantly, lacZ-positive cells can also be detected by flow cytometry. In the protocol described here, the lacZ measurement can be combined with immunofluorescent staining allowing to precisely determining the identity of the lacZ-positive cells.

Table 12.3 Surface antigens supporting analysis of the hematopoietic system

Surface markers	Cell type
Spleen	
$CD4^+$	T helper cells
$CD8^+$	Cytotoxic T cells
IgD^+	B cells
$NK1.1^+$	NK and NKT cells
Ter-119	Erythroblasts
$B220^+$ CD21high CD23low	Marginal zone B cells
CD23high CD21low	Follicular B cells
Bone marrow	
$B220^+$ IgM^-	Pre–pro B cells and later developmental stages
$CD19^+$	Pro B cells and later developmental stages
B220low IgM^+	Immature B cells
B220high IgM^+ IgD^+	All mature B cells
Mac-1^+ Gr-1^+	Granulocytes
Mac-1^+ Gr-1^-	Monocytes
Ter-119	Erythroblasts
$NK1.1^+$	NK and NKT cells
$CD4^+$	T helper cells
$CD8^+$	Cytotoxic T cells
Lin^- c-kit^+ Sca-1high	Hematopoietic stem cells
Lymph nodes	
$B220^+$ $CD19^+$	B cells
$CD4^+$	T helper cells
$CD8^+$	Cytotoxic T cells
Thymus	
Lin^-	$B220^-$, $CD4^-$, $CD8^-$, $Mac1^-$, Gr-1^-, $Ter119^-$
Lin^- $CD25^-$ $CD44^+$	DN1
Lin^- $CD25^+$ $CD44^+$	DN2
Lin^- $CD25^+$ $CD44^-$	DN3
Lin^- $CD25^-$ $CD44^-$	DN4
$CD4^-$ $CD8^-$	Double-negative T cells
$CD4^+$ $CD8^+$	Double-positive T cells
$CD4^+$	CD4 single-positive T cells
$CD8^+$	CD8 single-positive T cells
Peyer's patches	
$CD4^+$	T-helper cells
$CD8^+$	Cytotoxic T cells

3.3.1. Materials

FDG: Fluorescein Di-(B-D-galactopyranoside) (Sigma). Dissolve 5 mg of FDG in a mixture of 304.8 μl of H_2O, 38.1 μl of dimethyl sulfoxide (DMSO) (Sigma), and 38.1 μl of ethanol (8:1:1) to obtain a 20-m*M* solution. Add 3429 μl of H_2O (10:1) as soon as the FDG is completely dissolved to obtain a 2-m*M* working solution. Store aliquots of around 300 μl at –20° in the dark.

Procedure

1. If immunofluorescence staining is combined with lacZ staining, the cells are stained as described (see Section 3.2.5). Use around 3×0^6 cells per staining, since the described method can lead to cell mortality and hence loss of cells. After staining, the cells are pelleted by centrifugation at 300×*g* at RT and resuspended in 20 μl of FACS-PBS.
2. The cell suspension is admixed with 20 μl FDG to each sample, and incubated for 75 sec in a 37° water bath to allow FDG uptake. The uptake is then quickly terminated by the addition of 200 μl of ice-cold FACS-PBS. Since exceeding the 75-sec incubation time with FDG significantly increases cell death, avoid handling more than five samples at the same time.
3. The cell suspensions are incubated for 2 h on ice in the dark and then measured in the flow cytometer. Excluding dead cells by the addition of propidium-iodide is recommended (see Section 3.2.5).

Flow cytometry and histology allow determination of the number, size, distribution, and differentiation of distinct cell populations. Additional assays further help to pinpoint potential functional defects of blood cell lineages. Standard procedures include *in vitro* colony formation assays with stem/progenitor cells, which permit evaluation of the formation of erythroid and myeloid cells; co-culture assays of bone marrow precursors with stromal cells that permit determination of differentiation and proliferation of B cells; and bone marrow precursor cell differentiation into dendritic cells, which permits determination of T-cell proliferation and dendritic cell analysis *in vitro*.

3.4. Generation of dendritic cells from bone marrow

Bone marrow–derived dendritic cells represent an elegant tool to study antigen presentation and T-cell proliferation. Furthermore, dendritic cells are also used to study cell migration, polarity, phagocytosis, adhesion, and podosome formation (Calle *et al.*, 2006), among other processes. Our

protocol for generating dendritic cells is a slightly modified procedure originally described by Lutz *et al.* (1999).

3.4.1. Materials

R10 medium: RPMI-1640 (Gibco) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 m*M* of L-glutamin (all from PAA), and 10% heat-inactivated FCS (Gibco).

GM-CSF: rmGM-CSF (Peprotech)—20 ng/ml correspond to 200 U/ml. Alternatively, cell culture supernatant collected from Ag8653 myeloma cells transfected with the murine GM-CSF cDNA can be used (Zal *et al.*, 1994).

Freezing medium: Heat-inactivated FCS is supplemented with 10% DMSO. Freezing medium is always freshly prepared.

LPS: Lipopolysaccharides from *Escherichia coli* (Sigma). A stock solution of 1 mg/ml is prepared with PBS.

Procedure

1. At day 0, bone marrow cells are isolated as described in Section 3.2.3, resuspended in 10 ml of R10 medium, and counted.
2. Around 2.5×10^6 cells in a total volume of 10 ml of R10 medium containing 20 ng/ml GM-CSF or 10% GM-CSF supernatant are transferred to a 10-cm Petri dish. Dendritic cells should be cultured in bacterial-grade Petri dishes, since they strongly adhere to the plastic surface of cell culture dishes, which prevents their differentiation.
3. Ten milliliters of R10 containing 20 ng/ml GM-CSF or 20% GM-CSF supernatant are added at day 3 to the bone marrow culture. At day 6, 10 ml of the medium are carefully removed by tilting the plate slightly and slowly sucking off the medium. Stirring and shaking should be avoided when taking the plates out of the incubator. The removed medium is replaced with 10 ml of fresh R10 containing 20 ng/ml of GM-CSF or 20% GM-CSF supernatant.
4. At day 8, one of the following two possibilities is selected. First, the immature dendritic cells can be frozen for later usage: The cells from a 10-cm cell culture dish are collected by gentle pipetting, centrifugation at 300×*g*, and the cell pellet is resuspended in 1 ml of freezing medium. The 1-ml suspension is transferred into a freezing tube and quickly transferred to a –80° freezer. Second, the immature dendritic cells are brought to maturation: The cells from a 10-cm culture dish are collected as described above, resuspended in 10 ml of R10 containing 20 ng/ml GM-CSF or 10% GM-CSF and 200 ng/ml LPS, and cultured overnight in a 6-cm cell culture dish.

3.5. *In vitro* T-cell proliferation assay

Several possibilities are available to trigger and determine T-cell proliferation. First, T cells can be treated with ionophores and phorbol esters (e.g., ionomycin and phorbol-12-myristate-13-acetate [PMA]). Ionomycin increases the intracellular calcium concentration by facilitating calcium transport through the plasma membrane. Using this procedure, protein kinase C (PKC) can be activated in a phospholipase C–independent manner. Additionally, phorbol esters such as PMA activate PKC by mimicking the action of diacylglycerol (DAG). Second, T-cell proliferation can also be induced by cross-linking T-cell receptors (TCR) with antibodies to the CD3 T-cell coreceptor. This treatment mimics antigen-dependent TCR cross-linking, and thereby evokes signaling from the TCR. Third, antigen-dependent T-cell stimulation can be rather easily examined by crossing the integrin knockouts with a mouse strain expressing a transgenic TCR. A transgenic MHC class II–restricted TCR is expressed in the OT-II.2 mouse strain, which generates $CD4^+$ T cells specific for the $OVA_{323–339}$ peptide from chicken ovalbumin (Barnden *et al.*, 1998). Upon intercrossing gene-targeted mice with OT-II.2 transgenic mice, mutant $CD4^+$ T cells can be stimulated by co-culture with $OVA_{323–339}$–loaded dendritic cells. A similar system exists with the OT-I mice for $CD8^+$ T cells (Clarke *et al.*, 2000).

We usually monitor division of the stimulated T cells by determining the dilution of the carboxy-fluorescein diacetate succinimidyl ester (CFSE). CFSE diffuses freely into cells where it is converted by esterases into a membrane-impermeant dye, which becomes covalently bound to cellular proteins and is then capable to emit a fluorescence signal that can be assessed with a flow cytometer equipped with 488-nm excitation and emission filters. The CFSE fluorescence signal is halved during each cell division.

An alternative procedure to monitor cell division is measuring the incorporation of H^3-thymidine (Krishnamoorthy *et al.*, 2006), which is more sensitive but does not permit the analysis of the proliferation on a single-cell basis.

3.5.1. Materials

Magnetic sorting of T cells: $CD4^+$ T Cell Isolation Kit, LS MACS columns, and magnetic separation unit (all from Miltenyi).

ACK buffer: 150 m*M* NH_4Cl, 1 m*M* $KHCO_3$, 0.1 m*M* EDTA, pH 7.3.

MACS buffer: PBS containing 0.5% bovine serum albumin and 2 m*M* EDTA. The MACS buffer is degassed by applying vacuum or sonification.

anti-CD3e: Purified hamster anti-mouse CD3e monoclonal antibody (BD Biosciences, clone 145–2C11).

CFSE: Carboxyfluorescein diacetate succinimidyl ester (CFDA, SE) (Molecular Probes). A 6-m*M* stock solution is prepared in DMSO and stored at – 20° in the dark.

$OVA_{323–339}$ peptide: The sequence of the $OVA_{323–339}$ peptide is H-ISQAVH AHAEINEAGR-OH. A stock of 2 mg/ml is prepared in PBS and 100 μl aliquots are stored at –20°.

Dendritic cells: 1.5×10^6 bone marrow–derived dendritic cells matured overnight with 200 ng/ml LPS (see Section 3.4) are resuspended in R10 medium to a final concentration of 0.4×10^6/ml.

PMA: Phorbol-12-myristate-13-acetate (Calbiochem). A 1-mg/ml stock is prepared in DMSO, and 20-μl aliquots are stored at –20°. A 1:300 dilution in R10 medium is the working dilution and is always freshly prepared.

Ionomycin: Ionomycin calcium salt from *Streptomyces conglobatus* (Calbiochem). A 1-mg/ml stock is prepared in DMSO and 20-μl aliquots are stored at 4° in the dark. A 1:50 dilution in R10 medium is the working dilution and is always freshly prepared.

3.5.2. Magnetic sorting of T cells

For high sorting purities, it is critical to cool the cells and solutions on ice.

Procedure

1. The spleen is dissected and a single cell suspension prepared (see Section 3.2.4). The cell suspension is pelleted by centrifugation at 300×*g*, and then thoroughly resuspended in 5 ml of ACK buffer and incubated at RT for 5 min. Afterward, 10 ml of PBS is added to the cell suspension and centrifuged.
2. After centrifugation, the cell pellet is resuspended in 10 ml of MACS buffer, counted, and 4×10^7 cells are centrifuged. The cells are labeled and sorted using the $CD4^+$ T Cell Isolation Kit according to the manufacturer's instructions. With this kit, non-$CD4^+$ T cells are labeled indirectly magnetically with a cocktail of biotin-conjugated antibodies and magnetic anti-biotin beads, and are subsequently depleted. Therefore, the isolated $CD4^+$ T cells remain untouched.

3.5.3. CFSE staining of T cells

Procedure

1. The sorted $CD4^+$ T cells are resuspended in PBS in a concentration of 1×10^7/ml. The CFSE stock solution is diluted 1:240 with PBS and added 1:50 to the cell suspension to reach a final concentration of 0.5 μM. The mixture is briefly vortexed to ensure even distribution. The T-cell suspension is incubated for 10 min at RT in the dark.
2. The reaction is stopped by the addition of 5 ml of ice-cold R10 medium. The cells are centrifuged at 300×*g*, and then taken up in R10 medium to obtain a final concentration of 1×10^6/ml.

3.5.4. Proliferation assay

Procedure

1. Fifty microliters of 10 μg/ml of anti-CD3e in PBS is added to three of the positive control wells (Fig. 12.5) of a 96-well, round-bottom plate, and the plate is then incubated for 1 h at 37°.
2. The dilutions of the OVA peptide are prepared according to Fig. 12.5 in a 12-well plate. Each dilution step must be thoroughly mixed before proceeding to the next dilution.
3. One hundred microliters of the peptide dilution are added to the respective wells (Fig. 12.5). The wells in row 5, which lack OVA peptide, serve as negative controls. Next, 50 μl of dendritic cells (DCs) (0.4×10^6/ml) and 50 μl of CFSE-labeled T cells (1×10^6/ml) are then added to each well.
4. The anti-CD3e–coated wells are washed carefully two times with 200 μl of PBS. Then 100 μl of R10 medium are supplemented with 2 μl of PMA (1:300 dilution) and 100 μl of CFSE-labeled T cells (1×10^6/ml) are added to each well. This will result in a final PMA concentration of 33 ng/ml. To the remaining "positive control wells," 100 μl of R10 medium is supplemented with 2 μl of PMA (1:300 dilution) and 2 μl of ionomycin (1:50) dilution, and 100 μl CFSE-labeled T cells (1×10^6/ml) is added. This will result in a final ionomycin concentration of 200 ng/ml.
5. The plate is incubated for 72 h at 37° in a regular cell-culture incubator. For each measurement, the cells from three wells are collected and pooled by thorough pipetting. The CFSE signal is determined in the fluorescein channel of a flow cytometer. It is often convenient to analyze additional parameters such as activation markers, for example. In this case, an immunofluorescence staining of the cells with antibodies against markers such as CD25, CD44, and CD69 antibodies must be performed as described in Section 3.2.5 before the flow cytometric analysis.

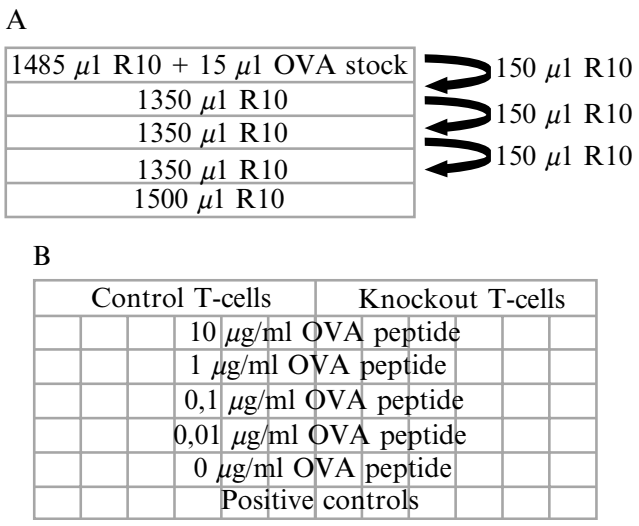


Figure 12.5 Pipetting scheme for peptide dilutions and T-cell proliferation assays.

3.6. *In vivo* T-cell proliferation assay

The intravenous injection of T cells together with antigen-loaded dendritic cells into mice is the most physiological way to examine antigen-dependent proliferation of TCR transgenic T cells. The two injected cell types will move to the spleen where they interact in a physiological environment and trigger T-cell proliferation.

3.6.1. Materials

Magnetic sorting of T cells, ACK buffer, MACS buffer, CFSE, and $OVA_{323–339}$ peptide. See Section 3.5.

Dendritic cells: 2.5×10^6 bone marrow–derived dendritic cells/mouse, matured overnight with 200 ng/ml of LPS. They are prepared as described in Section 3.4.

3.6.2. Magnetic sorting of T cells

Magnetic sorting of the cells is performed as described in Section 3.5. Around 3×10^6 T cells are needed per mouse. Therefore, 5×10^7 splenocytes should be sorted to obtain enough cells for three experiments.

3.6.3. CFSE staining of T cells

Procedure

1. The sorted $CD4^+$ T cells are resuspended in PBS to obtain a concentration of 2×10^7/ml. The CFSE stock solution is diluted 1:600 with PBS and added 1:1 to the cell suspension to obtain a final concentration of 5 μM. The mixture is vortexed briefly to ensure even distribution. The T-cell suspension is incubated for 10 min at 37°.
2. The reaction is stopped by the addition of 10 ml of ice-cold R10 medium. The cells are then centrifuged at $300 \times g$ and taken up in PBS to obtain a final concentration of 15×10^6/ml.

3.6.4. Proliferation assay

Procedure

1. Around 5 to 15×10^6 matured dendritic cells are resuspended in 3 ml of R10 medium containing 20 ng/ml of GM-CSF or 10% GM-CSF supernatant and 20 μg/ml of Ova peptide. They are transferred in a 50-ml tube and incubated for 4 h in a cell culture incubator. A control batch of dendritic cells should be incubated without the peptide. After the incubation, the dendritic cells are washed twice with 10 ml of PBS, and are taken up in PBS after a subsequent centrifugation to obtain a dendritic cell concentration of 12.5×10^6/ml.

2. About 3×10^6 of CFSE-labeled OT-II T cells resuspended in 200 μl PBS are injected into the tail vein of a mouse (see Section 3.1). Sex-matched mice of 6 to 10 weeks of age are used as recipients. Around 4 h later, 2.5×10^6 of Ova-loaded dendritic cells resuspended in 200 μl of PBS are injected into the same recipients.
3. Three days later the spleens are dissected and a single cell suspension is prepared as described in Section 3.2.4. To analyze T-cell proliferation by flow cytometry, 5×10^6 splenocytes are stained with the desired markers and measured in a flow cytometer. Care should be taken to adjust the gains and compensations of the cytometer to avoid leakage of the CFSE signal into other channels. Furthermore, it is important to include a control with unloaded dendritic cells to determine the fluorescence intensity of nondividing T cells.

3.7. Induction of experimental autoimmune encephalomyelitis in C57/BL6 mice

Experimental autoimmune encephalomyelitis (EAE) is a very well-established animal model representing the human disease multiple sclerosis (MS). Over the years it has become increasingly clear that integrins play an important role for the development of both EAE and MS (Yednock *et al.*, 1992). Nonetheless, the exact function of integrins during the disease-relevant processes of T-cell activation and proliferation and extravasation into the brain are still not clear. Aspects like T-cell adhesion to endothelial cells can be analyzed *in vitro* in adhesion assay (Reiss *et al.*, 1998), as well as *in vivo* by intravital microscopy (Vajkoczy *et al.*, 2001). But to analyze the overall contribution of genetically altered hematopoietic cells to the development of EAE, it is necessary to induce EAE in knockout mice. This has been done in many mutant mouse strains that are deficient in adhesion molecules with a potential role in inflammatory conditions (Kanwar *et al.*, 2000; Kerfoot *et al.*, 2006).

3.7.1. Materials

Complete Freund's Adjuvant (CFA): 50 mg of *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories) is first thoroughly ground with 1 ml of Incomplete Freund's Adjuvant (Sigma) using mortar and pestle, and then suspended with an additional 9 ml of Incomplete Freund's Adjuvant to obtain a total volume of around 10 ml, and finally stored at 4°. The suspension must always be homogenized with a syringe prior to usage.

$MOG_{35–55}$ peptide: The sequence is H-MEVGWYRSPFSRVVHLYRNGK-OH. A stock of 2 mg/ml is prepared in PBS and 500-μl aliquots are stored at –20°.

Syringes, adaptor, and needles: 2× 1-ml glass tuberculin syringes with Luer lock tip and a female Luer lock to female Luer lock syringe adaptor (Sigma), and disposable 23G and 26G needles.

Pertussis toxin: Pertussis toxin (List Biological Laboratories). The vial containing pertussis toxin powder is reconstituted according to the manufacturer's instructions to a 100 ng/μl-stock solution and stored at 4°.

3.7.2. Procedure

1. For every mouse, 100 μl MOG in PBS and 100 μl CFA are mixed using two syringes that are connected with an adaptor. The two solutions are mixed until a white emulsion forms, which is hard to push from one syringe to the other. The mixed emulsion is kept on ice. The mouse is anesthetized with Isoflurane and 100 μl of the emulsion are injected with a 23G needle subcutaneously into the tail base as well as the neck of the mouse.
2. To prepare 400 ng of pertussis toxin, add 4 μl of the stock solution to 200 μl PBS. The toxin is injected intraperitoneally using a 26G needle.
3. The mice are assessed daily for clinical EAE symptoms and weight loss. Clinical scoring should be performed blinded if possible, and according to the established classification as, for example, described by Krishnamoorthy *et al.* (2006).

3.8. Mononuclear cell isolation from the central nervous system

The first step to analyze the diseased mice is often histological analysis of the central nervous system (CNS). With H&E and luxol fast blue staining of CNS sections, infiltration of immune cells and demyelination of nerves can be assessed (Krishnamoorthy *et al.*, 2006). Additionally, mononuclear cells can also be isolated from the CNS by a Percoll density-gradient centrifugation, and subsequently be analyzed by flow cytometry.

3.8.1. Materials

Percoll: Medium for density centrifugation with a density of 1.130 g/ml (Fluka).

10× PBS: 80 g NaCl, 2 g KCl, 2 g KH2PO4 and 14.4 g Na2HPO4 × 2 H_2O are dissolved in 1 liter of distilled water.

Medium: DMEM cell culture medium (Gibco), supplemented with 2% FCS and 2.5% HEPES (1-M stock solution, pH 7.0).

Procedure

1. For each gradient, 9 ml of Percoll are mixed with 1 ml of 10× PBS to obtain an isotonic Percoll stock solution (IPSS) with a density of 1.123 g/ml. The IPSS can be stored at 4° for about 2 weeks. For each gradient, 4 ml of IPSS are mixed with 2.23 ml of PBS to obtain Percoll with a density of 1.08 g/ml.

2. All glass- and plasticware should be coated with serum-containing medium by wetting them once. This prevents cells sticking to surfaces.
3. The brain is carefully dissected out from the skull. The spinal column is transected in the cervical and lumbar part. The spinal cord is flushed out with 10 ml of PBS by inserting the end of a truncated 18G needle connected to a 10-ml syringe into the spinal column and applying gentle pressure with the syringe. Both the brain and spinal cord are rinsed with PBS to remove blood and put into a cell strainer, which is placed in a Petri dish together with 5 ml of ice-cold medium. To maintain the tissue at 4°, the Petri dish is kept on ice.
4. The brain is homogenized by gently squeezing it with the piston of a plastic syringe through the cell strainer. The piston should be only pressed, not ground, since grinding will result in increased mortality of T lymphocytes. The cell suspension is transferred from the Petri dish into a 15-ml tube by filtering it again through the cell strainer. To obtain all remaining cells floating in the Petri dish, the Petri dish is rinsed again with 5 ml of ice-cold medium, and then the suspension is combined with the other cells in the 15-ml tube. The volume is adjusted to 12.5 ml with the medium, and the suspension is mixed with 5.4 ml of IPSS.
5. Five milliliters of Percoll with a density of 1.08 g/ml are carefully underlayed by putting the tip of a 5-ml plastic pipette filled with 5.5 ml of the latter to the very bottom of the tube and slowly releasing the liquid. The last 0.5 ml should not be released from the pipette since this would often result in disturbance of the layering.
6. The gradient is centrifuged at 1200×*g* for 30 min at 20°. Afterward, the myelin debris, which is on top, is sucked away. The mononuclear cells that accumulated at the interface of the two solutions are sucked away with a medium-coated Pasteur pipette and transferred into a medium-coated 50-ml Falcon tube. The recovered suspension is filled up to 50 ml with medium and centrifuged at 300×*g* for 10 min at 4°. The pelleted cells can now be resuspended—for instance in FACS-PBS—and analyzed by flow cytometry as described in Section 3.2.5.

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