β1 integrins: zip codes and signaling relay for blood cells

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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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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

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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*.

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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
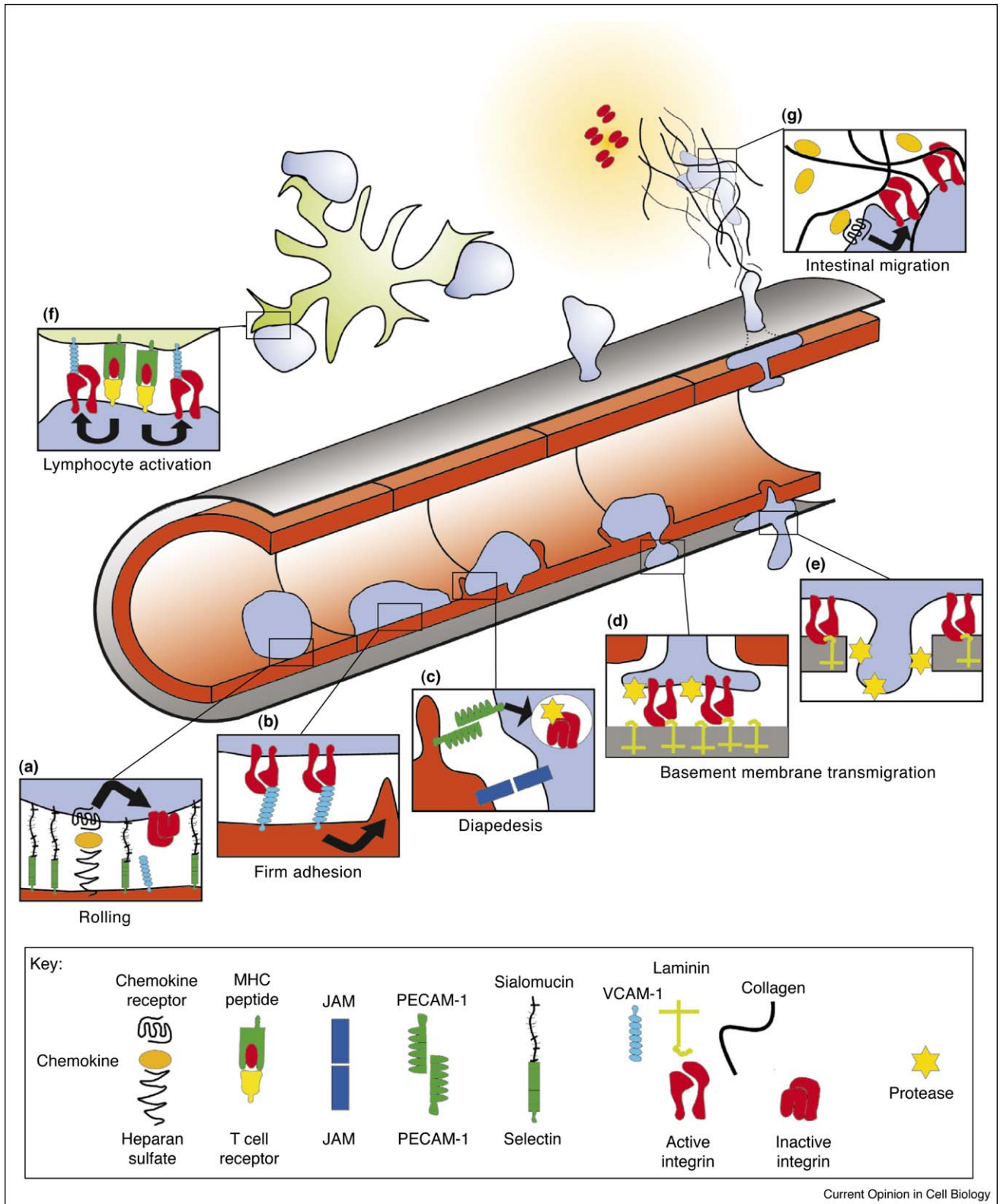
Roles of β1 integrins during extravasation, interstitial migration and lymphocyte activation. **(a)** During selectin-mediated rolling, the extravasating cell senses chemokines that are immobilized on the surface of the endothelium, leading to the inside-out activation of integrin α4β1. **(b)** The α4β1–VCAM-1 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 inflammatory 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

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- • of special interest
- •• of outstanding interest

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