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New approaches to blockade of α4-integrins, proven therapeutic targets in chronic inflammation

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ABSTRACT

The recruitment of leukocytes into tissue is a pivotal step in inflammation. α4-Integrins are adhesion receptors on circulating leukocytes that mediate attachment to the endothelium and facilitate their migration into the inflamed tissue. This multistep process is mediated by the interaction of α4-integrins with their counter receptors VCAM-1 and MadCAM-1 that are expressed on endothelial cells. α4-Integrins act as both adhesive and signaling receptors. Paxillin, a signaling adaptor molecule, binds directly to the α4 cytoplasmic tail and its binding is important for cell migration. Blocking the adhesive functions of α4-integrins has been shown to be an effective therapeutic approach in the treatment of autoimmune diseases, but also carries the risk of defects in development, hematopoiesis and immune surveillance. Interfering with α4 signaling by inhibiting the α4–paxillin interaction decreases α4-mediated cell migration and adhesion to VCAM-1 and MadCAM under shear flow. These in vitro effects are accompanied by a selective impairment of leukocyte migration into inflammatory sites when the α4–paxillin interaction is blocked in vivo. Thus, blockade of α4-integrin signaling may offer a novel strategy for interfering with the functions of these receptors in pathological events while sparing important physiological functions.

Contents

1. Introduction

Autoimmune diseases are characterized by an inappropriate response of the immune system against self which leads to inflammation-induced dysfunction and ultimately to the destruction of the affected tissue. In rheumatoid arthritis for example, the immune system attacks the synovium [1] while in inflammatory bowel disease (Crohn's disease) the intestinal mucosa is affected [2–4]. In multiple sclerosis T cells, B cells, macrophages and microglia mount a concerted attack against the myelin sheath surrounding the nerve fibers in the brain and spinal cord [5–8], whereas in type 1 diabetes mellitus

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destruction of the insulin producing β-cells of the pancreatic islets is mediated largely by T lymphocytes [9–11].

Although the exact causes of most chronic inflammatory diseases are unclear, leukocyte accumulation in the affected tissues or organs contributes to the pathogenesis of the disease [12,13]. The recruitment of leukocytes from the blood into the tissue at sites of inflammation is regulated by sequential engagement of adhesion and signaling molecules on leukocytes and endothelial cells such as α4-integrins and their ligands [14,15]. In this review we discuss recent findings on the role of α4-integrins in chronic inflammatory diseases and the impact of anti α4-integrin therapy to treat these disorders. Furthermore, we describe the importance of α4-integrin signaling in the immune response and thus in autoimmune diseases and highlight the possible usefulness of α4-integrin signaling as a therapeutic target.

2. α4-Integrins in adhesion and migration: function blocking antagonists

Integrins are cell surface receptors, which mediate cell adhesion and migration and regulate cell growth and survival. They are heterodimers consisting of α and β subunits. Each subunit contains an extracellular domain involved in ligand binding, a single transmembrane domain, and a cytoplasmic domain, which regulates integrin function. Integrins function as bi-directional signaling molecules [12,16], and binding to their ligands results in intracellular signals and conversely, cellular signaling events can modulate the affinity of integrins for extracellular ligands.

The α4-integrins, α4β1 (very late antigen-4: VLA-4) and α4β7 are most prominent on mononuclear leukocytes, but can also be expressed on neutrophils [17]. α4β1 mediates cells adhesion to vascular cell adhesion molecule-1 (VCAM-1) and to an alternatively spliced form of the extracellular matrix protein fibronectin (FN) [18–21]. α4β7 is important in lymphocyte homing to mucosal tissue by adhering to the gut homing receptor mucosa addressin cell adhesion molecule (MadCAM) [22] and it also binds to VCAM-1 and FN [23,24].

α4-Integrins are essential for embryogenesis, hematopoiesis, lymphocyte homing and the recruitment of leukocytes to sites of inflammation [14,21]. α4-Integrins are involved in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis [25], diabetes type 1 [26,27], inflammatory bowel disease [28–31] and multiple sclerosis. Migration of circulating leukocytes from the blood into sites of inflammation is a multistep event that involves sequential leukocyte–endothelial interactions. This process includes initial tethering to and rolling along the vascular endothelial surface, leukocyte stimulation, primarily of integrin molecules, firm adhesion and spreading to the endothelium, and finally migration across the endothelium (diapedesis) [21,32–34]. α4-Integrins and their endothelial counter receptors have a unique role in this multistep cascade because they are the only molecules known to mediate both rolling (when the integrins are in a low-affinity state) and arrest (when they are in a high-affinity state) [35,36].

Because of the important role of α4-integrins in cell trafficking during inflammatory processes and in autoimmune diseases they may be useful as targets in the treatment of these disorders. A multitude of papers have been published during the last decade describing monoclonal antibodies or small molecules that are directed against α4-integrins or their endothelial ligands (reviewed by [37,38]).

Monoclonal antibodies directed against α4-integrins or their cell adhesion molecule ligands have been shown to be effective modulators in animal models for autoimmune diseases such as asthma [39], rheumatoid arthritis [40–43], inflammatory bowel disease [44] or diabetes type 1 [45].

Early studies provided first evidence for the importance of anti-α4 antibodies in inflammatory bowel disease. Administration of HP1/2, an anti-α4 antibody that inhibits α4 binding to VCAM-1 and FN [46] to the cotton-top Tamarin, a primate that develops spontaneous acute and chronic colitis that resembles ulcerative colitis, resulted in significant attenuation of inflammation [31]. Further studies on the effect of antibodies against α4-integrin, α4β7 integrin or its ligand MadCAM-1 in animal models of colitis report reduced T cell mediated intestinal inflammation and abrogated symptoms of colitis [28–31]. The promising result of these pre-clinical studies in animals led to human trials using humanized α4-integrin antibodies. LDP-02 (MLN02), a α4β7 blocking antibody was successfully used in a phase I/II clinical trial of ulcerative colitis and Crohn's disease [47,48].

Efficacy of antibodies directed against α4-integrin was also studied in models for autoimmune encephalomyelitis (EAE), an autoimmune disorder with similarities to multiple sclerosis. In Lewis rats, the antibody HP1/2 completely prevented the development of paralysis in 75% of the treated animals. In those that developed the disease, paralysis was delayed and its severity was reduced [49]. Another antibody, AN100226m, was studied in a guinea pig model of EAE and administration prevented leukocyte infiltration and suppressed clinical and pathological features of EAE [50]. The humanized version of this antibody, natalizumab [51–53], has been successful in the treatment of multiple sclerosis and Crohn's disease in humans [54,55]. However, the mechanisms by which natalizumab exerts its beneficial effects are poorly understood. It is known though that it binds to α4 and thereby inhibits the interaction between α4β1 and VCAM-1 and between α4β7 and MadCAM-1 [56–58]. It has been suggested, that by inhibiting the interaction of α4β1 and VCAM, natalizumab blocks leukocyte trafficking across the blood–brain barrier and thereby moderates inflammation within multiple sclerosis lesions. In Crohn's disease, the major players of the inflammatory process are neutrophils. Since α4-integrin expression is very low on circulating neutrophils [21], it is believed, that in Crohn's disease natalizumab functions by inhibiting T lymphocytes that induce cytokines and chemokines needed to sustain neutrophil recruitment [59].

To date, approximately 8000 patients have received natalizumab for the treatment of multiple sclerosis or Crohn's disease. However, the occurrence of three cases of progressive multifocal leucoencephalogy (PML) and their association with anti-α4 therapy [60–62] led to suspension of natalizumab from sales and clinical trials in February 2005 [63,64]. Clinical trials have been revived in February 2006 and in June 2006 the FDA approved an application for resumed marketing of natalizumab with a special restricted distribution program [65]. Nevertheless, it is possible that in some patients long-term α4-integrin

blockade can lead to impaired immune surveillance of the central nervous system (CNS) by inhibiting α4-integrin mediated immune cell trafficking to the CNS and thus allowing JC virus replication [60–62,66].

Although clinical trials using α4-integrin blocking antibodies look very promising this approach also carries limitations such as high cost, potential immunogenicity of the antibodies and the requirement for intravenous administration. Thus, small molecule antagonists of the α4-integrin interaction with VCAM-1 or MadCAM appear attractive in the treatment of autoimmune disorders, since their synthesis is less expensive and they can be orally administered.

Adhesion antagonists have been designed to inhibit binding of α4β1 to VCAM-1 (reviewed by [37]) and include peptide inhibitors and VCAM-1 binding site mimics.

Peptides based on the amino acid sequences found at protein–protein interaction sites make excellent leads for antagonist development. Early reports of α4-integrin peptide antagonists were published about a decade ago and were based on an alternatively spliced connecting segment of fibronectin (CS-1) that acts as a ligand for α4β1 and has been proven to be involved in leukocyte adhesion and migration [21]. A series of overlapping peptides of CS-1 was synthesized and their ability to inhibit α4β1-dependent cell adhesion to fibronectin (FN) was measured [67]. This led to the minimal tripeptide epitope LDV which inhibited cell adhesion to fibronectin. At the same time a hexapeptide comprising the LDV peptide which inhibited α4-integrin binding to FN, VCAM and MadCAM was discovered by Cytel.

The RGD motif that interacts with several integrins has also been in the focus of generating inhibiting peptides of α4β1 [68,69]. A variety of extracellular matrix proteins, including collagen [68], fibrinogen [70], vitronectin [70,71] and fibronectin [72] contain the RGD motif. From RGD peptides as a lead, cyclic RCD peptides arose that were patented for a multitude of autoimmune indications [69].

Peptidomimetic small molecule antagonists of VLA4 based on the connecting segment 1 peptide sequence of FN have been shown to be potent blockers of integrin adhesive function in vitro and show efficacy in murine contact hypersensitivity and in the sheep allergic airways model and therefore are possible candidates for clinical intervention in human asthma [73].

A dual α4β1 and α4β7 small molecule antagonist (TR14035) was recently published that was orally active in a model of allergic asthma in Brown Norway rats. It decreased the number of eosinophils, mononuclear cells and neutrophils in the bronchoalveolar lavage fluid and led to marked reduction of lung inflammatory lesions [74].

Taken together, antibody or small molecules that block the ligand-binding site are a proven approach to the inhibition of α4-integrin function. Although this therapeutic approach may be effective, it also carries the possibility of serious side-effects:

1. Mechanism-based toxicities consequent to blockade of α4-integrin functions are likely to include defects in placentation, heart development [75], and development of certain cells of the hematopoietic lineages, e.g. B cells [76,77].
2. Anti-integrin antibodies can elicit immune responses. For example, 11% of patients developed antibodies to natalizumab during a 6-month trial [58]. Furthermore, low molecular weight integrin antagonists can induce antigenic changes in integrins [78–81] including α4β1 [82]. These antigenic changes may lead to drug-induced antibodies and consequent cytopenias [83–85].
3. Antibody or peptide integrin inhibitors can have unexpected agonistic [86] or trans-dominant effects [78]. These effects have been proposed to account for adverse consequences of GPIIb–IIIa antagonists [87–89]. Similarly, anti α4 antibodies can co-stimulate T cells [90] and such effects might account for the enhanced Th1 responses elicited by such antibodies administered during symptomatic experimental allergic encephalomyelitis [91].
4. Long-term blockade of the α4 binding site bears the risk of opportunistic infections including PML [63,64].

3. α4-Integrin signaling: an alternative approach

Integrin α4-subunits regulate cell migration, cytoskeletal organization and gene expression in a distinct manner from other integrin α-subunits. They promote cell migration and antagonize cell spreading and contractility. These biological activities are a function of the α4 cytoplasmic domain [92,93]. The cytoplasmic tail of α4-integrin binds tightly to paxillin, a cytoplasmic adaptor protein, and paxillin binding is required for the ability of α4-integrin to enhance migration [94,95]. Additional evidence comes from a mutated Jurkat T cell line that expresses α4-integrin with a point mutation (Y991A), which selectively disrupts paxillin binding. In this cell line α4β1-dependent migration but not α4-dependent static adhesion, is disrupted [96]. Another signal transduction pathway that is affected by this mutation is trans-regulation of αLβ2 by α4β1 [96]. Engagement of α4β1 by ligands such as VCAM-1 stimulates αLβ2-dependent cell adhesion and migration which is relevant to the immune response [97–100]. This form of trans-regulation in T cells requires the binding of paxillin to the α4-integrin cytoplasmic domain and is abolished by disruption of this interaction by the α4(Y991A) mutation. The α4–paxillin interaction mediates trans-regulation by enhancing the activation of tyrosine kinases, focal adhesion kinase (FAK) and/or proline-rich tyrosine kinase-2 (Pyk2). Disruption of the paxillin–α4 interaction results in much less α4β1 mediated phosphorylation of Pyk2 and FAK, and blocked α4β1 stimulation of αLβ2-dependent migration [96]. Furthermore, α4β1 binding to VCAM-1 or FN stimulates c-Src phosphorylation and promotes c-Src catalytic activation in an FAK-independent manner. This linkage between α4 and Src does not require paxillin binding to α4 since α4WT and α4Y991A expressing $FAK^{-/-}$ cells show equal levels of membrane ruffling and motility in haptotaxis and random motility assays [101]. Transgenic mice that are homozygous for a mutation in the α4-integrin cytoplasmic tail (α4Y991A) that disrupts paxillin binding are viable. Their viability and fertility stands in stark contrast to the embryonic lethal α4-integrin null mice. Mice with disrupted α4–paxillin interaction show defective recruitment of mononuclear leukocytes in thioglycollate-induced peritonitis (Fig. 1, republished with copyright permission by JCI). Importantly, these mice have

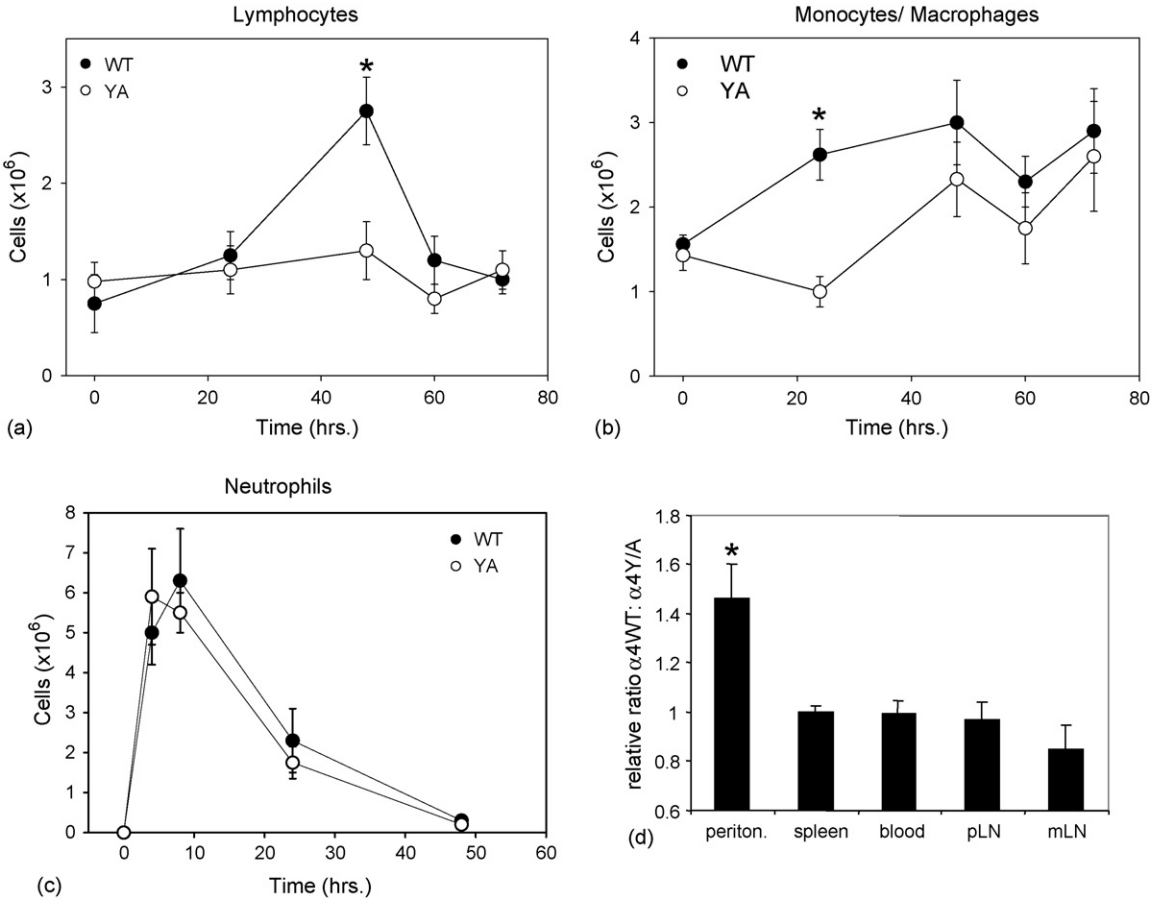

Fig. 1 – The recruitment of mononuclear leukocytes to the peritoneum in response to thioglycollate is impaired in mice with disrupted α4-integrin–paxillin interaction. (a–c) WT and α4(Y991A) mice were injected intraperitoneally with thioglycollate, and peritoneal lavage fluid collected at the indicated time points. Total cell number in the lavage fluid was measured with a hemocytometer, and differential cell counts were performed on cytospin slides after modified Wright–Giemsa staining. Results are shown for total lymphocyte (a), monocyte/macrophage (b), and neutrophil (c) counts. *P = 0.013, two-tails Student's t-test. Results are mean ± S.E.M. of four to eight mice for each time point. (d) Ratios of adoptively transferred WT/α4(Y991A) splenic lymphocytes found in the spleen, blood, peripheral LN (pLN), mesenteric LN (mLN), and thioglycollate-induced inflamed peritoneal cavities (periton.) of recipient WT mice. Recipient WT mice with thioglycollate-induced peritonitis were injected with CSFE labeled splenic mononuclear cells from WT or α4(Y991A) mice to test whether the defect in peritoneal lymphocytosis was ascribable to defective homing of the mutant leukocytes. Ratios of differentially labeled cells were assessed after 24 h by flow cytometry and normalized to the starting input ratio. Results are mean ± S.E.M. of eight mice from three separate experiments. P = 0.037, WT vs. α4(Y991A), one-tailed Student's t-test (figure published in Ref. [102]).

normal hemograms, normal abundance of hematopoietic precursors and unimpaired homing of hematopoietic progenitor cells to the bone marrow. These findings lend support to the idea that blockade of α4-integrin signaling can impair recruitment of leukocytes to sites of inflammation while averting the adverse effects of α4-integrin loss on development and hematopoiesis [102].

Paxillin is the founding member of a family of related proteins that contains two additional members, leupaxin and Hic-5 [103]. They contain N-terminal LD motifs and C-terminal LIM-domains, which mediate protein–protein interactions [104,105]. Because of the high degree of sequence similarity within the LD- and LIM-domains, it is anticipated that some of the functions of paxillin, leupaxin and Hic-5 overlap. In addition to α4-integrin, paxillin also interacts with α9-integrin [94], which is most closely related to α4 and promotes transendothelial neutrophil migration [106]. A principal role of paxillin is to transmit signals from integrins to assure efficient cell migration.

The association of paxillin with the α4 tail is regulated by the phosphorylation state of Ser988 in the α4 tail [107]. Jurkat T cells expressing a phosphorylation-mimicking (α4S998D) α4 variant show reduced cell migration and enhanced cell spreading due to the disruption of paxillin binding to α4. In contrast, Jurkat T cells that express the non-phosphorylatable α4S998A mutant have a spreading defect. Surprisingly, this

mutation led to an unexpected suppression of cell migration [108]. This can be explained by the fact that efficient α4-mediated cell migration requires precise spatial control of α4 phosphorylation by protein kinase A (PKA) and hence of paxillin binding to the α4-integrin tail. α4 phosphorylation by PKA only occurs at the leading edge of the migrating cell which leave α4-integrin unbound to paxillin resulting in Rac activity and the formation of stable lamellipodia. This spatial regulation of the α4–paxillin interaction contributes to suppression of lamellipodia at the sides and rear but not at the leading edge of migrating cells and thus to more efficient cell migration [109].

It is clear now that binding of paxillin to the α4 tail inhibits adhesion-dependent lamellipodium formation by blocking Rac activation and the region responsible for reduction in Rac activation has been mapped to the LD4 domain of paxillin. Furthermore, Arf–GAP has been shown to be responsible for inhibition of Rac activation by decreasing Arf activity. The localized formation of the α4–paxillin–Arf–GAP complex mediates the polarization of Rac activity and directional migration [110].

These data suggest that integrin signaling might be a favorable therapeutic target for the treatment of chronic inflammatory disorders and that multiple potential targets exist within these signaling pathways. Intracellular integrin regulatory pathways are cell type-specific [16] and may therefore contain targets for cell type-specific blockade of α4 function, such as the α4–paxillin interaction (Fig. 2). Furthermore, blockade of integrin signaling can leave ligand binding function partially intact [96,99,111]. Consequently, only partial inhibition of integrin function may occur, even with full blockade of the target. This may provide a more beneficial therapeutic ratio because mechanism-based toxicities can be easily averted. Thus, intracellular approaches may provide new and therapeutically advantageous targets for inhibition of α4-integrin in autoimmune diseases.

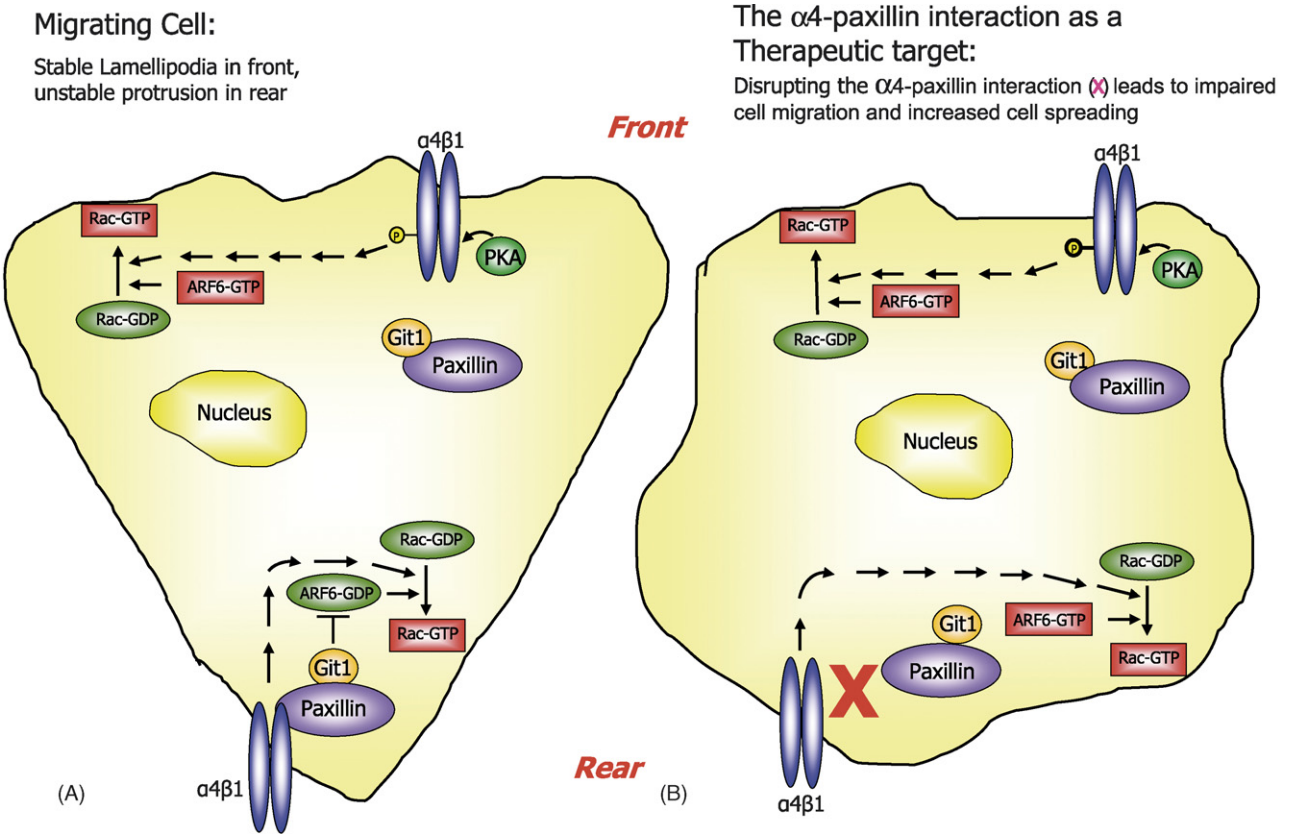

Fig. 2 – (A) Mechanism for the spatial localization of Rac activity to enhance cell migration. Formation of a stable lamellipodium at the front of migrating cells requires localization of Rac activation to the leading edge. Restriction of α4-integrin phosphorylation to the leading edge limits the interaction of α4 with paxillin to the sides and rear of a migrating cell. The α4–paxillin complex inhibits stable lamellipodia, thus confining lamellipod formation to the cell anterior. Binding of paxillin to the α4-integrin subunit inhibits adhesion-dependent lamellipodium formation by blocking Rac activation. The paxillin LD4 domain mediates this reduction in Rac activity by recruiting the ADP-ribosylation factor GTPase-activating protein (Arf–GAP) Git1 that decreases Arf activity, thereby inhibiting Rac. Finally, the localized formation of the α4–paxillin–Arf–GAP complex mediates the polarization of Rac activity and promotes directional cell migration. (B) α4-integrin signaling as a therapeutic target. A mutation in the cytoplasmic tail of α4-integrin (Y991A) leads to disruption of the α4–paxillin interaction and thereby to reversion of inhibition of Rac activity at the rear and lateral edges of the cell. This results in decreased cell migration and enhanced cell spreading. Small molecule inhibitors that specifically target the α4–paxillin interaction might have the same effect and are therefore very promising for therapeutic intervention. X represents the site of possible therapeutic intervention.

The first small molecule that inhibits the α4-integrin–paxillin interaction has been identified by screening a positional scanning library and was published in 2002. It efficiently blocks the interaction of α4-integrin with paxillin in vitro and impaired α4-integrin mediated leukocyte migration [112]. Thus, small molecule interference with the α4–paxillin interaction is feasible.

Downstream of integrins, paxillin can bind several signaling effectors to mediate migration. These effectors include the non-receptor kinase FAK [103,113,114] and its Pyk2, protein-phosphatase–PEST (PTP–PEST) [115,116] and Git1/paxillin kinase linker (PKL) [117]. FAK which is widely expressed, is recruited to focal adhesions by paxillin and adhesion stimulates FAK tyrosine phosphorylation, which can then promote cell migration [118–121]. The localization of FAK to focal adhesions seems to play a key role in FAK signaling [122] and is mediated by the focal adhesion targeting domain (FAT) of FAK [123,124]. A solution structure of the FAT domain has been described, showing that the α-helical LD2 motif of paxillin binds to the FAT domain through charge–charge and hydrophobic interactions, determining the chief residues of the LD2 peptide, involved in the interaction as R147, L148, E151, and L152 [125]. Thereby LD2 binds to α-helices H1 and H4 of FAT. This structural information is a valuable starting point to synthesize specific small molecule inhibitors that mimic the α-helical LD2 motif of paxillin and thereby interfere with FAK binding.

4. Conclusions

Blocking α4-integrin functions has been proven useful to decrease the severity or delay the development of chronic inflammatory disorders in animal models of disease and in humans. Nevertheless, the promise of integrin-directed therapeutics has been limited by mechanism-based toxicities and even led to fatal results in a few cases. Recent findings suggest that α4-integrin signaling might be a promising target for the treatment of autoimmune diseases. Interfering with downstream, intracellular protein–protein interactions for example that are involved in α4-mediated cell migration raises the possibility of inhibiting cell migration but leaving α4-mediated cell adhesion intact, and thereby obviating certain side-effects. Intriguing evidence comes from a transgenic mouse that expresses α4-integrin with a mutation that disrupts paxillin binding. These mice exhibit defective recruitment of mononuclear leukocytes into thioglycollate-induced peritonitis, but show intact lymphohematopoiesis. Although the long-term effects of blocking α4-integrins or their signaling are not known yet and need to be investigated in more detail, α4-integrin signaling seems to be a promising target for the treatment of autoimmune diseases.

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