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Brief report

Monoclonal antibodies to human laminin α4 chain globular domain inhibit tumor cell adhesion and migration on laminins 411 and 421, and binding of α6β1 integrin and MCAM to α4-laminins

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ABSTRACT

α4-Laminins, such as laminins 411 and 421, are mesenchymal laminins expressed by vascular and lymphatic endothelial cells, leukocytes and other normal cell types. These laminins are recognized by α6β1 and α6β4 integrins and MCAM (CD146), and promote adhesion and migration of the cells. α4-Laminins are also expressed and secreted by some tumor cells and strongly promote tumor cell migration. Moreover, the abluminal side of blood and/or lymphatic vessels and the nerve perineurium, common tracks of tumor cell dissemination, express α4-laminins, and these laminin isoforms, when expressed in the stroma, may contribute to tumor invasion. In the present study, we examined ten mAbs to human laminin α4 chain for their reactivity with the isolated laminin α4 globular domain, their ability to inhibit tumor cell adhesion and migration on laminins 411 and 421, and their effect on the binding of α6β1 integrin and MCAM to both α4-laminins. Most of the mAbs reacted with the laminin α4 globular domain, but only two, mAbs FC10 and 084, significantly inhibited tumor cell adhesion and migration on laminin-411. When used in combination, these antibodies practically abolished the cell adhesion and migration on laminin-411 and significantly reduced the cellular responses on laminin-421. Accordingly, mAbs FC10 and 084 significantly inhibited the binding of purified α6β1 integrin and MCAM to laminins 411 and 421. These results indicate that mAbs to the laminin α4 globular domain are able to inhibit tumor cell adhesion and migration on laminins 411 and 421, and that α6β1 integrin and MCAM bind α4-laminins at very close sites on the globular domain. These reagents contribute to a better understanding of the biology of α4-laminins and may have a therapeutic potential in malignant and inflammatory diseases.

1. Introduction

Laminins are a large family of αβγ heterotrimeric proteins, which have both structural and signaling functions (Miner and Yurchenco, 2004; Durbeej, 2010). They are major components of basement membranes (BMs) and strong promoters of cell adhesion and migration, as well as regulators of cell differentiation, proliferation, and survival. Presently, there are over 15 known laminin isoforms, which assemble as αβγ heterotrimers after subunit combinations from five laminin α chains (α1–α5), three laminin β chains (β1–β3), and three laminin γ chains (γ1–γ3) (Miner and Yurchenco, 2004; Durbeej, 2010). Laminins are presently denominated according to their chain composition; for example laminin α4β2γ1, previously known as laminin-9, is currently denominated as laminin-421 (Aumailley et al., 2005).

Laminin expression, particularly the α chain, is developmentally regulated, and cell- and tissue-type specific. Cells respond to laminins through the interaction of the α chain with integrins and other cell surface receptors (Durbeej, 2010). Nearly half of the 24 integrins in mammals bind laminins in an isoform-specific fashion; however, α3β1, α6β1, α7β1, and α6β4 integrins are considered the "classical" laminin receptors. BCAM (basal cell adhesion molecule) (CD239) and MCAM (melanoma cell adhesion molecule) (CD146), cell-surface molecules of the Ig superfamily, are additional laminin receptors (Durbeej, 2010; Flanagan et al., 2012; Ishikawa et al., submitted).

The laminin α4 chain was the fourth laminin α chain identified. This truncated α chain of nearly 200 kDa was originally cloned in humans, and then in mouse (Richards et al., 1994; Iivanainen et al., 1995; Liu and Mayne, 1996; Richards et al., 1996; Friesser et al., 1997; Iivanainen et al., 1997). By immunohistochemistry, α4-laminins have been localized

Abbreviations: LM, laminin; laminin-411, laminin α4β1γ1; MCAM, melanoma cell adhesion molecule; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

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in tissues of mesenchymal origin, such as the basement membranes (BMs) of vascular endothelial and smooth muscle cells, skeletal and cardiac muscle cells, and adipocytes (Miner et al., 1997; Talts et al., 2000; Petäjäniemi et al., 2002). α4-Laminins, such as laminins 411 (formerly laminin-8), 421 (formerly laminin-9) and 423 (formerly laminin-14), are expressed by vascular and lymphatic endothelial cells, smooth muscle cells, adipocytes, fibroblasts, leukocytes, platelets, and other mesenchymal cells (Friesser et al., 1997; Miner et al., 1997; Geberhiwot et al., 1999; Libby et al., 2000; Pedraza et al., 2000; Talts et al., 2000; Geberhiwot et al., 2001; Petäjäniemi et al., 2002; Hansen and Abrass, 2003; Matsuura et al., 2004; Wondimu et al., 2004; Fried et al., 2005; Vainionpää et al., 2007a). LMα4 chain-deficient mice are viable, but present some cardiovascular and neurological abnormalities (Miner and Yurchenco, 2004; Durbeej, 2010).

Tumor cells from gliomas and melanomas and from carcinomas after epithelial to mesenchymal transition are able to express and secrete α4-laminins (Fujiwara et al., 2001; Patarroyo et al., 2002; Fujiwara et al., 2004; Kawataki et al., 2007; Vainionpää et al., 2007b; Takkunen et al., 2008; Oikawa et al., 2011). Moreover, some cancer cells use the abluminal side of blood and/or lymphatic vessels and the nerve perineurium, which express α4-laminins, as dissemination tracks (Lugassy et al., 2002; Kawataki et al., 2007; Lugassy et al., 2009; Sroka et al., 2010; Oikawa et al., 2011). Tumor cells could also interact with α4-laminins expressed in the tumor stroma (Vainionpää et al., 2007b; Franz et al., 2010). In vitro studies with laminin-411, either natural or recombinant, and modules of the laminin α4 globular domain have demonstrated adhesive and migration-promoting activity of these proteins for a large variety of normal and tumor cells, as well as neurite-promoting activity (Geberhiwot et al., 1999; Kortesmaa et al., 2000; Pedraza et al., 2000; Talts et al., 2000; Fujiwara et al., 2001; Geberhiwot et al., 2001; Gonzales et al., 2001, 2002; Gu et al., 2003; Hansen and Abrass, 2003; Fujiwara et al., 2004; Wondimu et al., 2004; Fried et al., 2005; Li et al., 2006; Lian et al., 2006; Ishikawa et al., submitted). These cellular responses were primarily mediated by α6β1 integrin, while α3β1, α6β4, and αVβ3 appear to constitute additional receptors. Accordingly, purified α6β1 integrin has been reported to bind α4-laminins (Nishiuchi et al., 2006; Ishikawa et al., submitted). Recently, laminin-421 was found to have grater migration-promoting abilities for melanoma cells and other tumor cells than laminin-411 (Ishikawa et al., submitted). Moreover, MCAM (CD146), a cell-surface protein whose expression is induced during tumor progression, and closely correlates to tumor invasion and metastasis, was identified as a novel receptor for α4-laminins, particularly laminin-421 (Flanagan et al., 2012; Ishikawa et al., submitted).

Development of reagents specific for laminin chains is essential for the investigation of the multiple laminin isoforms. Unfortunately, early polyclonal antibodies to mouse laminin-111 (former laminin-1 or EHS laminin), the prototype laminin, which was first described in 1979 (Miner and Yurchenco, 2004; Durbeej, 2010), cross-react with most laminin isoforms, and are unable to dissect the complexity of the laminin family. Since monoclonal antibodies (mAbs) to laminins are mostly chain-specific, these reagents are most valuable in studies of laminin isoforms and, together with recombinant laminins, largely contribute to define the biology of laminins. In early studies, we developed and characterized nine novel mAbs to human LMα4 chain as tools for immunohistochemistry, immunoprecipitation, and Western blotting (Petäjäniemi et al., 2002; Wondimu et al., 2004). More recently, we generated five novel mAbs to human LMα5, and used them for similar purposes (Wondimu et al., 2013). Notably, one of the latter antibodies strongly inhibited integrin-mediated cell adhesion and migration on laminins 511 and 521.

In the present study, we investigated the reactivity of one novel, mAb 084, and the nine previously developed LMα4 mAbs with laminin-411 and the newly generated recombinant laminin-421, and the isolated LMα4 globular domain. We also tested the effect of the antibodies on tumor cell adhesion and migration on laminins 411 and 421, and on the binding of purified α6β1 integrin and MCAM to these laminin isoforms. By doing so, we have identified function-blocking mAbs to LMα4 chain.

2. Results

2.1. mAbs to LMα4 chain react variably with intact recombinant laminins 411 and 421 and the isolated LMα4 globular domain

As an initial step in the identification of function-blocking mAbs to LMα4 chain, the recombinant human laminins 411 and 421 were analyzed by Western blotting with well-defined laminin chain-specific mAbs (Fig. 1A). Bands of 230 kDa (LMα4), 230 kDa (LMβ1), and 220 kDa (LMγ1), and of 230 kDa (LMα4), 190 kDa (LMβ2), and 220 kDa (LMγ1) were detected for laminins 411 and 421, respectively, indicating that the laminins, including their α chain, were intact. The LMα4 chain is often proteolytically processed in vivo, losing the carboxy-terminal G4 and G5 modules of the globular domain, and suffering a reduction in size from 230 kDa to 180 kDa (Talts et al., 2000).

By ELISA, all mAbs to LMα4, except mAb 5D8, reacted with laminins 411 and 421, but at different levels (Fig. 1B). Notably, the reactivity of mAb FC10 with laminin-421 was slightly lower than that with laminin-411, suggesting that the β chain modulates accessibility of the binding of the mAb to the LMα4 chain. The mAbs were often used for ELISA at 1 μg/mL but they also reacted with α4-laminins at much lower concentrations (0.2 μg/mL and 0.04 μg/mL) as demonstrated in titration studies (Fig. S1A). However, none of the mAbs to LMα4 reacted with α5-laminins at any concentration (Fig. S1B). As expected, equal reactivity was detected with mAb 2E8 to LMγ1 chain, whereas mAbs DG10 (LMβ1) and S5F11 (LMβ2) discriminated between laminins 411 and 421. The ability of most of the mAbs to LMα4 to immunoprecipitate and work in Western blotting has been previously reported (Petäjäniemi et al., 2002; Wondimu et al., 2004). In the present study, mAb FC10 and, to a lower extent, mAb BH2 were found to react by Western blotting, but to a lesser degree than mAb 6C3. mAb 084 was unreactive under the present experimental conditions (Fig. S2). In a preliminary epitope mapping, the reactivity of the LMα4 mAbs with the isolated LMα4 globular domain was also tested by ELISA (Fig. 1C); five antibodies, 6A12, 6C3, 8C10, 9B2 and 084, were reactive.

2.2. mAbs FC10 and 084 to LMα4 chain largely inhibit α6β1-integrin-mediated tumor cell adhesion to laminin-411 and, to a lower extent, laminin-421

In additional studies, the function-blocking activity of the LMα4 mAbs was analyzed in adhesion assays of BE melanoma cells to laminins 411 and 421 (Fig. 2). These cells readily attached to both of the intact laminins, but they did not attach to the isolated LMα4 globular domain (Fig. 2A). Cell adhesion to laminin-421 was lower than to laminin-511 but slightly higher than to laminin-411 (Fig. S3A). Although 30 nM of the various proteins was used in most experiments, cell adhesion was still detected at 15 nM, but not at 7.5 or 3.8 nM (Fig. S3A). The cell adhesion on laminins 411 and 421 was exclusively and largely mediated via α6β1 integrin, respectively, and completely inhibited by EDTA (Fig. 2B).

Among the LMα4 mAbs, FC10 and 084 inhibited cell adhesion to laminin-411 by over 40% when used alone at 20 μg/mL; when tested in combination, they exerted an additive effect, inhibiting nearly 65% of the cell adhesion (Fig. 2C). Titration studies with these antibodies demonstrated that mAb 084 was more inhibitory than mAb FC10 and that their additive effect was already observed at 5 μg/mL (Fig. S3B). mAb BH2 also significantly reduced the cell adhesion, but to a lesser extent (by nearly 25%). Lower but significant inhibition was detected with mAbs 6C3 and 9B2. On laminin-421, the cell adhesion was less sensitive to the antibodies, and was significantly reduced by 15% with the combination of mAbs FC10 and 084 (Fig. 2C).

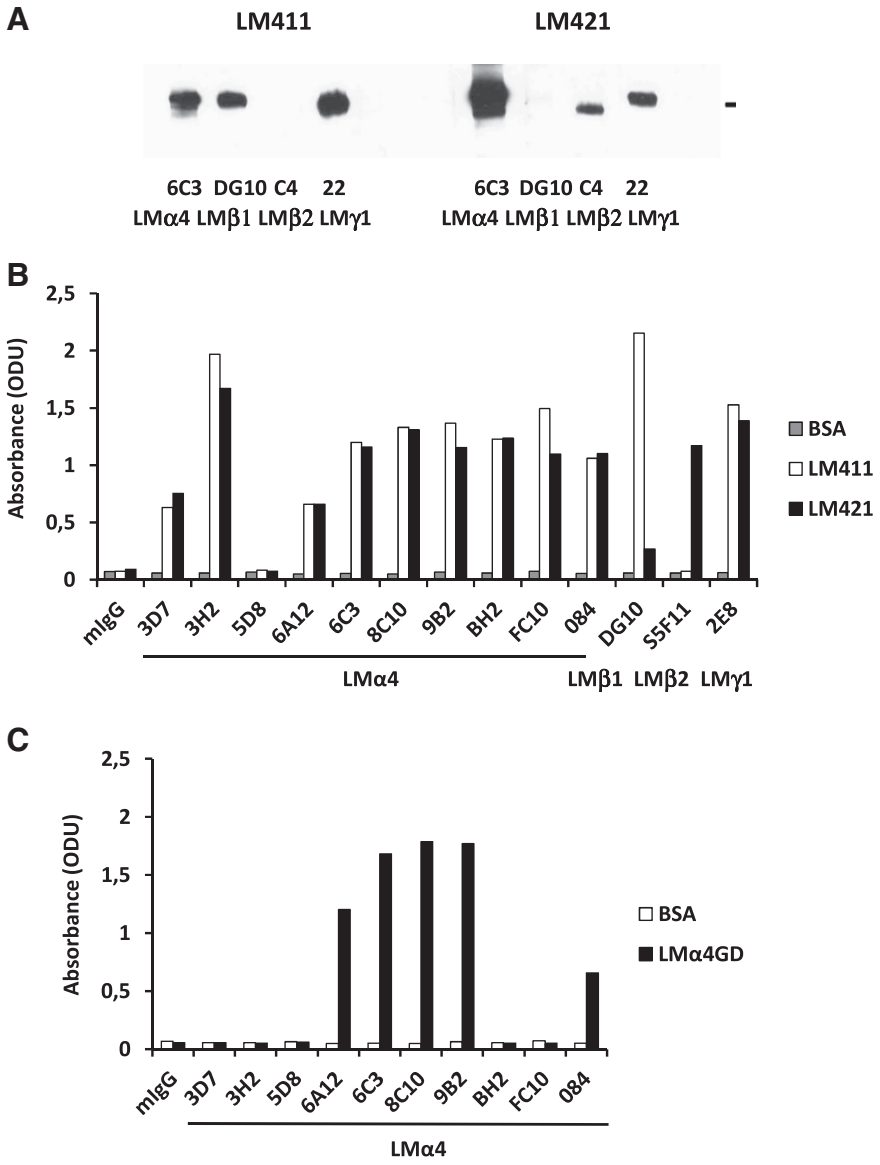

Fig. 1. Characterization of recombinant human laminins 411 and 421 and reactivity of LMα4 mAbs against these laminins and the isolated LMα4 globular domain. A) Western blots of recombinant laminins 411 and 421. Bands of 230 kDa (LMα4), 230 kDa (LMβ1), and 220 kDa (LMγ1), and of 230 kDa (LMα4), 190 kDa (LMβ2), and 220 kDa (LMγ1) were detected for laminins 411 and 421, respectively, with the chain-specific antibodies. Marker of 199 kDa is shown to the right. B) Reactivity of mAbs to laminin chains with laminins 411 and 421, as detected by ELISA. C) Reactivity of LMα4 mAbs with the isolated LMα4 globular domain, as detected by ELISA.

2.3. mAbs FC10 and 084 to LMα4 chain abolish α6β1-integrin mediated tumor cell migration on laminin-411 and strongly inhibit the cell migration on laminin-421

In cell migration assays, laminins 411 and, to a greater extent, 421 strongly promoted migration of BE melanoma cells (103 and 192 cells/field, respectively), when compared to bovine serum albumin (1 cell/field). The cell migration on laminin-411 was almost exclusively mediated by α6β1 integrin, whereas, on laminin-421, although α6β1 was still the predominant integrin, α3β1 integrin also appeared to contribute together with another unidentified adhesion receptor (Fig. 3A). Among all ten mAbs to LMα4 chain, mAbs FC10 and 084 alone each significantly inhibited the cell migration on laminin-411 by nearly 60%, and together by nearly 100% (Fig. 3B). mAb BH2 alone reduced the cell migration by nearly 40%, but this effect did not reach statistical significance. On laminin-421, the combination of mAbs FC10 and 084 significantly reduced the cell migration by nearly 60%; separately, their effect was less than 10% (Fig. 3B). Migration of A549 lung adenocarcinoma cells and HT1080 fibrosarcoma cells on laminins 411 and 421, also predominantly mediated by α6β1 integrin (Ishikawa et al., submitted), was similarly inhibited by mAbs FC10 and 084, but to a lesser extent than the inhibition of BE melanoma cell migration (Fig. 3C and D).

2.4. mAbs FC10 and 084 significantly reduce the binding of purified α6β1 integrin and MCAM to laminins 411 and 421

α6β1 integrin and MCAM participate in cell adhesion/migration on α4-laminins through their direct binding to the LMα4 globular domain of laminins 411 and 421 (Flanagan et al., 2012; Ishikawa et al., submitted; present study). Thus, the effect of function-blocking mAbs BH2, FC10 and 084 to LMα4 chain was tested in solid-phase binding assays of purified α6β1 integrin and MCAM to laminins 411 and 421 (Fig. 4). mAb 3H2 was used as non-inhibitory antibody control.

The function-blocking antibodies significantly reduced the binding of α6β1 integrin to laminin-411 (5–20%) and to laminin-421 (10–30%) when used at 20 μg/mL (Fig. 4A). Similarly, the same antibodies significantly inhibited MCAM binding to laminin-411 (15–60%) and to laminin-421 (6–40%) (Fig. 4B). For both α6β1 and MCAM binding,

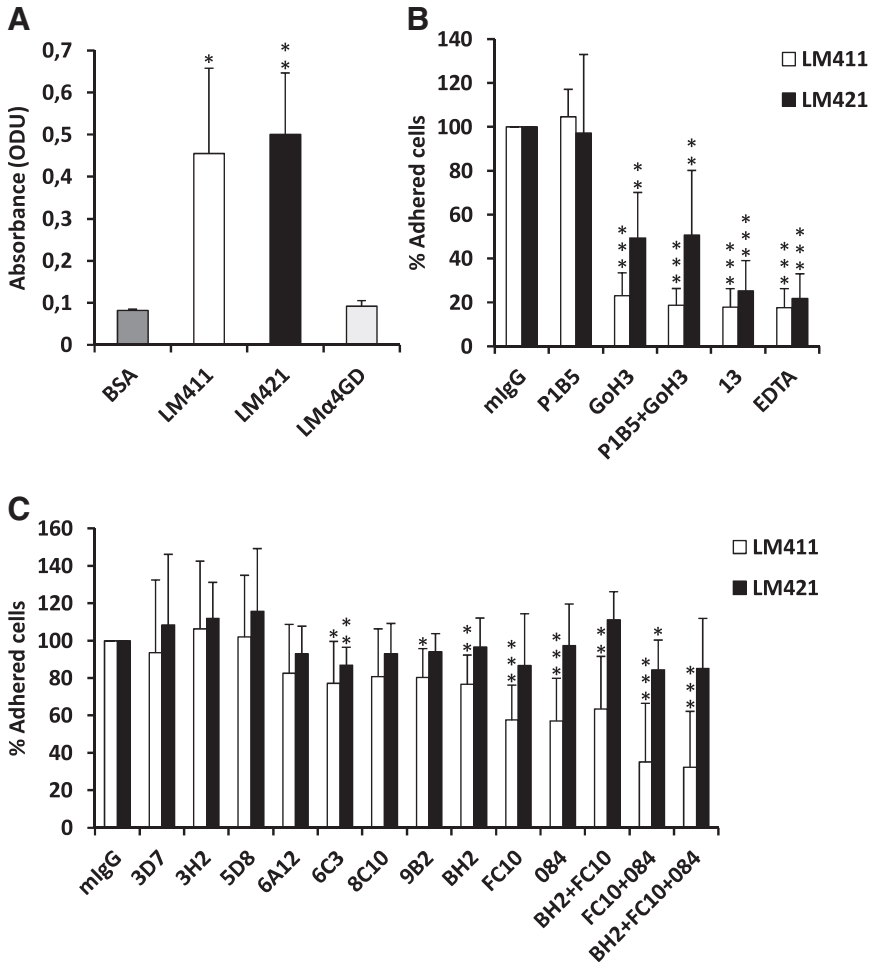

Fig. 2. LMα4 mAbs FC10 and 084 inhibit adhesion of BE melanoma cells to laminins 411 and 421. A) Adhesion of BE cells to laminins 411 and 421, and to the isolated LMα4 globular domain. B) Effect of mAb to integrin chains on the adhesion of BE cells to laminins 411 and 421. mAbs P1B5 (INTα3), GoH3 (INTα6), and 13 (INTβ1) were used (ODU for mIgG on laminins 411 and 421 were 0.57 and 0.47, respectively). C) Effect of mAbs to LMα4 chain on the adhesion of BE cells to laminins 411 and 421 (ODU for mIgG on laminins 411 and 421 were 0.78 and 0.46, respectively). Mean and standard deviation of at least three experiments are shown. The level of significance compares laminins to BSA and antibodies to mIgG control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

mAbs FC10 and 084 alone were similarly active (more so than mAb BH2), while they demonstrated the greatest inhibition when in combination. The inhibitory effect of the antibodies did not increase when their concentration was doubled.

2.5. Sheep antibodies to the LMα4 globular domain and mAbs 084 and BH2 interfere with the binding of mAb FC10 to laminins 411 and 421

α6β1 integrin and MCAM are known to bind the LMα4 globular domain of laminins 411 and 421 (Ishikawa et al., submitted). Considering that mAbs FC10 and 084 hindered this binding (Fig. 4), it was plausible to assume that both mAbs recognized the LMα4 globular domain. However, in contrast to mAb 084, mAb FC10 was unable to react with the isolated LMα4 globular domain (Fig. 1C). As a further attempt to localize the mAb FC10 epitope, this mAb was biotinylated, and tested in competition experiments with sheep polyclonal antibodies to the LMα4 globular domain, as well as with other mAbs to the LMα4 chain.

Sheep antibodies to the LMα4 globular domain successfully competed for binding of biotinylated-FC10 to laminins 411 and 421, hindering the interaction by nearly 60% (Fig. 5A), suggesting that the FC10 epitope is located at or near the LMα4 globular domain. Moreover, among the LMα4 mAbs, 084 significantly reduced the binding of biotinylated-FC10 to both laminins 411 and 421, and mAb BH2 showed a similar inhibition of binding to laminin-411 (Fig. 5B). As expected, non-biotinylated FC10 largely competed out binding of biotinylated-FC10 to laminins 411 and 421 (Fig. 5B).

Interference of mAb FC10 binding by mAb 084, which was shown to bind to the LMα4 globular domain (Fig. 1C), provides further evidence to support that mAb FC10 binds at or near the LMα4 globular domain. Consequently, the competitive effect of mAb BH2 on FC10 binding similarly suggests that mAb BH2 also binds at or near the LMα4 globular domain. Altogether, these results indicate the topographic proximity of the epitopes defined by function-blocking mAbs BH2, FC10, and 084 at and/or near the LMα4 globular domain.

3. Discussion

In the present study, we identify two major function-blocking mAbs against the human LMα4 chain, FC10 and 084, which inhibit α6β1 integrin-mediated adhesion and migration of tumor cells on laminins 411 and 421. The two antibodies were rather similarly effective and demonstrated an additive effect in combination. Furthermore, we confirm the reactivity of mAb 084 with the LMα4 globular domain, and report that mAb FC10, and probably mAb BH2, bind at or near the globular domain of the LMα4 chain. We also describe that mAbs FC10 and 084 similarly hinder the binding of α6β1 integrin and MCAM to the α4-laminins, demonstrating the binding of the two receptors to close sites in the LMα4 globular domain.

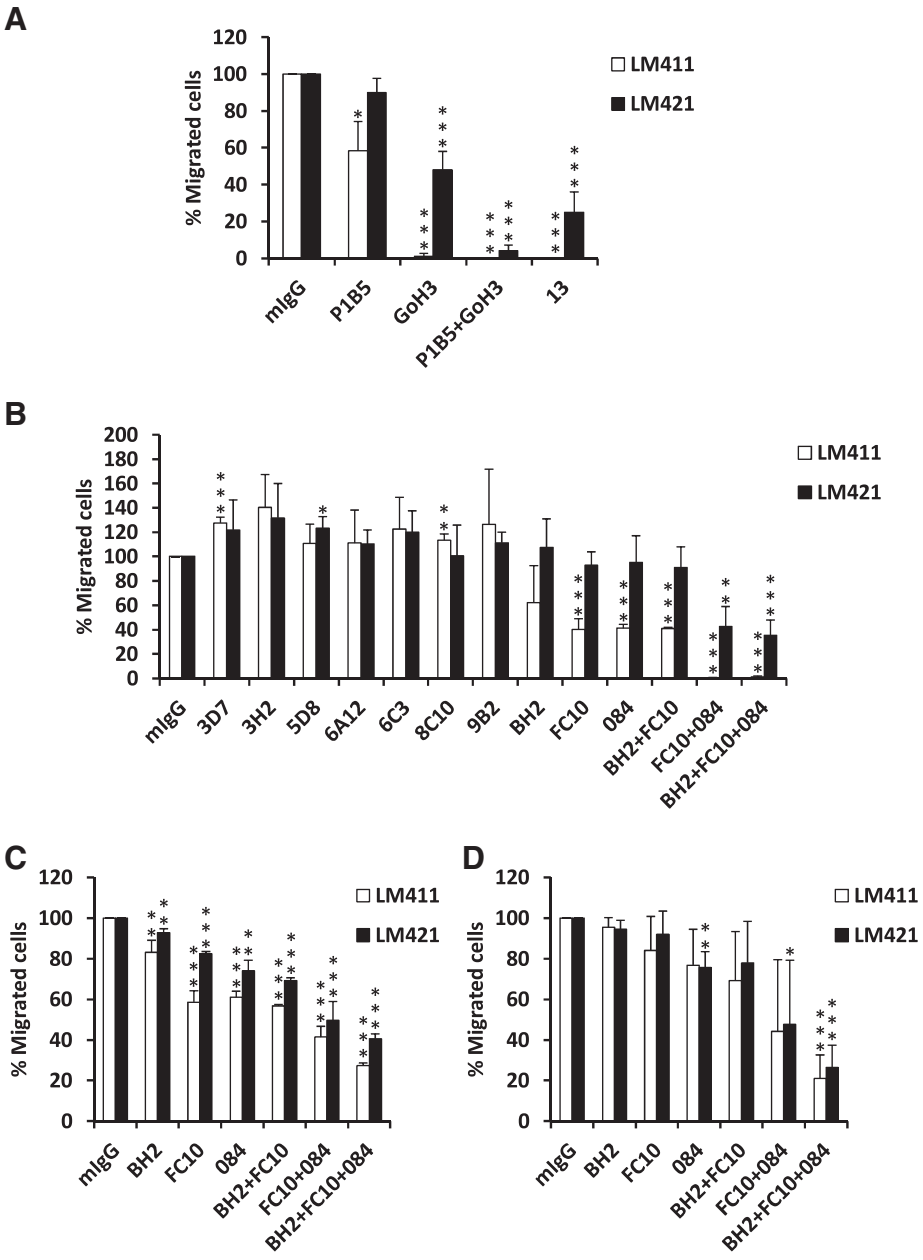

Fig. 3. LMα4 mAbs FC10 and 084 inhibit integrin-mediated migration of tumor cells on laminins 411 and 421. A) Effect of mAbs to integrin chains on the migration of BE cells on laminins 411 and 421. mAbs P1B5 (INTα3), GoH3 (INTα6), and 13 (INTβ1) were used. B) Effect of mAbs to LMα4 chain on the migration of BE cells on laminins 411 and 421 (mIgG in A and B corresponds to 103 and 192 cells/field on laminins 411 and 421, respectively). C) Effect of function-blocking mAbs to LMα4 chain on the migration of A549 lung adenocarcinoma cells on laminins 411 and 421 (mIgG corresponds to 359 and 353 cells/field on laminins 411 and 421, respectively). D) Effect of function-blocking mAbs to LMα4 chain on the migration of HT1080 fibrosarcoma cells on laminins 411 and 421 (mIgG corresponds to 295 and 323 cells/field on laminins 411 and 421, respectively). Mean and standard deviation of at least three experiments are shown. The level of significance compares antibodies to mIgG control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

mAbs to LMα4 chain reacted variably with laminins 411 and 421 by ELISA, ranging from minimal binding with mAb 5D8 to strong reactivity with mAb 3H2. The reason for the poor reactivity of the former antibody under the present experimental conditions is unknown. However, all other nine mAbs readily and specifically reacted with the α4-laminins. Five mAbs, 6A12, 6C3, 8C10, 9B2, and 084, were able to bind to the isolated LMα4 globular domain. Although mAb FC10 was unreactive, this mAb seemed to bind to the LMα4 globular domain, but only in the context of other laminin domains, since its binding to laminins 411 and 421 was successfully competed by sheep polyclonal antibodies to the LMα4 globular domain, and by mAb 084. Thus, it is likely that the laminin β and/or γ chains contribute to the formation of the FC10 epitope.

In comparison to the known roles of laminin-411, the biological role of laminin-421 is poorly understood. This laminin isoform, which is found in lymphatic vessels and neuromuscular synaptic clefts in skeletal muscle (Sunderland et al., 2000; Vainionpää et al., 2007a), may also be present in blood vessels, but the simultaneous expression of several α and β chains in these anatomical structures limits the interpretation of the immunohistochemical data (Petäjäniemi et al., 2002). In the present study, laminins 411 and 421 showed rather similar adhesive properties for BE melanoma cells, while the isolated LMα4 globular domain was completely inactive. Notably, the cell adhesion on laminin-411 was exclusively mediated by α6β1 integrin, whereas on laminin-421, cells appeared to use α6β1 integrin and a β1 integrin different from α3β1. Separately, mAbs FC10 and 084 partially inhibited the cell adhesion on

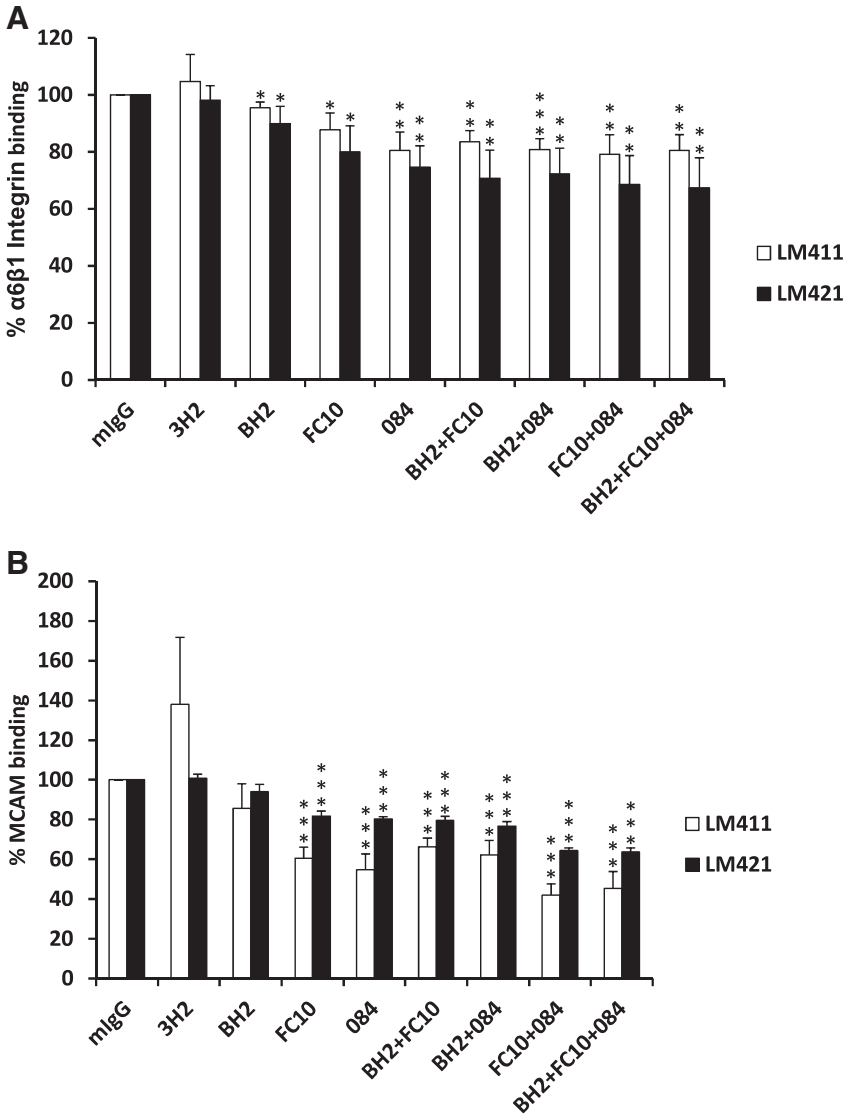

Fig. 4. LMα4 mAbs FC10 and 084 reduce the binding of purified α6β1 integrin and MCAM to immobilized laminins 411 and 421. A) Effect of function-blocking mAbs to LMα4 chain on α6β1 integrin binding to laminins 411 and 421 (ODU for mIgG on laminins 411 and 421 were 1.61 and 1.77, respectively). B) Effect of function-blocking mAbs to LMα4 chain on MCAM binding to laminins 411 and 421 (ODU for mIgG on laminins 411 and 421 were 0.339 and 1.327, respectively). Mean and standard deviation of at least three experiments are shown. The level of significance compares antibodies to mIgG control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

laminin-411, but they were strongly inhibitory in combination. In contrast, the two mAbs together only partially inhibited cell adhesion to laminin-421.

Interestingly, a similar pattern of inhibition by the mAbs was noted for cell migration. Thus, cell migration on laminin-411 was exclusively mediated by α6β1 integrin, and completely inhibited by the combination of mAbs FC10 and 084. On the other hand, cell migration on laminin-421 was primarily mediated by α6β1 integrin, with some contribution of α3β1 integrin, and the mixture of mAbs FC10 and 084 largely, but not completely, inhibited the cell migration. In addition to the effect on BE melanoma cells, mAbs FC10 and 084 similarly inhibited migration of A549 lung adenocarcinoma cells and HT1080 fibrosarcoma cells on laminins 411 and 421, although to a lower extent. As BE cells, A549 cells and HT1080 cells also primarily use α6β1 integrin to migrate on the α4-laminins (Ishikawa et al., submitted).

Direct interference of the binding of purified α6β1 integrin and MCAM to immobilized laminins 411 and 421 by mAbs FC10 and 084 was demonstrated in solid-phase binding assays. Notably, the pattern of inhibition by the various mAbs was similar for both α6β1 integrin and MCAM, indicating that the two receptors bind at close sites in the LMα4 globular domain. A similar phenomenon has been reported for the binding of α3β1 integrin and BCAM to the LMα5 globular domain (Kikkawa et al., 2007, 2013). Accordingly, we have observed that MCAM interferes with the binding of α6β1 integrin to α4-laminins (unpublished data). Since the binding inhibition by the mAbs to LMα4 chain was only partial, it is plausible to assume that the corresponding epitopes are located nearby, but not at the exact binding sites for α6β1 integrin and MCAM; alternatively, both α6β1 integrin and MCAM could use more than one binding site on the α4-laminins.

In a structural model of the intact LMα4 globular domain, the LG4–LG5 modules bend onto the LG1–LG3 modules and modulate receptor binding. Notably, proteolytic processing of the α4-laminin globular domain occurs in vivo and results in the removal of the carboxy-terminal LG4–LG5 modules (Talts et al., 2000). Current studies aim to provide more precise epitope mapping of the LMα4 globular domain with the various mAbs, and to identify mAbs that discriminate LG1–LG3 modules from LG4–LG5 modules.

Until now, only a single function-blocking mAb to LMα4 has been reported, mAb 2A3 (Gonzales et al., 2001, 2002). This IgM antibody,

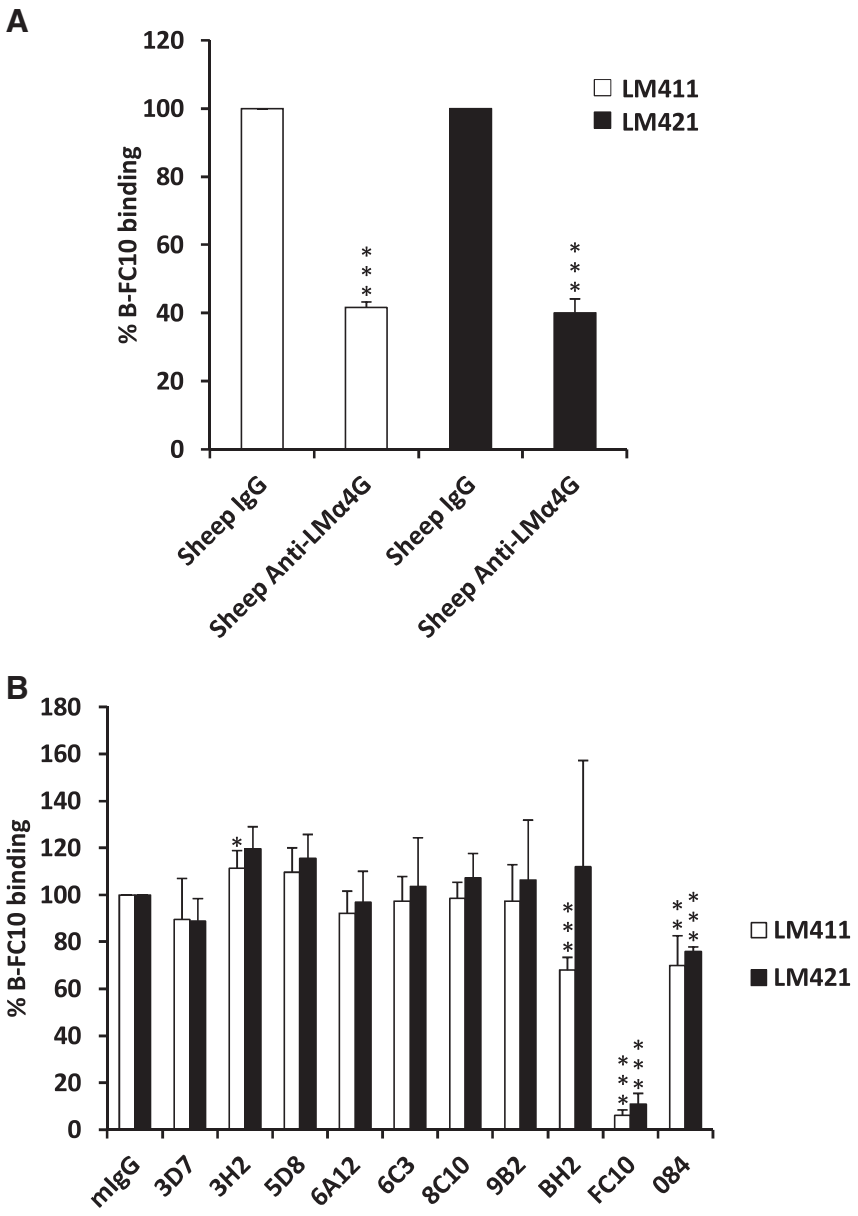

Fig. 5. Polyclonal antibodies to the LMα4 globular domain and mAbs 084 and BH2 interfere with the binding of mAb FC10 to laminins 411 and 421. A) Polyclonal antibodies to the LMα4 globular domain largely compete with the binding of mAb FC10 to recombinant laminins 411 and 421 (ODU for sheep IgG on laminins 411 and 421 were 1.47 and 0.84, respectively). B) mAbs 084 and BH2, but not other mAbs to LMα4 chain, reduce the binding of mAb FC10 to laminins 411 and 421 (ODU for mIgG on laminins 411 and 421 were 1.60 and 0.84, respectively). Competing antibodies were used at 20× higher concentrations than biotinylated-FC10. Mean and standard deviation of at least three experiments are shown. The level of significance compares sheep anti-LMα4 globular domain antibodies to sheep IgG (A) and mAbs to mIgG (B) on FC10 binding (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

generated by immunization of mice with human LMα4 LG1–LG2 modules, was able to inhibit motility of vascular endothelial cells and angiogenesis. However, the reactivity of this antibody with isolated α4-laminins, or its effects on the adhesion and migration of tumor cells, has not yet been reported.

Altogether, the present study describes mAbs FC10 and 084 as function-blocking antibodies that bind at or near the globular domain of α4-laminins. These reagents may constitute valuable tools for a better understanding of the role of α4-laminins in cell and tumor biology. These antibodies could be used to identify α4-laminins as active components, such as inducers of neurite outgrowth, in complex extracellular matrices. As inhibitors of tumor cell adhesion and migration, they may be used to inhibit tumor invasion and metastasis, thus, having a therapeutic potential in malignant diseases. Down-regulation of laminin α4 chain expression has been reported to inhibit glioma invasion in vitro and in vivo (Nagato et al., 2005). Moreover, in recent in vitro studies we found that tumor cells can use endogenous α4-laminins to migrate following stimulation (unpublished data). Since leukocytes similarly interact with α4-laminins for adhesion, migration, and extravasation (Pedraza et al., 2000; Geberhiwot et al., 2001; Wondimu et al., 2004), antagonists of α4-laminins may also have a beneficial effect in inflammatory disorders.

4. Material and methods

4.1. mAbs to human LMα4 chain and other proteins

mAbs FC10, BH2, 3D7, 3H2, 5D8, 6A12, 8C10 and 9B2 against human laminin α4 chain were generated by hybridoma technology in two independent immunizations, as previously described (Petäjäniemi et al., 2002; Wondimu et al., 2004). mAb 839084, here referred to as mAb 084, was generated by immunizing mice with isolated recombinant

human LMα4 globular domain and purchased from R&D Systems (Abingdom, UK). All antibodies were mouse IgG, and used in purified form. Recombinant human laminins 411 and 421 and the isolated laminin α4 globular domain were obtained from BioLamina (Stockholm, Sweden) and R&D Systems, respectively. The laminins were produced in a mammalian expression system as full-length molecules and purified by affinity chromatography (Kortesmaa et al., 2002). Purified sheep antibodies to the human laminin α4 globular domain and the sheep IgG negative control were purchased from R&D Systems.

4.2. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting

Recombinant laminins 411 and 421 were separated by SDS-PAGE under reducing conditions in a 6% polyacrylamide gel, and electroblotted to a PVDF membrane. The blots were incubated with specific primary mAbs to LMα4 (6C3), LMβ1 (DG10), LMβ2 (C4), and LMγ1 (22), and detected with a HRP-conjugated secondary Ab (Dako, Copenhagen, Denmark) (Kawataki et al., 2007). A generic ECL reagent kit (GE Healthcare Biosciences) was used for chemiluminescence.

4.3. Cell lines

BE melanoma cells, A549 lung adenocarcinoma cells and HT1080 fibrosarcoma cells were grown and maintained in RPMI-1640 (BE and HT1080) or MEM (A549) media with 10% fetal bovine serum, supplemented with HEPES buffer, antibiotics, and L-glutamine (Oikawa et al., 2011; Ishikawa et al., submitted).

4.4. Enzyme-linked immunosorbent assay (ELISA)

For conventional ELISA, 96-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 10 nM of laminin-411, laminin-421, or the laminin α4 globular domain. Following blocking with 1% bovine serum albumin (BSA), mAbs were added at a final concentration of 1 μg/mL and allowed to interact for 1 h at 4 °C. After three washes with phosphate buffered saline (PBS), bound antibodies were detected using secondary, goat antibodies to mouse Ig, coupled to horseradish peroxidase (HRP) (Dako, Glostrup, Denmark), and the enzyme activity was measured using orthophenylenediamine (Sigma-Aldrich). The plates were analyzed using a generic ELISA reader (at 492 nm). In addition to the mAbs to LMα4 chain, mAbs DG10 (LMβ1) (Geberhiwot et al., 2000), S5F11 (LMβ2) (Wewer et al., 1997), 2E8 (LMγ1) (Geberhiwot et al., 2000) and 4C7 (LMα5) (Geberhiwot et al., 2000) were also used.

In competition studies, biotinylated mAb FC10 (1 μg/mL) was mixed with unlabeled antibodies (20 μg/mL) before interacting with the immobilized laminins.

4.5. Cell adhesion and migration assays

BE melanoma cells, A549 lung adenocarcinoma cells and HT1080 fibrosarcoma cells were used for cell adhesion and migration assays. The function-blocking integrin (INT) mAbs P1B5 (INTα3) (Millipore, Solna, Sweden), GoH3 (INTα6) (R&D Systems), and 13 (INTβ1) (BD Biosciences, Stockholm, Sweden), together with mAbs to the LMα4 chain were also used in these assays.

The cell adhesion assay was performed in 96-well flat-bottomed polystyrene plates (Maxisorp, Nunc), coated overnight at 4 °C with 30 nM of laminin-411, laminin-421, or the laminin α4 globular domain. Following three washes with sterile PBS and blocking with 1% BSA for 1 h, 100 μL of cell suspension (10^6 cells/mL in RPMI-1640 medium with 0.1% BSA) was added to the coated wells. Cell adhesion was allowed to progress for 60 min at 37 °C. Thereafter, the wells were washed five times with pre-warmed medium, and adhered cells were fixed with 4% formaldehyde for 15 min, and stained overnight with 0.5% toluidine blue (Sigma-Aldrich, St. Louis, MO, USA). The following day, the plate was washed five times with deionized water, then 100 μL of 2% SDS was added to each well to release the dye. A generic ELISA-reader (620 nm wavelength) was used to measure the optical density. To test the effect of mAbs, cells or coated wells were pre-incubated with integrin or laminin antibodies (20 μg/mL), respectively, for 15 min before cells were added to the wells for the cell adhesion assay.

Cell migration was performed in polycarbonate Transwell culture inserts with 8-μm pore size membranes in 24-well plates (Costar, Cambridge, MA, USA). The membrane, which separates upper and lower compartments, was coated on its bottom side with laminin 411 or 421 (30 nM) for 2 h at 37 °C, and then washed three times with sterile PBS. Thereafter, the inserts were blocked with 0.1% BSA for 30 min and meticulously washed three to five times with sterile PBS. Cells (1×10^6 cells/mL in RPMI-1640 medium with 0.1% BSA) were pre-treated for 15–20 min with integrin-blocking mAbs (20 μg/mL), then 100 μL of this suspension was added to each insert. Alternatively, 20 μg/mL of laminin mAb was added to the cell suspension and the lower chamber, which contained 600 μL RMPI-1640 with 0.1% BSA. Cells were allowed to migrate at 37 °C for 18 h, the membranes were then fixed in 2% glutaraldehyde, and stained with hematoxylin. Following removal of cells from the upper side of the membrane with cotton sticks, cells attached to the lower side were counted using a microscope. Mean cell number of three different representative fields (400×) was calculated. Cell migration in the presence of mouse IgG was defined as 100% control. No apparent differences in cell number were observed when cells were re-suspended in RPMI-1640 medium with 0.1% BSA and incubated on BSA- or laminin-coated surfaces for 18 h, the experimental conditions of the cell migration assay, suggesting no contribution of cell proliferation to the cell migration results.

4.6. Effect of mAbs to laminin α4 chain on the binding of soluble α6β1 integrin and MCAM to immobilized laminins 411 and 421

The binding of purified recombinant human α6β1 integrin (R&D Systems) to immobilized recombinant human laminins 411 and 421 (BioLamina) was measured by ELISA. The integrin, a noncovalently-linked heterodimer of 140–170 kDa and 110–140 kDa chains, was prepared in Chinese Hamster Ovary cells. Briefly, 96-well Maxisorp plates (Costar) were coated overnight at 4 °C with 30 nM laminins. After blocking with 1% BSA, α6β1 integrin (at 2.5 μg/mL) was added for 1 h. Following three washes, biotin-labeled goat anti-human integrin β1 antibodies (R&D Systems) were added at 1 μg/mL for 1 h. After three additional washes, HRP-conjugated streptavidin (Dako) was added at a 1:500 dilution and the enzyme activity was measured using orthophenylenediamine. The plate was analyzed using a generic ELISA reader (at 492 nm). Phosphate-buffered saline (PBS) containing 2 mM $MgCl_2$ was used throughout the assay, including in the washing buffer (0.1% BSA in PBS/$MgCl_2$) and as the integrin buffer.

For MCAM binding, 2.5 μg/mL of recombinant human CD146/Fc chimera (Sino Biological, Beijing, China) was added to the immobilized laminins (30 nM) for 1 h and, after three washes, bound MCAM was detected with HRP-conjugated goat anti-human IgG at 1:500 (Sigma-Aldrich). The enzyme activity was measured using orthophenylenediamine, and the plate was analyzed in an ELISA reader, as described above.

The effect of mAbs to LMα4 chain on the binding of α6β1 integrin and MCAM to laminins 411 and 421 was tested by pre-incubating the immobilized laminins with the antibodies at 20 μg/mL before adding the integrin or MCAM. Unbound mAbs were present during receptor–ligand interaction.

4.7. Statistical analysis

Results are presented as mean and standard deviation. Significant differences were determined using paired Student's *t*-tests. For all

analyses, $p < 0.05$ was considered statistically significant: *, $p < 0.05$; **, $p < 0.01$; ***, and $p < 0.001$.

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Authors' contribution

T.I. performed most experiments with some participation of Z.W., S.I. and Y.O. S.I. and I.V. contributed with mAbs. M.P. designed the experiments and interpreted the results. The manuscript was drafted by M.P. and edited by all authors, except I.V. who died in 2009.

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