Monoclonal antibodies directed against cadherin RGD exhibit therapeutic activity against melanoma and colorectal cancer metastasis

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ABSTRACT

Purpose: New targets are required for the control of advanced metastatic disease. We investigated the use of cadherin RGD motifs, which activate the α2β1 integrin pathway, as targets for the development of therapeutic monoclonal antibodies (mAbs).

Experimental design: CDH17 fragments and peptides were prepared and used for immunization and antibody development. Antibodies were tested for inhibition of β1 integrin and cell adhesion, proliferation and invasion assays using cell lines from different cancer types: colorectal, pancreatic, melanoma and breast cancer. Effects of the mAbs on cell signaling were determined by Western blot. Nude mice were used for survival analysis after treatment with RGD-specific mAbs and metastasis development.

Results: Antibodies against full-length CDH17 failed to block the binding to α2β1 integrin. However, CDH17 RGD peptides generated highly selective RGD mAbs that blocked (CDH17 and VE-cadherin)-mediated β1 integrin activation in melanoma, breast, pancreatic and colorectal cancer cells. Antibodies provoked a significant reduction in cell adhesion and proliferation of metastatic cancer cells. Treatment with mAbs impaired the integrin signaling pathway activation of FAK in colorectal cancer, of JNK and ERK kinases, in colorectal and pancreatic cancers, and of JNK, ERK, Src and AKT in melanoma and breast cancers. *In vivo*, RGD-specific mAbs increased mouse survival after inoculation of melanoma and colorectal cancer cell lines to cause lung and liver metastasis, respectively.

Conclusions: Blocking the interaction between RGD cadherins and α2β1 integrin with highly selective mAbs constitutes a promising therapy against advanced metastatic disease in colon cancer, melanoma and, potentially, other cancers.
TRANSLATIONAL RELEVANCE

Many unsuccessful attempts have been made in the last decades to target integrins with RGD peptides for cancer therapy. Here, we have demonstrated that cellular cadherin RGD motifs are equally efficient and more selective targets for integrin inhibition in metastatic cells than RGD peptides from the extracellular matrix proteins. We describe the development and functional characterization of cadherin RGD-specific monoclonal antibodies that have demonstrated an extraordinary efficiency in blocking β1 integrin activation and protecting mice against liver and lung metastasis from colorectal cancer and melanoma, respectively. Our data support that these highly promising results can be also extended to breast, pancreatic and other tumors. In summary, these highly selective monoclonal antibodies open a new avenue for the treatment of metastasis in different types of cancer.
INTRODUCTION

There is a necessity to find new therapeutic targets to control metastatic spread in cancer. Therapeutic monoclonal antibodies (mAbs) against EGFR or VEGF(R) are in clinical use for the treatment of advanced metastatic colorectal cancer. However, their impact on the final outcome of patients with metastasis is still limited (1), probably due to the difficulty of assessing the EGFR status of the patients (2,3), adverse effects related to the wide distribution of these molecules in healthy tissues and the lack of response in patients with KRAS mutations (4).

Cadherin 17 (CDH17), also known as liver-intestine (LI)-cadherin, is present in fetal liver and the gastrointestinal tract, exhibiting elevated expression during embryogenesis (5). CDH17 localizes to the basolateral domain of hepatocytes and enterocytes, where it mediates intercellular adhesion in a Ca^{2+}-dependent manner to maintain tissue integrity in epithelia (6). In colon cancer, CDH17 is expressed at low levels in primary tumors or in regional lymph nodes metastasis, as well as in poorly-differentiated colon cancer tumors (7). However, CDH17 is overexpressed in advanced colorectal cancer liver metastasis (8), where it correlates with poor prognosis (9). It is also highly expressed in gastric cancer, esophagus carcinoma, pancreatic cancer (10) and hepatocarcinoma (11). CDH17 facilitates liver colonization and metastasis in orthotopic mouse colorectal cancer models after intra-splenic injection (9,12). CDH17 binds α2β1 integrin through a RGD motif and induces β1 integrin activation, which leads to increased cell adhesion to Matrigel and type IV collagen, and increased proliferation (12). The CDH17 RAD mutant does not induce integrin signaling but rather leads to a reduction in tumor growth and liver colonization (12). In colorectal cancer metastatic cells, we have described a non-conventional situation in which α2β1 integrin binds to cadherins in a RGD-dependent
manner, making it conformationally activatable and allowing it to bind to type IV collagen. This finding modifies the classical notion that α2β1 integrin binds type I collagen using the GFOGER motifs in a RGD- and conformation-independent way (13,14). Previous data support a preferential trans activation model for cadherin/integrin interaction, although cis interaction cannot be ruled out (12). However, no activation effects were observed for the RGD motif on αv or α6β4 integrins.

RGD motifs are also present in vascular-endothelial (VE)-cadherin, CDH6 (foetal kidney (K)-cadherin) and CDH20. VE-cadherin is expressed in aggressive human melanoma cell lines and cutaneous melanomas (15), but not in poorly aggressive cell lines isolated from the same tumors (16). VE-cadherin has been involved in vasculogenic mimicry (the ability to form novel blood vessel–like structures) in uveal melanomas (16). It is also expressed in Ewing sarcoma (17), and promotes breast cancer progression (18). Recently, we have demonstrated that knocking-down VE-cadherin suppresses the lung colonization capacity of melanoma or breast cancer cells inoculated in mice, while pre-incubation with VE-cadherin RGD peptides promotes lung metastasis for both cancer types (19). Like CDH17 RGD peptides, VE-cadherin RGD peptides causes β1 integrin activation, suggesting that the mechanisms of action for both cadherins are similar (12).

We hypothesized that blocking of the cadherin RGD motifs would provoke an inhibition of liver and lung metastasis through α2β1 integrin inhibition. Here, we investigated the use of 9-mer peptides containing the CDH17 RGD motif and their flanking sequences to retrieve highly selective mAbs with anti-metastatic activity in different cancer cell types expressing CDH17 and VE-cadherin. We developed a panel of CDH17 RGD-specific mAbs that inhibited β1 integrin activation, cell adhesion and proliferation in colorectal and pancreatic cancer cells. This blocking effect was also effective in VE-
cadherin-mediated β1 integrin activation of melanoma and breast cancer cells. RGD-specific mAbs were able to induce a significant increase in mouse survival after intravenous and intrasplenic injection of highly metastatic cells from melanoma and colorectal cancer causing lung and liver metastasis, respectively. Consequently, blocking the interaction between RGD cadherins and α2β1 integrin represents a promising therapy for distinct cancer metastases.
MATERIALS AND METHODS

Cell lines, peptides, antibodies and siRNAs

Metastatic KM12SM human colon cancer cells were obtained directly from Dr. I. Fidler (MD Anderson Cancer Center), whereas MDA-MB-468 triple-negative human breast cancer cells and metastatic BLM human melanoma cells were kindly provided by Dr. J. Teixidó (CIB-CSIC). KM12SM cells were authenticated by short tandem repeat analysis. SKBR3 breast cancer cells were obtained from Dr. A Villalobos (IIB-CSIC). RKO, Colo320, HT29 human colon cancer cells, A375 human melanoma cells and the pancreatic cancer cell line PANC1 were purchased from the ATCC and passaged fewer than 6 months after purchase for all the experiments. BLM, SKBR3 and MDA-MB-468 were not authenticated in our laboratory. All cell lines were tested regularly for Mycoplasma contamination and cultured in DMEM (Invitrogen) containing 10% FCS (Invitrogen) and antibiotics at 37 °C in a 5% CO₂ humidified atmosphere.

CDH17 polyclonal antibodies (H-167 and C-17), RhoGDIα (G-2), VE-cadherin (BV9) and FAK (A-17) were purchased from Santa Cruz Biotechnology. CDH17 antibody (#141713) and Src (AF3389) were from R&D systems. Blocking anti-β1 (#P5D2), α4 (ALC 1/1) and α5 (P1D6) integrin antibodies were from Abcam. β1 integrin antibody specific for high affinity conformation (HUTS-21) and pY397-FAK (#611722) were from BD transduction laboratories. Antibodies against phospho-Src (#2101), JNK (56G8), phospho-JNK (G9), ERK1/2 (L24F12) and phospho-ERK1/2 (#9101) were from Cell Signaling Technology. Anti-α3 integrin (AB1920) was from EMD Millipore. The antibody against α1 (TS 2/7) integrin was a kind gift from Dr. C. Bernabeu (CIB-CSIC).

CDH17-derived peptides (VSLRGDTRG, SLRGDTR and LRGDT), VE-cadherin domain 2 (QGLRGDSGT), VE-cadherin domain 3 (SILRGDYQD), CDH6
DQDRGDGSL, CDH16 (RAIRGDTEG) and CDH20 (DMDRGDGSI) peptides were synthesized using solid phase chemistry with a Focus XC instrument (APPtec). In the CDH17 9-mer VSLRGDTRG, Tyr at position 600 was replaced for a Val to facilitate synthesis and hydrophilicity. The cyclic RGD peptide Cilengitide was from Selleckchem.

SiRNAs against human α1 (SASI_Hs01_00067020), α3 (SASI_Hs01_00196571), and α2 integrin (19) subunits were purchased from Sigma and transfected using Jet Prime reagent (Polyplus).

**Immunization and preparation of mouse mAbs**

All animal experiments in this study were conducted according to the national RD 53/2013 and EU Directives 2010/63/EU. All animal protocols were approved by the ethics committee of the Instituto de Salud Carlos III (CBA22_2014-v2) and Community of Madrid (PROEX 278/14). Four female Balb/c mice were immunized 3x intraperitoneally using ovalbumin (OVA)-conjugated CDH17 peptide (VSLRGDTRG) as antigen, the first time together with 50 μg of peptide-OVA emulsified in Freund's complete adjuvant, and the next two times with 25 μg of peptide-OVA emulsified in Freund's incomplete adjuvant. Then, mAbs were prepared according to standard procedures (20). The selection of clones was carried out using an indirect ELISA against the CDH17 protein expressed in *E. coli*, and the peptide VSLRGDTRG coupled to BSA as described (20), flow cytometry against KM12SM cells and β1 integrin activation assays. These antibodies were used at 10 μg/mL in the different experiments. mAbs were grown in HAT media, purified by Protein G and dialyzed against PBS for final testing and characterization.

**Flow cytometry**
For flow cytometry, 2.5×10⁵ KM12SM cells in 100 μL were incubated with immune sera or control serum (diluted 1:50) or with the different antibodies (10 μg/mL) for 30 min at 4 °C. After incubation, the cells were washed with 2 mL of PBS and incubated in the dark at 37 °C for 30 min in the presence of Alexa Fluor® 488 goat anti-mouse IgG antibodies (Thermo Fisher Scientific). Fluorescence was analyzed in a Coulter Epics XL cytofluorometer. At least 10,000 events per sample were acquired and cells were identified on the basis of their specific forward (FSC) and side (SSC) light scattering properties. Mean fluorescence intensities (MFI) for the indicated antibodies are shown inside each panel.

**Analysis of high-affinity conformation for β1 integrin.**

Cells were detached with 2 mM EDTA in PBS, washed with PBS, resuspended in DMEM and incubated with a CDH17 9-amino acid peptide (VSLRGDTRG) containing the RGD motif for 25 min at 37 °C in presence of immune or control serum (diluted 1:50) or the indicated antibodies (10 μg/mL). Peptides were used at 1 μg/mL in the different experiments (at approx. 1 μM). After incubation, cells were subjected to flow cytometry assays using antibodies specific for β1 integrin in high-affinity conformation and Alexa Fluor® 488 goat anti-mouse IgG antibodies. Fluorescence was analyzed in a Coulter Epics XL cytofluorometer. Experiment was repeated for other CDH17 RGD peptides (7 and 5 amino acids), VE-cadherin domains 2 and 3 and CDH6.

**Antibody confocal microscopy and internalization assay**

KM12SM cells were cultured on Matrigel-coated cover slides. Then, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 1% Triton X-100. After washing,
cells were incubated for 40 min with primary antibodies (H167, 25.4.1 at 10 µg/mL; 6.5.2, 6.6.1, 12.4.1 and control antibody at 30 µg/mL) in PBS with human gamma-globulin (40 µg/mL) at room temperature. Cells were then incubated 25 min with secondary antibodies coupled with Alexa-488 and 4,6-diamidino-2-phenylindole (DAPI). Samples were mounted with Mounting Fluorescence Medium (Dako, Copenhagen, Denmark) and images were captured using a TCS-SP5-AOBS confocal microscope with 63× oil immersion objective.

For internalization assays, KM12SM cells were cultured on Matrigel-coated cover slides and treated with the indicated mouse CDH17 antibodies (10 µg/mL) for 1 h. Then, cells were fixed, permeabilized and stained with rabbit anti-LAMP1 antibody (MyBioSource) followed by incubation with secondary antibodies (anti-mouse IgG coupled with Alexa 488, and anti-rabbit IgG coupled with Alexa 568). Zoom images were taken with 100x oil immersion objective.

**Tubule formation**

Cancer cells (5 x 10^3) were resuspended in serum-free DMEM and added upon 96-well plates previously coated with Matrigel (50 µL). After 24 h incubation, images of the wells were taken under a microscope with 10x phase contrast and tubules from 5 different wells were counted by two different observers (21). Tubules were defined as tubes when formed by multiple singles cells arranged in a row (1 cell thickness) or a thick bundle (thickness of several cells) connecting two cell 'islands'.

**Cell signaling analysis by Western blot**

Cells were incubated 3 h in serum-free DMEM, detached with 2mM EDTA, washed and treated with anti-RGD mAbs (10 µg/ml) for 40 min. Then, cells were added to plates
previously coated with Matrigel (0.4 μg/mm²) for 30 min in the presence of either control IgG or the mAbs. Finally, cells were detached as before and lysed with lysis buffer (1% Igepal, 100mM NaCl, 2mM MgCl₂, 10% glycerol, protease inhibitors (Complete Mini, Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich) in 50mM Tris-HCl). Protein extracts were separated in SDS-PAGE and transferred to nitrocellulose membranes, incubated with primary antibodies, washed and incubated with HRP-conjugated secondary antibodies (Sigma-Aldrich). Membranes were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

MTT assays
Cancer cells were seeded at 1x10⁴ cells/well on 96-well plates and incubated for 48 h at 37°C in DMEM with 0.5% serum in the presence of the indicated antibodies, followed by 1h incubation with thiazolyl blue tetrazolium bromide (MTT) (0.6 mg/mL) (Sigma-Aldrich). Cell viability was determined by absorbance at 560 nm and comparison with control cells collected ad initium. MTT assays were used as a surrogate of cell proliferation as no increase in cell detachment or cell death was observed after mAb treatments.

Cell adhesion and invasion assays
Adhesion and invasion assays were performed as previously described (22). Briefly, cancer cells were labeled with BCECF-AM (Sigma-Aldrich), detached with EDTA/PBS and incubated in serum-free DMEM in the presence of the indicated antibodies (10 μg/mL) for 10 min at 37 °C. Then, 6 x 10⁴ cells in 100 μL were added to 96-well plates previously coated with Matrigel (0.4 μg/mm²) or type I collagen (Millipore) (0.3 μg/mm²), and the plates were incubated for 25 or 30 min at 37 °C, respectively. Subsequently, non-adhered
cells were removed by three washes with DMEM. Bound cells were lysed with 1% SDS in PBS, and the extent of the adhesion was quantified using a fluorescence analyzer POLARstar Galaxy (BMG Labtechnologies).

For invasion assays, 6x10^4 cells were loaded onto 8 mm pore-size filters coated with 35 µL of Matrigel (1:3 dilution; BD Biosciences) in Transwell plates (Sigma-Aldrich) in the presence of antibodies. The lower compartment of the invasion chamber was filled with 5% serum DMEM. After 24 h, non-invading cells were removed from the upper surface of the filter, and cells that migrated through the filter were fixed with 4% paraformaldehyde, stained with crystal violet and counted under a microscope.

**Metastasis experiments in nude mice**

The Ethics Committees of the Consejo Superior de Investigaciones Científicas (Madrid, Spain) and Community of Madrid approved all protocols used. Swiss nude mice (Charles River) (n=4-6 per condition) were inoculated in the spleen with 1.5 x 10^6 KM12SM cells in 0.1 mL PBS. A day after inoculation, mice were subjected to removal of the spleen to avoid local growth of the tumors. Then, these mice were inoculated intravenously with control antibodies or with anti-RGD antibodies 25.4.1 and 6.6.1 (50 mg/Kg of weight, divided in 7 doses) starting 2 days after inoculation and for 2 weeks. Mice were daily inspected for signs of disease, such as abdominal distension, locomotive deficit, or tumor detectable by palpation. When signs were visible, mice were euthanized, subjected to necropsy, and inspected for metastasis in liver.

For liver and lung colonization assessment, mice were inoculated in spleen or tail vein respectively, with 1 x 10^6 KM12SM, BLM or MDA-MB-468 cells, and euthanized 96 h after inoculation. RNA was isolated from liver using TRIzol (Thermo Fisher Scientific),
retrotranscribed and 0.3 mg cDNA subjected to PCR with Taq DNA polymerase (Thermo Fisher Scientific) to amplify human GAPDH as previously described (19). As a control, a 20 cycle amplification of murine β-actin was performed.

**Statistical analyses**

Data were analysed by Student’s t test (2 conditions) or by one-way ANOVA followed by Tukey-Kramer multiple comparison test (more than 2 conditions). Histograms showed the average of the assessed values and the error bars showed the standard deviation (SD). Each experiment was carried out at least three times. Survival curves were plotted with Kaplan–Meier technique and compared with the log-rank test. The minimum acceptable level of significance in all tests was $P < 0.05$. 


RESULTS

CDH17 RGD peptide induces β1 integrin blocking antibodies

Different commercial CDH17-specific antibodies were first tested for their capacity to inhibit β1 integrin activation and cell proliferation in metastatic colon cancer cells. CDH17 polyclonal antibodies (H167, C-17 or 141713) raised against the ectodomain or the carboxy terminal domain of CDH17 failed to inhibit β1 integrin activation induced by CDH17 RGD peptides (Fig. 1A). We therefore prepared mouse polyclonal and monoclonal antibodies against the domain 6 polypeptide of CDH17, which contains the RGD domain. These antibodies inhibited β1 integrin activation to a limited extent (<50%) (Fig. 1A). In contrast, mouse polyclonal antibodies from mice immunized with CDH17 peptide VSLRGDTRG inhibited β1 integrin activation at about 80-90%. These data confirmed the hypothesis that RGD-specific antibodies inhibited CDH17 RGD peptide-induced activation of β1 integrin. In addition, RGD peptide-specific antibodies significantly inhibited cell adhesion (Fig. 1B) and proliferation (Fig. 1C), whereas full-length CDH17 or domain 6 antibodies did not affect cell adhesion or proliferation of CDH17+ colon cancer cells. The adhesion to Matrigel was β1 integrin dependent, as confirmed using β1 integrin blocking antibodies. In summary, a selective immune response against the CDH17 RGD motif displayed β1 integrin blocking activity together with a clear reduction in cell adhesion and proliferation.

Cilengitide, a cyclic RGD peptide, fails to bind and activate α2β1 integrin

From our previous data, it seemed counterintuitive to use RGD peptides for cancer therapy as they might activate α2β1 integrin in cancer epithelial cells. Cilengitide, a cyclic RGD pentapeptide targeting integrins αvβ3 and αvβ5, was unsuccessfully tested in clinical trials for glioblastoma (23). We did not observe any effect of Cilengitide on β1 integrin
activation either as a ligand, probably due to the different flanking sequences and conformation, or as an inhibitory drug (Fig. 1D). Therefore, not all RGD sequences are appropriate for α2β1 integrin activation.

**Development of RGD-specific mAbs for inhibition of cell adhesion and proliferation in colorectal and pancreatic cancer**

Mouse monoclonal antibodies (mAbs) were prepared against the CDH17 RGD 9-mer peptide and functionally tested for their inhibitory capacity of β1 integrin activation. After initial anti-peptide ELISA, four clones 6.5.2, 6.6.1, 12.4.1 and 25.4.1 were selected for characterization (Supplementary Table S1). Only mAbs 6.6.1 and 6.5.2 were positive for immunoprecipitation (Supplementary Fig. 1A). mAbs did not work in Western blot (data not shown) and were only weakly positive for flow cytometry analysis of surface CDH17, except for antibodies 6.61 and 12.4.1 (Supplementary Fig. 1B). By confocal microscopy, the staining capacity of the RGD antibodies was good, except for antibody 12.4.1 (Fig. 2A). We thus tested antibody internalization after CDH17 binding, as a potential tool for toxin payload delivery to tumors. We observed a similar internalization in lysosomes (assessed by colocalization with the lysosomal marker LAMP-1) for the anti-RGD mAb 25.4.1 and the control anti-CDH17 antibodies (Fig. 2B), suggesting an endocytosis of CDH17 after antibody binding.

The four purified mAbs were functionally active and inhibited the activation of β1 integrin (Fig. 2C), cell adhesion (55-68%) (Fig. 2D) and cell proliferation (Fig. 2E) in KM12SM colon cancer cells and in PANC1, a CDH17+ pancreatic cancer cell line (12). We obtained similar results with HT-29, a different CDH17+ colorectal cancer cell line (Supplementary Fig. S2A). In contrast, colorectal Colo320 cells that are negative for
CDH17 expression were not affected by the RGD mAbs (Supplementary Fig. S2B). Collectively, these results support that the RGD mAbs preferentially recognize the conformational epitopes in the native CDH17 and underscore their value for blocking cell adhesion and proliferation in colorectal and pancreatic cancer cell lines that express CDH17.

**Blocking effect of mAbs on β1 integrin activation by different cadherin RGD peptides**

Next, we investigated the minimal length of the RGD motif necessary to induce β1 integrin activation in combination with the peptide-blocking capacity of the specific mAbs. In RKO colon cancer cells, we compared the 7-mer SLRGDTR and the 5-mer LRGDT with the positive control 9-mer peptide VSLRGDTRG. Peptides were used at 1 μg/mL in the different experiments. In the 9-mer peptide-treated cells, mAbs caused a strong inhibition of β1 integrin: antibody 25.4.1 caused a complete inhibition, followed by 6.6.1 (90% inhibition) and then 12.4.1 and 6.5.2 to a lesser degree (Fig. 3A). Shorter peptides induced integrin activation, but to a lesser extent (50%). The 7-mer peptide was partially inhibited by mAbs 6.6.1 and 25.4.1 and was totally blocked by 12.4.1(Fig. 3B, C). The 5-mer had a minor inhibitory effect, likely due to the short length of the peptide supporting only a weak interaction with the binding site of the antibody (Fig. 3B, C).

To study the applicability of our mAbs to other RGD cadherins in other tumors, we examined the capacity of VE-cadherin and the CDH6, CDH16 and CDH20 RGD peptides to activate β1 integrin in metastatic melanoma and breast cancer cell lines that did not express CDH17 (Supplementary Fig. S3). Incubation with 9-mer RGD peptides from VE-cadherin (D2), VE-cadherin (D3), CDH6 and CDH20 (but not CDH16), caused β1 integrin activation in all cancer cell lines (Fig. 3D). We therefore investigated whether mAbs could
block the β1 integrin activation caused by VE-cadherin and CDH6 RGD peptides in RKO cells. VE-cad (D2) was partially blocked by the mAbs 6.6.1 and 12.4.1, whereas the VE-cad (D3) site was fully blocked by 6.6.1, 12.4.1 and 25.4.1 (Fig. 3E, F). For CDH6 RGD, we observed a complete β1 integrin activation inhibition by mAb 12.4.1, a partial blocking effect for mAbs 6.6.1 and 6.5.2 and no effect for 25.4.1 in RKO cells (Fig. 3G). Overall, the mAb blocking effects were more significant for the VE-cad (D3) motif, which displays a flanking sequence YSI/L (RGD) similar to CDH17.

**CDH17 RGD monoclonal antibodies inhibit VE-cadherin-triggered α2β1 integrin activation in melanoma and breast cancer cells**

To confirm the broad applicability of cadherin RGD-specific antibodies for other cancer metastatic cell lines, we tested two melanoma (BLM and A375) and two breast (MDA-MB-468, a triple negative cell line, and SKBR3) cancer cells expressing VE-cadherin but not CDH17 (Supplementary Fig. S3) (19). RGD mAbs inhibited the high-affinity conformation of β1 integrin (Fig. 4A, Supplementary Fig. S4A), causing a significant reduction in cell adhesion (Fig. 4B, Supplementary Fig. S4B), proliferation (Fig. 4C, Supplementary Fig. S4C) and invasion capacity through extracellular matrix, particularly in breast cancer cells (Fig. 4D, Supplementary Fig. S4D). A blocking anti-β1 integrin antibody was used as a positive control and antibodies against full-length VE-cadherin and control antibody, as negative controls (Fig. 4). Interestingly, the antibody effects on adhesion, proliferation and invasion were not associated to the reduction of cadherin expression in the cell surface (Fig. 4E), interference with tubule formation (Fig. 4F, Supplementary Fig. S5A) or alterations in endothelial permeability (Supplementary Fig. S5B). These results suggest that the RGD antibodies inhibit β1 integrin activation in VE-cadherin+ cancer cell lines without
interfering with cadherin homotypic cell-cell interactions, similar to RGD-independent cell aggregation mechanisms observed in CDH17+ cells (12).

To confirm that the blocking capacity of the RGD mAbs was specific for α2β1 integrin, we investigated for other α integrin subunits in the cancer cells. We detected the presence of a significant expression of α1, α2 and α3 on the surface of both, KM12SM and BLM cells (Supplementary Fig. S6A). Then, we performed siRNA silencing experiments for α1, α2 and α3 integrins in both cell lines (Supplementary Fig. S6B, C). Whereas RGD antibodies caused a significant reduction of adhesion to Matrigel and type I collagen in KM12SM and BLM cells silenced for α1 and α3 integrins, they did not cause any alteration in α2-silenced cells (Supplementary Fig. S6D). Together, these results confirm the specific blocking capacity of the RGD mAbs on α2β1 integrin.

**CDH17 RGD antibodies arrest integrin signaling on colorectal, pancreatic, melanoma and breast cancers**

To investigate whether the integrin signaling pathway was affected by the blocking RGD mAbs, representative KM12SM, PANC1, BLM and MDA-MB-468 cell lines were cultured on Matrigel in the presence of the four RGD mAbs or a control IgG. In KM12SM colon cancer cells, the four RGD mAbs diminished the activation of FAK, JNK and ERK kinases, which correlate with a decrease in cell adhesion and proliferation, but they did not affect Src or AKT activation (Fig. 5). In PANC1 pancreatic cancer cells, the mAbs provoked a clear decrease in JNK, ERK and Src activation but had no effect on FAK or AKT. The mAbs (except for 6.5.2) inhibited JNK, ERK and Src activation in BLM (melanoma) and MDA-MB-468 (breast) cancer cell lines, without affecting FAK activation. AKT activation was only reduced in melanoma. Src and AKT activities have been correlated with cell
invasion (19). In summary, RGD mAbs caused different type-specific effects on cell signaling after blocking the integrin pathway activation in the four tested metastatic cell lines. These results confirm cell type-specific differences observed in VE-cadherin cell signaling on migration and invasion with respect to CDH17 colon cancer cells on cell adhesion.

**RGD-specific mAbs showed anti-metastatic capacity *in vivo.***

For “*in vivo*” metastasis experiments, we examined first the effect of the antibodies on liver and lung homing capacity caused by colon and melanoma cancer cells, respectively. Tumor cells were inoculated in spleen (for liver metastasis) or tail vein (for lung metastasis) of nude mice together with the RGD mAbs. Mice were euthanized at 96 h post-inoculation to avoid unspecific binding to target organs. mRNA was then extracted from liver and lung. In melanoma and breast cancer cells treated with mAb 12.4.1 no human GAPDH was detected in lung or liver (Fig. 6A). Furthermore, in colon cancer cells, liver homing was completely inhibited after tumor cells were treated with the RGD antibodies (Fig. 6A).

We next investigated the effects of RGD-specific mAbs on mouse survival after liver (colon) or lung metastasis (melanoma). For colon cancer, highly metastatic KM12SM cells were inoculated in the spleen to induce liver colonization through the hepatic portal vein. After 48h, mice were treated on alternate days for 2 weeks, with a total dose of 50 mg of antibody per kg of mouse weight. When signs of disease were evident, animals were euthanized and livers were removed for visual inspection of metastatic nodules. Otherwise, surviving animals were sacrificed at days 80 and 90 for lung and liver metastasis, respectively. Kaplan-Meier survival curves showed that all control mice died by day 50, while those inoculated with mAb 6.6.1 were still alive at this time point; further, only half
of them developed liver metastasis by 90 days, and the rest survived to the experimental endpoint (Fig. 6B). mAb 25.4.1 caused an intermediate survival (50 days), whereas the half-life of mice inoculated with control antibody was 27 days. The number of metastatic nodules observed in liver was substantially reduced or abolished in half of the treated mice (Fig. 6C). For lung metastasis in melanoma, mice inoculated with mAbs 6.6.1 and 25.4.1 presented a prolonged survival in most of the treated animals. Lung inspection revealed a large number of metastases in control mice, but none in treated mice at the experimental end-point (Fig. 6C). In summary, blocking the cadherin RGD-induced activation of α2β1 integrin constitutes a promising strategy for treating lung and liver metastasis in melanoma and colorectal cancer.
**DISCUSSION**

After many unsuccessful attempts in the last decades to target integrins with RGD peptides for cancer therapy, we propose here a novel strategy. Rather than using extracellular matrix RGD motifs, we have demonstrated that cadherin RGD motifs are efficient and selective targets for α2β1 integrin inhibition in metastatic cells of four different types of tumors. We have shown the protective effects of cadherin RGD-specific antibodies using two different cancer cell types (colorectal and melanoma) and two metastatic settings (lung and liver metastasis). In addition, RGD mAbs were effective in blocking the integrin pathway activation in pancreatic and breast cancer cell lines. The capacity to inhibit the metastatic capacity of different cancer cell lines enhances the value and promise of these mAbs as potential therapeutic agents to control metastatic spread in different solid tumors.

We proved that i) peptide sequences containing the CDH17 RGD motif elicited blocking antibodies for β1 integrin activation in metastatic cells, ii) the RGD peptide length required for integrin activation was as short as 7 amino acids, iii) mAbs raised against the CDH17 RGD motif were equally effective against β1 integrin activation with VE-cadherin and CDH6 RGD peptides, iv) RGD mAbs blocked the integrin signaling pathway cascades in a cancer-dependent manner and v) RGD-specific mAbs significantly delayed and decreased liver and/or lung metastasis in colorectal and melanoma cancers. Moreover, since these mAbs did not affect the cell-cell homotypic interactions of cadherins (12,19), their side effects on endothelial cells and vascular integrity might be avoided. The selectivity conferred by the cadherin RGD flanking sequences might avoid unspecific binding of the mAbs to the RGD motifs of the ECM proteins (fibronectin, collagen, etc).

Integrins are key molecules in multiple cellular processes required for cancer progression and metastasis (see (24) for a review). Integrin-targeted treatments for cancer
therapy have been the focus of intense research efforts in the last decades and integrin-targeted antibodies are under clinical evaluation for different diseases (24,25). Historically, αvβ3, α4β1 or α5β1 integrins have been preferentially associated with cancer metastasis therapy (26). However, growing evidence underscores the critical relevance of α2β1 integrin as a key regulator of cancer metastasis (12,19,27,28), despite some conflicting results with a mouse mammary tumor model (29,30). Note that mouse CDH17 and VE-cadherins do not have RGD motifs (12), suggesting that alternative signaling pathways are used for metastasis progression in mice. A key role for α2β1 integrin has been well established in melanoma, rhabdomyosarcoma, gastric, prostate and colon cancer metastasis (9,31-35). Therefore, we speculate that RGD mAbs might theoretically be applicable for all of these human tumors.

α2β1 integrin has been therapeutically targeted using blocking antibodies against α2 integrin subunit (GRB-500), which is currently in clinical trials for multiple sclerosis and ulcerative colitis (27). Another α2 integrin blocking mAb demonstrated high value in the inhibition of breast carcinoma cell growth (36). Regarding β1 integrin, volociximab (M-200), a monoclonal targeting α5β1 integrin is currently in clinical trials for solid tumors (37). In general, α2β1 integrin targeting is considered safe and tolerable (27). The RGD cadherin selectivity of our antibodies avoids the adverse effects caused by indiscriminate targeting of integrins using other therapeutic approaches, like cyclic RGD molecules (38).

A cyclic RGD peptide (cilengitide) targeting the ligand binding site of αvβ3 and αvβ5 integrins reached clinical trials for glioblastoma but did not reach significant disease outcome improvement (39). In our hands, cilengitide was completely ineffective in inducing or blocking α2β1 integrin activation (Fig. 1D).
Metastasis development has been associated with enriched cancer stem cell populations (40). β1 integrin is critical to preserve stem cell populations (41). Indeed, β1 and α6 integrins are enriched in cancer stem cells (24,40). Interestingly, these two integrins are abundantly present in KM12SM colorectal metastatic cancer cells (8). Other stem cell markers present in KM12SM cells are ALDH1, ALCAM and CD44 (9). These data suggest that the KM12SM metastatic cells have some stemness features.

Cancer cell types activate different integrin pathways for metastasis, therefore it was not surprising to observe differences in the blocking effects on downstream signaling caused by the mAbs in the different cancer types. Cadherin RGD-specific antibodies blocked the integrin signaling pathways in a cancer cell-dependent manner. As a general rule, proliferation pathways were always inhibited, whereas adhesion and invasion were cell type dependent. These data support that β1 integrin is a critical molecule for the activation of multiple pathways required for metastatic colonization.

In summary, our data support that antibodies against the cadherin RGD motifs reduced the proliferation, adhesion and invasion capacity of metastatic cancer cell lines by inhibiting the activation of α2β1 integrin. The extended mice survival demonstrates the potential therapeutic effect in cancer metastasis, specifically in colorectal cancer and melanoma. Moreover, it is likely that this capacity might be extended to other neoplasias as pancreatic and breast cancer. Based on the different biochemical properties (antigen recognition by ELISA, immunoprecipitation, confocal microscopy), yield and metastatic inhibition capacity, mAb 6.6.1 seems to be the most convenient antibody for further therapeutic developments. The next steps should include the humanization process of the most effective mAbs, particularly 6.6.1, for potential clinical application. Finally, the
crucial role of α2β1 integrin in fibrosis, platelet-mediated thrombosis and angiogenesis (29) might increase considerably the application range of cadherin RGD mAbs to other diseases.

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Author contributions

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LEGENDS TO FIGURES

Fig. 1 Testing and production of CDH17-specific antibodies to inhibit the activation of β1 integrin. Different strategies were followed for the generation and testing of inhibitory antibodies. (A) RKO cells were exposed to a 9 amino acid RGD peptide (1 µg/mL) from CDH17 and treated with the indicated antibodies. Then, cells were subjected to flow cytometry using a specific antibody for testing activated β1 integrin. (B) KM12SM cells were treated with the indicated antibodies and subjected to cell adhesion assays to Matrigel. (C) KM12SM cells were subjected to MTT assays in the presence of the indicated antibodies. (D) KM12SM were incubated for 1 h with the indicated concentrations of cilengitide and subjected to flow cytometry assays as in A. Activation of β1 integrin, cell adhesion or number of viable cells was significantly inhibited by the presence of the indicated anti-Cadherin RGD antibody (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Fig. 2. Phenotypic and functional characterization of CDH17 RGD-specific antibodies. KM12SM cells were subjected to (A) confocal microscopy or (B) antibody internalization assays using the CDH17 RGD-specific antibodies or the indicated commercial antibodies. MAbs recognized surface CDH17 at different extent and are further internalized in the lysosomal compartment. Then, KM12SM and PANC1 cells were treated with the indicated antibodies and subjected to: (C) flow cytometry assays to study β1 integrin in high affinity conformation, (D) cell adhesion assays to Matrigel and (F) MTT assays. Activation of β1 integrin, cell adhesion and number of viable cells was significantly inhibited by the presence of the indicated RGD-specific antibodies in colorectal and, at a lower extent, in pancreatic cancer cells (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
**Fig. 3 Effect of RGD peptide size and sequence on the activation of β1 integrin.** RKO cells were exposed to different CDH17 RGD peptides, ranging in length from (A) 9 amino acids, (B) 7 amino acids and (C) 5 amino acids or to peptide-free medium and treated with the indicated antibodies. Cells were subjected to flow cytometry assays to detect β1 integrin in high affinity conformation. (D) RKO, BLM and MDA-MB-468 cells were exposed to 9-mers containing the RGD motif from VE-cadherin domain 2 (VE-cadherin D2), VE-cadherin domain 3 (VE-cadherinD3), CDH6, CDH16, CDH17 or CDH20 and subjected to flow cytometry assays as above. RKO cell were exposed to 9 amino acid peptide containing the RGD motif and the flanking sequences of VE-cadherin domain 2 (E), VE-cadherin domain 3 (F) and CDH6 (G), treated with the indicated antibodies and subjected to flow cytometry assays as in A. Activation of β1 integrin was significantly inhibited by the presence of the indicated RGD antibody (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Peptides were used at 1 μg/mL in the different experiments.

**Fig. 4. RGD-specific antibodies inhibit activation of β1 integrin, cell adhesion, proliferation and invasion of melanoma and breast cancer cells.** BLM and MDA-MB-468 cells were treated with the indicated antibodies and subjected to: (A) flow cytometry assays to detect β1 integrin in high affinity conformation, (B) cell adhesion assays to Matrigel, (C) proliferation assays (MTT), (D) cell invasion assays through Matrigel, (E) flow cytometry assays to detect VE-cadherin expression and (F) tube formation assays. Activation of β1 integrin, cell adhesion, number of viable cells and number of invasive cells were significantly inhibited by the presence of the indicated anti-cadherin RGD antibody (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
**Fig. 5. Anti-cadherin RGD antibodies inhibit the integrin pathway cell signaling.** KM12SM, PANC1, BLM and MDA-MB-468 cells were treated with the indicated antibodies and added to Matrigel-coated plates for 30 min. Then, cells were lysed, and the extracts analyzed by Western blot using antibodies against FAK, JNK, ERK1/2, Src or AKT or their phosphorylated forms. RhoGDI was used as a loading control.

**Fig. 6. RGD mAbs increase mouse survival to liver and lung metastasis.** (A) KM12SM, BLM and MDA-MB-468 cells treated with control or anti-RGD 6.6.1 mAb were inoculated intra-tail vein or spleen in Swiss nude mice. At 96 h after inoculation RNA was isolated from lungs (for intra-tail inoculation) or liver (for spleen inoculated) and subjected to RT-PCR to amplify human GAPDH for homing detection. Anti-RGD antibodies specifically inhibited the homing in lung and liver, for melanoma, breast and colorectal cancer. (B) Kaplan–Meier survival results for nude mice inoculated with KM12SM or BLM cells in the spleen or intravenously, respectively. Starting at 48 h after inoculation, the indicated antibodies (50 mg/Kg of weight, divided in 7 doses) were administered intravenously for two weeks. When signs of illness were detected, mice were euthanized and examined for macroscopic metastases in liver. Survival was significantly enhanced by the indicated mAb (*, p < 0.05; ***, p < 0.001). (C) Representative pictures of livers and lungs from the inoculated mice after necropsy.