Chain specific antibodies for laminin-511

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Abstract

Mouse monoclonal antibodies (mAbs) that bind to specific chains of laminin-511 (LM-511) have been developed. Antibody 2F12 binds to the LMα5 chain, 3G10 binds to the LMβ1 chain and 3C12 binds to the LMγ1 chain. These antibodies can be used to purify LM-511, to detect LM-511 in cell extracts or to detect the location of LM-511 in tissue by immunohistochemistry. In combination, the antibodies recognize all three chains of LM-511 and combinations of the antibodies can be used to quantitate levels of LM-511 in physiological fluids. One of the antibodies (3G10) is a potent inhibitor of the activity of LM-511 in cell adhesion, spreading and proliferation assays.

Introduction

Laminins (LMs) are high molecular weight extracellular matrix (ECM) proteins that are the major components of the basement membranes in vertebrates (Timpl and Brown, 1996). The LMs act in conjunction with growth factors such as TGF-α to facilitate the proliferation of normal and cancerous epithelial cells (Pouliot et al., 2000; Sizeland and Burgess, 1991). Each LM molecule is a cross-shaped heterotrimer of disulphide-linkedα, β and γ chains. Several isoforms of each LM chain have been identified: five α (α1 - α5), three β (β1 - β3) and three γ (γ1 - γ3) chains (Colognato and Yurchenco, 2000). Currently, 16 LM trimers have been identified, each differing in chain composition, physical properties, tissue distribution and biological activities (Colognato and Yurchenco, 2000). Recent studies have shown that the LMα5, β1 and γ1 chains are widely expressed in epithelial basement membranes as well as those belonging to the vascular endothelium, neural structures and stromal myofibroblasts, whereas the α1 chain (present in LM-111 and LM-121) has a more restricted expression pattern found only in the basement membranes of distinct epithelial tissues (Ekblom et al., 1990; Falk et al., 1999; Hewitt et al., 1997; Klein et al., 1988; Maatta et al., 2001; Miner et al., 1997; Pedrosa-Domellof et al., 2000; Tani et al., 1999; Virtanen et al., 2000). The LM-511 (α5, β1, γ1) and LM-332 (α3, β3, γ2) are widely distributed in normal tissues.

The LMα5 chain, present in LM-511, LM-521 and LM-523 is expressed in a number of adult tissues including kidney, lung, placenta, heart, the central nervous system, skeletal muscle, pancreas, colon, breast and skin tissues (Champliaud et al., 2000; Chia et al., 2007; Durkin et al., 1997; Hewitt et al., 1997; Koch et al., 1999; Libby et al., 2000; Miner et al., 1995; Miner et al., 1997; Pouliot et al., 2002; Sorokin et al., 1997). The status of LM-523 as a basement membrane LM is in doubt, as its

expression has only been associated with nerve terminals (Libby et al., 2000). The LMγ3 chain appears to have a non-basement membrane distribution in most tissues (Koch et al., 1999; Libby et al., 2000; Son et al., 2000).

The LMα5 chain is expressed at moderate to high levels in almost all epithelial tumor basement membranes (Chia et al., 2007; Maatta et al., 2001). As the β2 chain is detected only occasionally in epithelial basement membranes, LM-511 rather than LM-521 appears to be the most widespread heterotrimer in normal and malignant tissues. The expression of the LM-511and LM-332 isoforms appears to be an important feature of many human cancers. Immunohistochemical staining of moderately differentiated colon adenocarcinoma cryosections using the 4C7 monoclonal antibody, which binds to the to the LG1 module of the α5 chain G domain (Ido et al., 2005), reveals a linear pattern of staining at the junction of normal and neoplastic tissues, indicating that LM-511/-521 are present at the invasive front of tumors (Moriya et al., 2001; Pyke et al., 1995). However, discontinuous staining of the α5 chain is also seen in neoplastic tissues of the colon and breast (Hewitt et al., 1997), indicating that level of α5 chain expression in the basement membrane of malignant tissues is perturbed.

The 4C7 antibody has been used previously in immunohistochemistry studies to detect the presence of the LMα1 chain in multiple tissue types (Hewitt et al., 1997; Korhonen and Virtanen, 1997; Sanes et al., 1990; Virtanen et al., 1995). However, other studies have clearly demonstrated that 4C7 actually detects human LM α5 chain rather than the LMα1 chain (Church and Aplin, 1998; Kikkawa et al., 1998; Tiger et al., 1997), indicating that many of the characteristics originally attributed to LM-111 on the basis of 4C7 reactivity need to be re-evaluated and should be reassigned to LM-511, LM-521 or LM-523.

While the 4C7 antibody can be used as a reagent for immunohistochemistry studies on frozen tissues, it reacts poorly with archival tissues that have undergone formalin-fixation and paraffin-embedding even with prior treatment with proteases. Formalin-fixation of tissues results in good penetration into the tissue and the preservation of tissue is very high, however, the antigen of interest can become denatured or hidden by formalin cross-links formed during this preservation process (Boenisch, 2001). Antigen retrieval can be employed to unmask these antigens. Techniques used include pressure cooking, microwaving, and heating sections in appropriate buffers and protease treatment (Cregger et al., 2006) An alternative method combining Zinc-Tris fixation buffer and enzymatic antigen retrieval was developed recently for paraffin-embedded tissues (Chia et al., 2007). This protocol results in robust staining of the basement membrane with the 4C7 antibody. Although this enables the 4C7 antibody to be used routinely for immunohistochemical analysis of paraffin-embedded tissues, it does not solve the problems for detecting LM in archival tissues that have been preserved using formalin-fixation.

Antibodies that bind to each of the LM-511 chains are required to distinguish between the three LM isoforms that contain the α5 chain (LM-511, LM-521 and LM-523). Such antibodies will be particularly useful for affinity purification of either native or recombinant human LM-511. Used in series, antibodies that recognize individual chains of LM-511 could be employed to specifically purify LM-511 from complex tissues such as human placenta. A recent report described a LMα5 specific monoclonal antibody (mAb) 8G9, with a specificity different to mAb 4C7, which is capable of inhibiting LM-511-dependent cell adhesion and migration of several cancer cell types (Wondimu et al., 2013).

In this report we describe the generation and characterization of several mAbs that specifically recognize the different chains of LM-511. The criteria for antibody selection were established prior to immunization of mice with purified recombinant LM-511. These included the ability to: (1) immunoprecipitate LM-511 from crude conditioned media for the use in the immunoaffinity based purification of either native and recombinant LM-511, (2) detect LM-511 chains in Western blot to aid in biosynthetic studies, (3) compatibility with immunohistochemistry allowing the investigation of the expression pattern of LM-511 during colon tumor progression, (4) possession of a neutralizing function for cell adhesion and spreading for the potential use in a therapeutic setting and (5) have an epitope different to that of the 4C7 antibody.

Materials and Methods

Cell culture

The LIM1215 human colonic carcinoma cell line, derived from a patient with inherited nonpolyposis colorectal cancer (Whitehead et al., 1985), was maintained in growth medium (GM) containing RPMI 1640 medium supplemented with penicillin (60μg/mL), streptomycin (100μg/mL) and 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO2 under humidified atmosphere. For all experiments cells between passages 15-25 were used. When starvation conditions were required serum-free media (SFM: RPMI 1640 medium supplemented with 20mM L-glutamine, 0.25μM transferrin and 0.1% (w/v) bovine serum albumin (BSA)) was used.

The recombinant human LM-511 (rhLM-511)-expressing cell line (Doi et al., 2002) was routinely maintained in Dulbecco's Modified Eagle's (DME) medium

supplemented with 10% (v/v) FBS and 0.5mg/mL geneticin (G418). Cells were used between passages 3-10.

Purification of recombinant laminin-511 (rhLM-511)

The rhLM-511 expressing cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FBS and 0.5mg/mL G418. Serum-free conditioned medium for the purification of rhLM-511 (rhLM-511-SCM) was produced by growing cells in T175cm2 flasks in 25mL of DMEM with 10% (v/v) FBS until approximately 50-60% confluent (2-3 days). The medium was then removed and cells washed twice with 25mL of HT-PBS (140mM NaCl, 20mM NaPO4, pH 7.2). Cells were then serum-starved in 25mL DMEM containing 0.5mg/mL G418, 1mM sodium pyruvate (Sigma-Aldrich) and insulin-transferrin-selenium supplement (Sigma-Aldrich) for up to 6 days. rhLM-511–SCM was harvested, clarified by centrifugation for 60min at 4°C, filtered through a 0.22µm filter-unit and stored at -20°C until required.

rhLM-511 was purified using immunoaffinity chromatography using immobilized 4C7 mAb (Engvall et al., 1986). 5mL of 4C7 at 1mg/mL was coupled to 5mL of NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare Bio-Sciences AB) overnight at 4°C. Unreacted sites were blocked following incubation with 1M ethanolamine at room temperature for 2h. The 4C7-NHS beads were equilibrated with HT-PBS (pH 7.2) prior to protein purification.

To allow for efficient binding to the 4C7-NHS beads, 100mL of rhLM-511-SCM was incubated in a batch wise manner with the beads overnight at 4°C with agitation. After washing the column with HT-PBS (eight column volumes), rhLM-511 was eluted from the immunoaffinity column at room temperature with 0.1M glycine (pH

2.5) and fractions (1mL) neutralized by the immediate addition of 100μL of 1M Tris-HCl (pH 8). Protein elution was monitored using Bradford reagent and dot blot assay. Fractions positive for rhLM-511 (3-14, total volume of 12mL) were concentrated 12-fold to approximately 1mL by evaporation and 0.5mL of the concentrated material was loaded at 0.5mL/min onto a TSK G3000SW size exclusion column (7.5 x 600mm) (LKB-Produkter AB, Bromma, Sweden) pre-equilibrated with HT-PBS (pH 6.5) at room temperature. 0.5mL fractions were collected. Individual fractions were characterized by both SDS-PAGE and mass spectrometric analysis.

Production of monoclonal antibodies

LM-511 mAbs were produced at the Walter and Eliza Hall Monoclonal Laboratory, Bundoora, Melbourne. Four mice (2 BALB/c and 2 C57BL6) were immunized three times with immuno-affinity purified rhLM-511. Mice received 30μg of purified rhLM-511 for the first two injections (the first with Freud's complete adjuvant and the second injection with Freud's incomplete adjuvant), and 20μg for the third injection. Mice were bled 12 days after the third injection of rhLM-511 and tested for binding to LM-511 in an ELISA assay. A BALB/c mouse showing high reactivity against rhLM-511 was selected for monoclonal antibody production. Spleen cells were isolated from this mouse and fused with the mouse myeloma cell line Sp2/0. The supernatants of each fusion were tested for their reactivity towards purified rhLM-511 using a LM-511 specific ELISA. Clones that showed reactivity towards rhLM-511 were investigated further, prior to cell expansion, antibody purification and characterization.

Purification of monoclonal antibodies

Hybridoma clones selected for expansion were grown until confluent in RPMI supplemented with 10% (v/v) FBS, 2mM glutamine, 2mM sodium pyruvate and 10μM β-mercaptoethanol. Cells were harvested, washed with HT-PBS, and seeded, at $6x10^4$ cells/mL in 450mL of RPMI containing reduced serum (1% (v/v FBS), in 2 liter plastic roller bottles (Corning Life Sciences). Cells were incubated at 37°C for 5-9 days and the media harvested and cleared of cell debris by centrifugation (10,000rpm, JA-10 Beckman rotor, 4°C, 60min), filtered through a 0.22μm filter-unit (Millipore) and stored at -20°C until required.

Batches (250mL) of the above conditioned medium were loaded at 2mL/min onto a 10mL Protein-A column pre-equilibrated with HT-PBS (pH 8.2) at 4°C. Antibodies were eluted from the column with 10mM sodium acetate (pH 4.5) at 1mL/min over 20min. One mL fractions were collected and immediately neutralized by addition of 100μL of 1M Tris-HCl (pH 8). For the 3C12 and 3G10 antibodies, peak fractions were collected and concentrated using a Vivaspin20 30,000kDa MWCO spin filtration unit and then loaded at 0.5mL/min onto a Superose 12 10/300GL size exclusion column pre-equilibrated with HT-PBS (pH 7.2). Antibodies were eluted at 0.5mL/min and 0.5mL fractions collected. The purity of final fractions was determined using SDS-PAGE analysis. The 2F12 and 4C10 antibodies were concentrated via evaporation and buffer exchanged into HT-PBS on a PD-10 desalting column to avoid antibody aggregation.

Isotype analysis

Isotype analysis of each monoclonal antibody was performed using a Mouse MonoAB ID Kit (HRP) (Zymed Laboratories). 96-well plates were coated with rhLM-511 (1μg/mL in HT-PBS) overnight at 4°C. Following blocking of the surface with 1% (w/v) BSA for 1h at 37°C, 100μL of each antibody (0.5μg/mL) was added to appropriate wells. After 30min at 37°C the wells were washed four times with HT-PBS, 0.05% (v/v) Tween-20 and rabbit anti-mouse subclass-specific antibodies were added. After 30 min incubation at 37°C and washing, HRP-conjugated goat anti-rabbit IgG was added to each well and the plates incubated for 30min at 37°C. Unbound antibodies were washed four times, as above, ABTS chromophore was added to each well and allowed to develop at room temperature for at least 15min. The absorbance was measured at 405nm in a Titertek Multiskan MCC/340 plate reader.

Immunoprecipitation and Western blot analysis

One microgram of each antibody was added to 200μL of rhLM-511-SCM and immunoprecipitated overnight at 4°C with agitation. 50% (v/v) Protein-A Sepharose slurry was added to the immunoprecipitation mix and incubated for an additional 2h at 4°C. After washing the immunoprecipitates were resolved on NuPAGE 3-8% gradient Tris-Acetate gels (Invitrogen) and the presence of rhLM-511 was detected by silver staining.

For Western Blot analysis, 500 ng of purified rhLM-511 were resolved under reducing conditions on either a 3-8% gradient Tris-Acetate gel or a Novex 4% Tris-Glycine gel (Invitrogen). Separated proteins were then transferred to an Immobilon P Transfer Membrane (Millipore) at 100V for 90min. Purified 2F12, 3C12, 3G10 and

4C10 as the primary probes were diluted to 1μg/mL with 1% (w/v) skim milk/TTBS (50mM Tris-HCl (pH 7.4), 150mM NaCl, 0.05% (v/v) Tween20) while goat anti-mouse HRP-conjugated secondary antibodies were diluted 1:3,000 with 1% (w/v) skim milk/TTBS. Detection was by ECL according to the manufacturer's instructions.

Biacore analysis

All biosensor experiments were performed at 25°C using a Biacore 2000 SPR biosensor. Surfaces were prepared by amine coupling chemistry (Nice and Catimel, 1999) using a CM5 sensor chip (Biacore Life Sciences) following activation with N-ethyl-N'-dimethylaminopropyl-carbodiimide (EDC) and N-hydroxysuccinimide (NHS). All experiments were performed in Biacore buffer (HBS: 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), 3.4mM EDTA, 0.15mM NaCl, 0.005% (v/v) Tween-20). A blank surface was prepared by activation of the chip surface with 35μL of EDC/NHS and immediate blocking of available binding sites with 35μL of 1M ethanolamine.

LM-511 mAbs surfaces were made by injection of 50μL of antibody (30μg/mL in 10mM sodium acetate pH 4.2) over an activated CM5 chip at a flow rate of 10μL/min. This was repeated using the Surface Preparation Wizard until a relative response of 8000 RU was achieved. Surfaces were then washed with 10mM HCl to remove any non-covalently bound material. Finally, the surface was blocked with 35μL of 1M ethanolamine (pH 8.5) at a flow rate of 10μL/min.

To determine if the LM-511 mAbs bound to a different epitope on the LM-511 molecule, a set of binding curves was generated using the Binding Analysis Wizard. A 3G10 antibody surface was loaded with of rhLM-511-SCM (60μL) at a flow rate of

10μL/min. This was followed by sequential injections of 60μL of the remaining LM-511 antibodies (2F12, 3C12, 3G10, 4C10 and 4C7) diluted to 20μg/mL in Biacore buffer. Injections were performed at a flow rate of 10μL/min. Regeneration was performed with a 10μL injection of 0.1M glycine (pH 2.5) at 20μL/min.

Immunohistochemistry

Immunohistochemistry was performed using normal and cancerous human colon tissues obtained from Peter MacCallum Cancer Centre Tissue Bank. Tissues were fixed for 48h at 4°C in Zinc-Tris fixation buffer (0.1M Tris-HCl (pH 7.4), 2.8mM calcium acetate, 36.7mM ZnCl2, 22.8mM zinc acetate) and processed for paraffin embedding. Sections (4μm) were re-hydrated and equilibrated in an antigen retrieval buffer (50mM Tris-HCl (pH 7.8), 0.1% (w/v) C_aCl_2, 150mM NaCl). Following digestion with trypsin (1mg/mL) at 37°C for 5min, each section was washed extensively with water and blocked for 30min in blocking buffer (HT-PBS, 3% (v/v) normal goat serum, 0.05% (v/v) Tween-20) at room temperature. Sections were incubated with specific primary antibodies (1-2μg/mL) overnight at 4°C under humidified atmosphere. Supernatant from ID4.5 (IgG2a) and 4C7 hybridomas were used as the isotype control and positive control respectively. Following overnight incubation, sections were washed three times with wash buffer (HT-PBS, 0.05% (v/v) Tween-20) and biotin-conjugated goat anti-mouse secondary antibodies (Vector Laboratories) were applied to the sections for 1h at room temperature. Sections were washed three times with wash buffer and once with HT-PBS. Endogenous peroxidases were inactivated following incubation in methanol and 0.3% (v/v) hydrogen peroxide for 30min at room temperature. Following washing with wash buffer the primary-secondary antibody complexes were detected using the ABC

reagent and DAB peroxidase substrate kit (Vector Laboratories). Slides were developed in parallel and each reaction was stopped by washing in HT-PBS before the appearance of non-specific staining in the isotype control sections. The sections were counterstained with hematoxylin, mounted onto coverslips in DPX neutral mounting medium (Chem-Supply) and photographed on a Zeiss Axioskop 2 microscope.

Inhibition assays

Wells of a 96-well tissue culture plate (Nalgene Nunc International) were coated overnight at 4°C with 50μL of 1μg/mL of rhLM-511, diluted in HT-PBS. rhLM-511 was pre-incubated at 56°C for 20min prior to coating. Excess rhLM-511 was removed and wells were washed twice (2x200μL HT-PBS) and non-specific binding sites were blocked with 2% (w/v) BSA in serum-free RPMI medium (SFM-RPMI) for 1hr at 37°C. LIM1215 cells were washed twice in SFM-RPMI and resuspended at $2x10^6$ cells/mL in RPMI-SFM. Cells were incubated with 10μg/mL calcein-AM (Molecular Probes, Invitrogen) in the dark for 45min at 37°C, 5% CO_2 with occasional agitation. Cells were washed twice with RPMI-SFM and resuspended at a density of $2x10^5$ cells/mL in RPMI-SFM. One hundred μL of labeled cells ($2x10^4$ cells) were added to the LM-511 pre-coated 96-well plates and allowed to adhere for 2½ hrs at 37°C in 5% CO2. When indicated, EGF was added at a concentration of 10ng/mL (diluted in RPMI-SFM). Following incubation, non-adherent cells were removed and wells washed twice with 200μL of RPMI-SFM and once with TBS (50mM Tris-HCl (pH 7.4), 150mM NaCl) containing 1mM $MgCl_2$ and 2mM C_aCl_2. Adherent cells were lysed with 100μL of 1% (w/v) SDS for 15min at room temperature and the fluorescence measured at an excitation wavelength of 482nm and

emission wavelength of 520nm using a FLUOstar OPTIMA (BMG Labtech). To test the inhibitory effect of the LM-511 antibodies, each antibody was diluted from 5ng/mL and 10μg/mL in RPMI-SFM and incubated with the pre-coated LM-511 surface for 30min at 37°C in 5% CO_2 prior to the addition of cells.

Cell spreading assay

Purified rhLM-511 was diluted to the desired concentration in HT-PBS and pre-incubated at 56°C for 20min before coating 96-well plates (50 μL/well) overnight at 4°C as described above. LIM1215 cells were seeded at low cell density ($2x10^4$ cells/mL in SFM-RPMI with or without EGF (10 ng/mL) purified from mouse submaxillary glands as described previously (Burgess et al., 1982). The plates were centrifuged at 1,500rpm, 4°C for 5min (Sigma 4K15 centrifuge) (Sigma Laborzentrifugen GmbH) to allow cells to settle and then incubated at 37°C in 5% CO_2 for 6hrs. Cell morphology was examined under a phase contrast microscope (EL WD 0.3) (Nikon, Kanagawa, Japan) at 20x magnification. The percentage of cell spreading was calculated by counting at least 100 cells/well. To test for the inhibitory activity of the LM-511 antibodies, 50μL of each antibody (5ng/mL up to 50μg/mL) were incubated with the pre-coated LM-511 surface for 30min at 37°C in 5% CO_2, before addition of cells.

Cell proliferation assay

Purified rhLM-511 was first pre-incubated at 56°C for 20min and 50 uL (1μg/mL) used to precoat 96-well tissue culture plates overnight at 4°C as described above. LM-511 aggregates on cold storage, unless it is preheated, it will not coat the tissue culture plate. LIM1215 cells were seeded at low cell density ($2x10^3$ cells/100 μL) in

SFM-RPMI with or without EGF (10ng/mL). Cells were incubated at 37°C in 5% CO_2 for 72hrs. Following incubation, media was removed and the cells gently washed twice with 200μL of HT-PBS. Cells were then incubated in the dark with 1mM calcein-AM in HT-PBS for 30min at 37°C in 5% CO2. Wells were washed twice with 200μL of TBS (50mM Tris-HCl (pH 7.4), 150mM NaCl) containing 1mM $MgCl_2$ and 2mM $CaCl_2$ to remove any residual calcein and cells were lysed with 100μL of 1% (w/v) SDS for 15min at room temperature. Specific fluorescence was measured as described for the adhesion assay. To test the inhibitory action of the 3G10 antibody, 50μL of antibody diluted from 100μg/ml to 1μg/ml in SFM-RPMI was incubated for 30min with the pre-coated LM-511 surface at 37°C in 5% CO_2, prior to the addition of LIM1215 cells.

Results and Discussion

Generation of LM-511 monoclonal antibodies

BALB/c mice were immunized with purified rhLM-511 (three injections, see Materials and Methods) and twelve days following the third injection, mouse spleen lymphocytes were isolated and fused with the mouse myeloma cell line Sp2/0 to generate hybridoma clones. All hybridoma clones were tested for their reactivity towards rhLM-511 in an ELISA prior to final selection and expansion of the chosen clones.

A number of hybridoma clones met the desired selection criteria; they appeared to inhibit rhLM-511 function in cell adhesion and spreading assays and/or immunoprecipitated rhLM-511 from crude conditioned media (data not shown). Four clones, 2F12, 3C12, 3G10 and 4C10, were selected for antibody purification,

isotype analysis and further characterization by immunohistochemistry and Western blotting.

Epitope and isotype analysis of purified monoclonal antibodies

The isotype of each LM-511 monoclonal antibody was determined using a Mouse MonoAB ID Kit (Zymed Laboratories). The well characterized 4C7 antibody (at 0.5μg/mL) is known to be of IgG2a isotype and to have a kappa light chain and was used as a control (Engvall et al., 1986). The 2F12, 3G10 and 4C10 antibodies were found to have an IgG1 isotype, while antibody 3C12 was as $IgG2_a$ (Figure 1A). Only low non-specific binding was seen with rabbit anti-mouse IgA antibodies. The rabbit anti-mouse IgA antibody also reacted to a small extent with the LM-511 surface (Figure 1A, red bar), further confirming that the low reactivity detected in wells treated with the rabbit anti-mouse IgA antibody was non-specific. All LM-511 mAbs generated in this study, along with the 4C7 antibody, contain the more common Kappa light chain (Figure 1B).

BIAcore analysis was employed to compare the epitopes of each of the LM-511 mAbs by investigating their potential to bind simultaneously to LM-511. One desired property of these antibodies was that they recognize a different epitope different to that recognized by 4C7. Such antibodies could potentially complement the 4C7 antibody and be used in applications where 4C7 is not suitable such as Western blot analysis and immunohistochemistry on tissue samples processed using formalin-fixation and paraffin-embedding. Further we were aiming to establish a sensitive sandwich ELISA using two antibodies: one for immunocapture and the other for sensitive and specific detection.

An epitope-binning method was developed using the BIAcore binding analysis wizard (BIAcore) that enabled sequential injections of each LM-511 antibody over a 3G10 antibody surface in complex with rhLM-511. The injection of rhLM-511-SCM over a 3G10 antibody surface resulted in strong binding due to the formation of a 3G10 antibody/rhLM-511 complex (Figure 2A, black bar). There was very little dissociation apparent immediately following injection of the rhLM-511-SCM (Figure 2, black asterisk) indicating strong binding. Injection of the 3G10 antibody (20μg/mL diluted in HBS) over the 3G10/rhLM-511 complex was then performed (Figure 2A, yellow bar). No additional binding of the 3G10 antibody to the pre-formed 3G10/rhLM-511 complex occurred, confirming that the binding epitope of the 3G10 antibody was saturated. However, further binding was observed when the other antibodies were injected, indicating interactions via a different epitope.

Injection of the 4C7 antibodies over pre-formed 3G10/rhLM-511 complexes resulted in binding showing the 3G10 monoclonal antibody has a different epitope to that of 4C7 (Figure 2A, orange bar). A further injection of the 4C10 antibody (Figure 2A, green bar) resulted in another binding event, indicating the epitope of the 4C10 antibody is different to that of both the 3G10 and 4C7 antibodies.

The 2F12 antibody did not bind to LM-511 when either 3G10 or 4C7 antibodies were bound (Figure 2B, dark blue bar), suggesting that the epitope recognized by the 2F12 antibody overlaps with either the 3G10 or 4C7 epitope. A subsequent injection of the 3C12 antibody over the 3G10/rhLM-511/4C7 complex (Figure 2B, light blue bar) resulted in binding, demonstrating that the 3C12 antibody has an epitope different to that recognized by 3G10 and 4C7.

To determine if the epitope of the 2F12 is similar to that of the 3G10 or 4C7, 2F12 was injected over 3G10/rhLM-511 complexes (Figure 2C, dark blue bar). This

resulted in positive binding indicating that the epitope recognized by 2F12 antibody overlaps with the 4C7 but not the 3G10 epitope and therefore localizes to the C-terminal globular domain of the α5 chain (Ido et al., 2005, 2006).

To confirm that the 2F12, 3C12, 3G10 and 4C10 antibodies react with epitopes that localize to different regions of the LM-511 molecule, the antibodies were injected in succession over preformed 3G10/LM-511 complexes (Figure 2C). All three LM-511 mAbs were able to bind to LM-511 that is orientated via the 3G10 antibody, again indicating they each have different epitopes. The 3G10 antibody bound relatively strongly to LM-511 as indicated by the lack of dissociation of the complex after the injection of LM-511 (Figure 2A, B and C, black asterisk). Similarly, the interaction between the 2F12 antibody and LM-511 was strong with a rapid on-rate, shown by the steep slope at the beginning of the antibody injection, and a stable baseline at the end of the 2F12 injection indicating that there is no dissociation of the antibody/protein complex (Figure 2C, dark blue asterisk). The 3C12 antibody also exhibited strong binding to LM-511 and had an even faster on-rate than the 2F12 antibody as shown by the extremely sharp slope at the beginning of the antibody injection (Figure 2C). This interaction was also stable and no dissociation between the 3C12 antibody and LM-511 was seen immediately after the antibody injection (Figure 2C, light blue asterisk). The interaction between the 4C10 antibody and LM-511 was less stable as shown by dissociation of the complex after the injection (Figure 2C, green asterisk).

A full kinetic analysis of the binding between LM-511 and the 2F12, 3C12, 3G10 and 4C10 antibodies would be helpful for the establishment of an ELISA. However, a kinetic analysis with immobilized LM-511 is difficult because of denaturation of the LM-511 during regeneration. Nevertheless, it should be possible to acquire the data

using multiple injections of each antibody without regenerating the surface. Alternatively, the sensor chip could be coupled with each antibody and the captured LM-511 used to determine the most sensitive secondary antibody.

Determination of LM-511 chain specificity

Western blot analysis was used to determine which LM-511 chains the 2F12, 3C12, 3G10 and 4C10 mAbs recognized. When rhLM-511 was resolved under reducing conditions on a 3-8% gradient Tris-Acetate pre-cast gel and probed with the 2F12 monoclonal antibody, the rhLM-511/antibody complexes detected clearly aligned with the α5 chain (Figure 3A). This result is consistent with the BIAcore analysis showing that 2F12 and 4C7 antibodies bind overlapping regions of LM-511 (Figure 2). It is interesting that 2F12 detects LM-511 on Western blots, but 4C7 does not detect denatured LM-511, suggesting the epitopes may overlap but they are not identical. Due to the similarity in the molecular weight of the β1 and γ1 LM chains (210kDa and 200kDa respectively), these subunits were resolved on a 4% Tris-Glycine pre-cast gel allowing for better separation of higher molecular weight protein bands (100 – 400kDa). Western blot analysis of rhLM-511 resolved under reducing conditions on a 4% Tris-Glycine pre-cast gel and subsequently probed with the 3C12 and 3G10 antibodies showed the epitope recognized by these antibodies resides on the γ1 and β1 chain respectively (Figure 3B). The purified 4C10 antibody did not recognize any of the LM-511 chains following SDS-PAGE under reducing or non-reducing conditions (data not shown). This indicates that the epitope of the 4C10 antibody is sensitive to any conformational changes and can only recognize LM-511 under native conditions.

The ability of each purified monoclonal antibody to immunoprecipitate LM-511 was examined using crude rhLM-511-SCM. The 2F12, 3C12 and 3G10 mAbs efficiently immunoprecipitated LM-511 from crude rhLM-511-SCM (Figure 4). Only a weak protein band representing the α5 chain could be seen in the rhLM-511-SCM immunoprecipitates performed with the 4C10 antibody (Figure 4). These observations, together with the dissociation of rhLM-511/4C10 complexes seen in the BIAcore analysis (Figure 2) indicate that the 4C10 antibody: rhLM-511 complex is of relatively low affinity.

Taken together, the results from these data demonstrate that the 2F12, 3C12 and 3G10 antibodies recognize the α5, γ1 and β1 chains respectively, and can efficiently precipitate LM-511 from crude rhLM-511-SCM. Thus these three antibodies could be used in combination for the purification of LM-511. 3G10 neutralizes LM-511 and would be expected to capture native LM-511. Although 2F12 and 3C12 can also capture rhLM-511, further studies are required to demonstrate that these antibodies can be used to purify native LM-511.

Inhibition studies

The action of each of the LM-511 mAbs on cell adhesion, spreading and proliferation was determined. Inhibition of these responses by LM-511 antibodies could identify new and biologically relevant sites on the LM-511 molecule required for adhesion, spreading and/or proliferation. For these assays, each antibody was pre-incubated with a rhLM-511 coated surface prior to the addition of calcein-labeled LIM1215 cells for 150min. The 3G10 antibody reduced the adhesion of LIM 1215 cells to LM-511 by 80% (Figure 5A). The 2F12 inhibited LIM1215 cell adhesion to LM-511 moderately (approximately 25% inhibition) while the 3C12 and 4C10 antibodies had

no effect on cell adhesion (data not shown). The 3G10 antibody resulted in almost complete inhibition of LIM1215 cell spreading on LM-511, even in the presence of EGF, a growth factor known to enhance LM-511-induced spreading of LIM-1215 cells at low density (Pouliot et al., 2000) (Figure 5B).

As the 3G10 monoclonal antibody strongly inhibits LM-511 induced cell adhesion and spreading, this antibody was also tested for its ability to inhibit LIM1215 cell proliferation. LIM1215 cells seeded at low density were assayed under four sets of conditions: untreated, stimulated with EGF, grown on a rhLM-511 surface or grown on a rhLM-511 surface and stimulated with EGF. The 3G10 antibody, was added to each plate for 30min prior to the addition of cells. Following 72hr incubation, cells were labeled with calcein-AM to quantitate the differences in the number of viable cells present at the end of the assay. When grown on a rhLM-511 surface LIM1215 cells (Figure 5C, dark blue line) proliferated more rapidly than cells on an untreated surface (Figure 5C, red line). Cell proliferation was also enhanced when LIM1215 cells were stimulated with EGF over 72hrs (Figure 5C, light blue line) and proliferation further increased when cells were grown in the presence of both rhLM-511 surface and soluble EGF (Figure 5C, green line). Treatment of the LM-511 coated surface with the 3G10 antibody, resulted in a decrease in cell proliferation under all culture conditions. Interestingly, cell proliferation was reduced by approximately 35% when LIM1215 cells were seeded on plastic and treated with 100μg/mL of 3G10. Even in the presence of EGF, 3G10 (10 μg/mL) inhibited proliferation of LIM1215 cells by up to 60%. Since LIM1215 cells produce LM-511 in culture (Pouliot et al., 2000), it is conceivable that 3G10 may inhibit proliferation by interfering with the interaction of LIM1215 cells with autocrine-derived LM-511 under these conditions.

In a recent report five antibodies were developed which recognized the LMα5 chain (Wondimu et al., 2013). Those antibodies were useful for immunohistochemistry, however, one of the antibodies (8G9) inhibited integrin-dependent adhesion and migration of several cancer lines on LM-511. When combined with mAb 4C7, mAb 8G9 also inhibited cell adhesion to LM-521. The mAb 8G9 / mAb 4C7 combination was not as effective for inhibiting the migration of the cancer cells on LM-521. It would be interesting to compare the biological specificities (i.e. adhesion, migration and proliferation) of combinations of mAb 3G10 and mAb 8G9. In particular, it is important to determine whether either mAb (3G10 or 8G9) can inhibit proliferation or induce the death of cells independently of their effects on migration or cell adhesion. The LIM1215 cells undergo cell density-dependent morphological changes in serum-free culture (Pouliot and Burgess, 2000; Sizeland and Burgess, 1991). At a high cell density, LIM1215 cells secrete sufficient TGF-α to induce cell spreading and proliferation (Sizeland and Burgess, 1991). Therefore the proliferation of LIM1215 cells at low cell density, even in the presence of EGF, is most probably driven in part by autocrine secreted LM-511. mAb 3G10 has an epitope that resides on the LM β1 chain and must be located in a region that is critical for the maintenance of a number of cell processes. Thus, the antibody could potentially be used as a therapeutic to inhibit the growth of tumors that secrete LM isoforms containing the β1 chain.)

Immunohistochemistry

Attempts to stain formalin-fixed archival tissues with the LM-511 antibodies were unsuccessful. However, as reported previously for breast tumor tissues, fixation of normal colon and colon cancer tissues with zinc-Tris buffer prior to paraffin-embedding was sufficient to maintain excellent tissue architecture as seen by H&E

staining (Figure 6A, B) while retaining the reactivity of all three chain-specific antibodies against LM-511: 2F12 (α5chain, Figure 6 C, D), 3G10 (β1 chain, Figure 6 E, F) and 3C12 (γ1 chain, Figure 6 G,H). When these tissues were stained with antibodies against other LM chains, the β2 chain (C4 antibody, (Sanes et al., 1990) (Hunter et al., 1989) was also detected (data not shown). So while LM-511 is expressed, other isoforms are also present. Interestingly, while all three subunits of LM-511 were restricted to epithelial and vascular basement membranes in normal tissues (Figure 6 C, E, G), in advanced tumor specimens their basement membrane expression appeared more fragmented (Figure 6 D, F, H) and diffuse expression of the LMα5 chain was also detected at the "invasive front" of tumor foci (Figure 6D, asterisk). Together, these observations suggest that changes in the distribution of LM-511 (or LM-521) may facilitate the escape of invasive cells that have breached the surrounding basement membrane and contribute to early metastatic spread.

Conclusions

We have developed and characterized several new chain-specific antibodies directed against LM-511 subunits. Four of these mAbs (3G10, 3C10, 3C12 and 4C10) react with different epitopes to that recognized by antibody 4C7. The 2F12 (LMα5), 3G10 (LMβ1) and 3C12 (LMγ1) antibodies can be used for Western blotting as well immunoprecipitation and therefore provide useful tools for the purification of LM-511 from biological specimens or cell lines. In conjunction with 4C7, these antibodies may also have prognostic application for the detection of LM-511 in the sera/biological fluids of cancer patients (Brocks et al., 1986; Saito and Kameoka, 2005; Skyldberg et al., 1999; Yudoh et al., 1994). Using a simple zinc fixation process these antibodies provide additional capabilities for studying the expression of

LM-511 in normal colon and colon cancer tissue samples at varying stages of tumor development. Whilst antibody 4C10 only recognizes native LM-511 (Biacore analysis), this antibody does not have high enough affinity for use in ELISA. Antibody 3G10 inhibits the effect of LM-511 on the adhesion, spreading and proliferation of the colon cancer cell line LIM1215. It will be interesting to study the anti-tumor/metastatic activity of antibody 3G10 on other colon cancer cells using invasion or xenograft assays.

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Figure Legend

Figure 1: Isotype analysis of LM-511 monoclonal antibodies
A mouse MonoAB ID Kit (see Materials and Methods) was used to identify the Isotype of each LM-511 monoclonal antibody.
(A) 96-well plates were coated overnight at 4°C with pre-treated (56°C, 20min) rhLM-511 (1μg/mL). After blocking with 1% (w/v) BSA, the LM-511 mAbs (2F12 ▭, 3C12 ▭, 3G10 ▭, 4C10 ▭ or 4C7 antibody (▭) were diluted to 0.5μg/mL and 100μL of diluent added to the plate. As a negative control rhLM-511 coated wells underwent the same incubation with no primary antibody (▭). The secondary incubation was performed with rabbit anti-mouse subclass-specific antibodies or rabbit serum as a negative control. ABTS chromosphere was added to each well following the completion of each assay and the absorbance of each reaction measured at 405nm.
(B) Rabbit anti-mouse kappa or lambda light chain specific antibodies were used to determine the type of light chain for each antibody (2F12 ▭, 3C12 ▭, 3G10 ▭ , 4C10 ▭ and 4C7 ▭).

Figure 2: Biacore epitope binning
Following activation of the CM5 Biacore chip with 35μL EDC/NHS, the 3G10 antibody (30μg/mL) diluted in 10mM sodium acetate (pH 4.2) was injected (10μL/min) over the surface until a relative response (RU) of 8000 was achieved. The surface was washed with 10mM HCl to remove any non-covalently material before blocking with 35μL of 1M ethanolamine (pH 8.5) at a flow rate of 10μL/min. 60μL of rhLM-511 conditioned medium was injected over the 3G10 antibody surface followed by the specified LM monoclonal antibody diluted to 20μg/mL in HBS. The asterisks indicate the dissociation phase following the end of each injection.
Cycle (A): rhLM-511-SCM → 3G10 antibody → 4C7 antibody → 4C10 antibody
Cycle (B): rhLM-511-SCM → 4C7 antibody → 2F12 antibody → 3C12 antibody
Cycle (C): rhLM-511-SCM → 2F12 antibody → 3C12 antibody → 4C10 antibody

Figure 3: Identification of chain specificity using Western blot
The individual chains of purified rhLM-511 (500ng) were separated by electrophoresis under reducing conditions on a 3-8% gradient Tris-Acetate pre-cast SDS gel (A) or a 4% Tris-Glycine pre-cast SDS gel (B). Western blots were performed with the 2F12 antibody (A, 1μg/mL) or the 3C12 and 3G10 antibodies (B, 1μg/mL). 500ng of purified rhLM-511 was resolved under the same conditions to identify the α5, β1 and γ1 bands following silver staining (red arrows).

Figure 4: Immunoprecipitation of LM-511 from crude rhLM-511-SCM with the 2F12, 3C12, 3G10, 4C10 and 4C7 antibodies

The immunoprecipitation capability of each LM-511 monoclonal antibody (2F12, 3C12, 3G10 and 4C10) was tested using crude rhLM-511-SCM. 1µg of each monoclonal antibody was added to 200µL of rhLM-511-SCM and incubated overnight at 4°C with agitation. As a positive control, 1µg of 4C7 antibody was used to immunoprecipitate LM-511 from rhLM-511-SCM. The resulting immunoprecipitates were resolved on 3-8% gradient Tris-Acetate gels. The presence of LM-511 in each of the immunoprecipitates was confirmed by silver staining of the gel. To identify the α5, β1 and γ1 chains (red arrows), 0.8µg of purified rhLM-511 was also resolved on a 3-8% gradient Tris-Acetate gel under the same conditions and silver stained.

Figure 5: Inhibition of LM-511 induced adhesion, spreading and proliferation of LIM1215 cells by monoclonal antibody 3G10

(A) The 3G10 antibody was added at the indicated concentrations to culture wells pre-coated with rhLM-511 prior to the addition of calcein-AM labeled LIM1215 cells. The inhibitory effect of the 3G10 antibody was determined by measuring the fluorescence excitation at 482nm and emission at 520nm (▭).

(B) The ability of the 3G10 antibody to inhibit LIM1215 cell spreading on coated rhLM-511 in the presence (—■—) or absence (—●—) of EGF was determined as described in the Materials and Methods section.

(C) LIM1215 cells were seeded in uncoated plates in the absence (—■—), or presence of EGF (10ng/mL) (—●—), or seeded on coated rhLM-511 (1µg/mL) in the absence (—▲—), or presence of EGF (—▼—). The 3G10 antibody was added to the culture wells at the indicated concentrations 30min prior to the addition of LIM1215 cells. Cells were allowed to proliferate for 72h at 37°C, and then labeled with 100µL of 1mM calcein-AM. The number of viable cells was determined by measuring the fluorescence of each sample at an excitation wavelength of 482nm and emission wavelength of 520nm.

Figure 6: Immunohistochemical staining of LM-511 chains in normal human colon and human colon adenocarcinoma tissues using LM-511 monoclonal antibodies. Normal human colon (A, C, E, G) and matched colon adenocarcinoma tissues (B, D, F, H) were obtained from the Royal Melbourne Hospital Tissue Bank. Specimens were fixed for 48h in zinc-Tris fixation buffer and processed for paraffin-embedding. Sections (4µm) were stained with hematoxylin and eosin (A, B) or immunostained as described in Materials and Methods for the detection of LMα5, LMβ1 and LMγ1 using the 2F12 (C, D), 3G10 (E, F) and 3C12 (G, H) antibodies respectively. LMα5, β1 and γ1 chains (brown staining) were detected in the epithelial basement membrane (EBM, arrows) and blood vessel walls (BV, arrowheads). LMα5 reactivity is also evident at the tumor invasive front (D, asterisks). Scale bar = 100 µm.

Figure 1

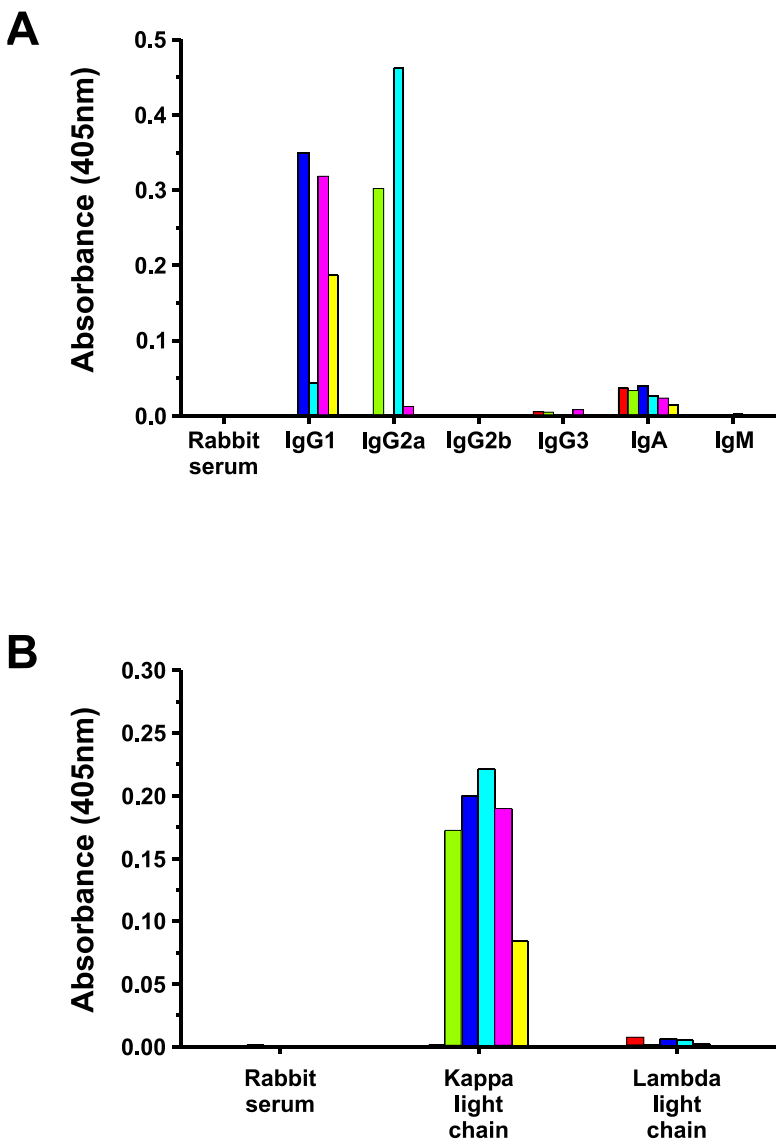

A
Absorbance (405nm)
0.5
0.4
0.3
0.2
0.1
0.0
Rabbit serum
IgG1
IgG2a
IgG2b
IgG3
IgA
IgM
B
Absorbance (405nm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Rabbit serum
Kappa light chain
Lambda light chain

Figure 2

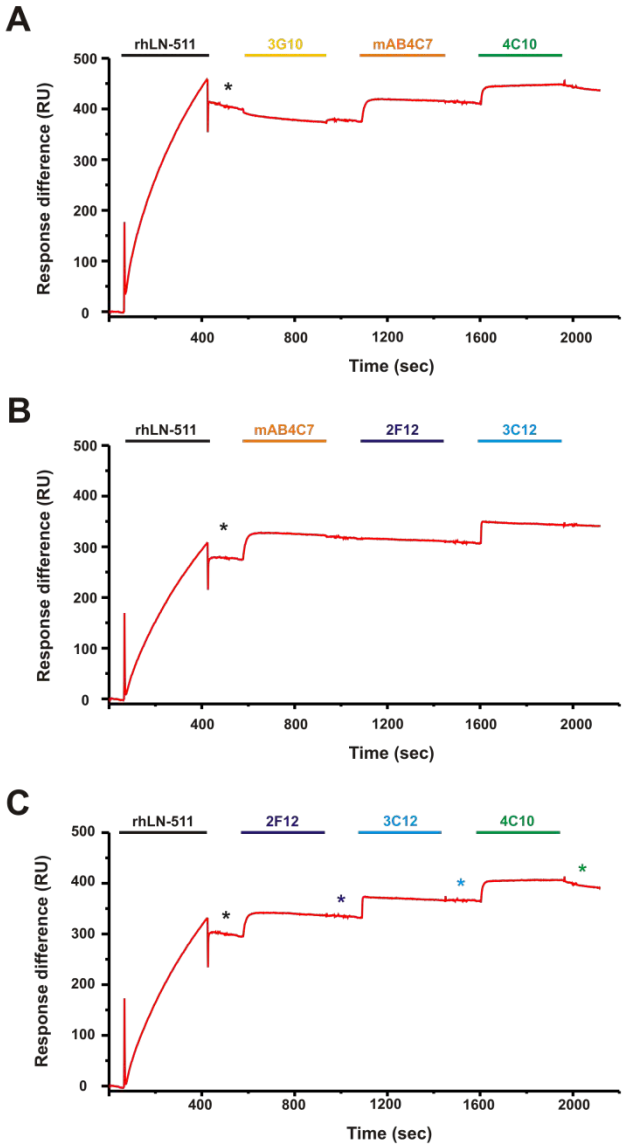

A
rhLN-511
3G10
mAB4C7
4C10
Response difference (RU)
Time (sec)
B
rhLN-511
mAB4C7
2F12
3C12
Response difference (RU)
Time (sec)
C
rhLN-511
2F12
3C12
4C10
Response difference (RU)
Time (sec)

Figure 3

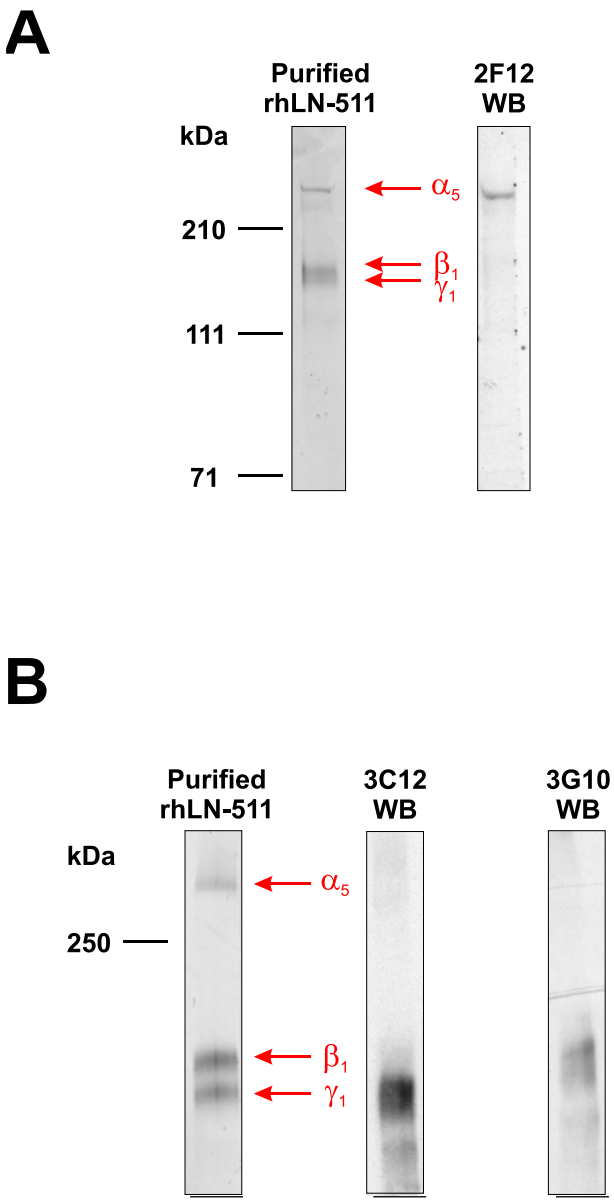

A
Purified rhLN-511
2F12 WB
kDa
210
111
71
α5
β1
γ1
B
Purified rhLN-511
3C12 WB
3G10 WB
kDa
250
α5
β1
γ1

Figure 4

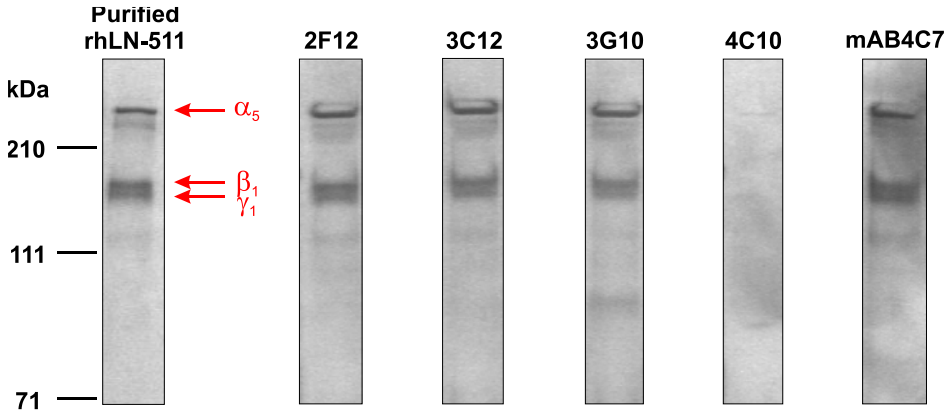
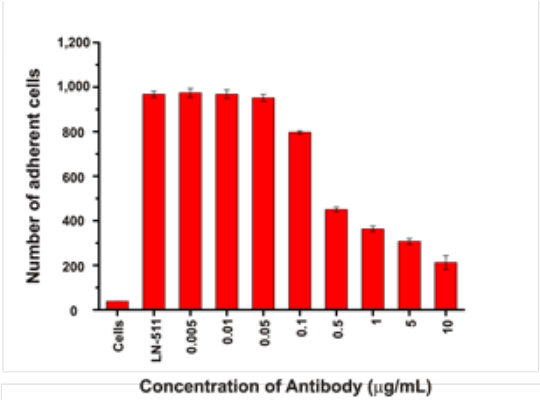
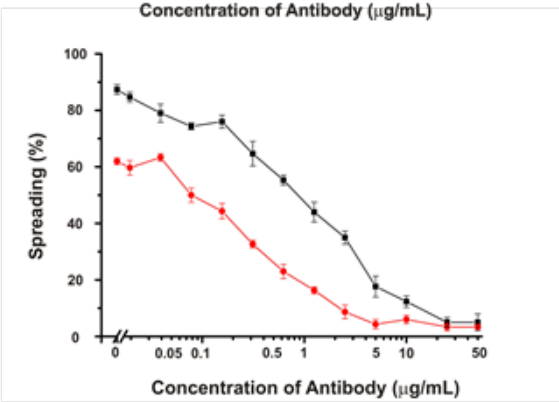

Purified
rhLN-511
2F12
3C12
3G10
4C10
mAB4C7
kDa
210
111
71
α5
β1
γ1

Figure 5

A


Number of adherent cells
1,200
1,000
800
600
400
200
0
Cells
LN-511
0.005
0.01
0.05
0.1
0.5
1
5
10
Concentration of Antibody (μg/mL)

B


Spreading (%)
100
80
60
40
20
0
0
0.05
0.1
0.5
1
5
10
50
Concentration of Antibody (μg/mL)

C

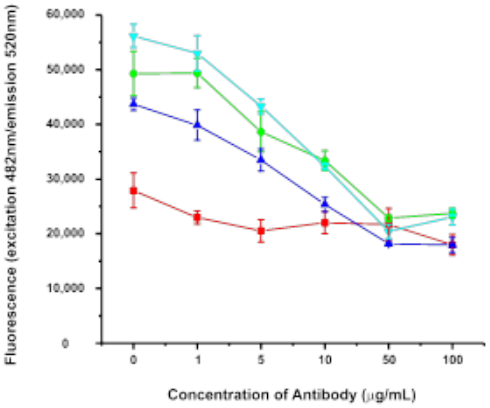

Fluorescence (excitation 482nm/emission 520nm)
60,000
50,000
40,000
30,000
20,000
10,000
0
0
1
5
10
50
100
Concentration of Antibody (μg/mL)

Figure 6