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Properties of Blocking and Non-blocking Monoclonal Antibodies Specific for Human Macrophage Galactose-type C-type Lectin (MGL/ClecSF10A/CD301)

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Monoclonal antibodies (mAbs) specific for the human macrophage galactose-type calcium-type lectin (MGL) were established. The recombinant extracellular domain of MGL was used to immunize a mouse, and 10 hybridoma clones were obtained. Binding of recombinant MGL to asialo-bovine submaxillary mucin was shown to be blocked by mAbs MLD-1, 4 and 6. Immunoprecipitation of MGL from lysates of COS-1 cells transfected with MGL cDNA (form 6A) was achieved with mAbs MLD-1, 4, 7, 8 and 16. Chimeric recombinant proteins between human MGL and mouse MGL1 were used to determine the location of the epitopes for these mAbs. mAbs MLD-8, 13, 15 and 16 interacted with the amino terminal side of the conserved WVDGTD sequence immediately upstream of QPD, whereas mAbs MLD-7, 12 and 17 interacted with the other side. mAbs MLD-1, 4, and 6 apparently required both sides of this boundary. mAbs MLD-15 and 16 were shown to recognize the protein products of alternatively spliced mRNA 6A/8A and 6C/8A, having deletions at the boundary of exons 7 and 8, in addition to full length and other spliced forms of MGL (6A, 6B and 6C), whereas the other mAbs bound only full length and forms 6A, 6B and 6C.

Key words: CD301/MGL, dendritic cell, lectin, monoclonal antibody, viral infection.

Abbreviations: BSM, bovine submaxillary mucin; CRD, carbohydrate recognition domain; C-type lectin, calcium-type lectin; DPBS, Dulbecco's PBS; ECL, enhanced chemiluminescence; GalNAc, *N*-acetylgalactosamine; HRP, horseradish peroxidase; mAb, monoclonal antibody; MGL, macrophage galactose-type C-type lectin; MGL-COS-1 cells, COS-1 cells transfected with MGL cDNA; Mock-COS-1, mock transfected COS-1 cells; MOPS, 3-(4-morpholino) propane sulfonic acid; TBST, Tris-buffered saline containing 0.1% Tween-20; PVDF, polyvinylidene difluoride.

INTRODUCTION

Macrophages and dendritic cells play important roles in innate and acquired immunity by capturing, clearing, processing, and presenting antigens, and by generating danger signals. These cells migrate and localize within distinct lymphoid tissue during their differentiation, activation, and senescence. To efficiently perform these functions, macrophages and dendritic cells are known to express a variety of carbohydrate recognition proteins, lectins (*1*). The macrophage galactose-type C-type lectin (MGL) is unique among C-type lectins expressed on macrophages and dendritic cells for its carbohydrate specificity towards galactose and *N*-acetylgalactosamine (*2–6*). MGLs, i.e. human MGL and mouse MGL1 and 2, are type 2 transmembrane glycoproteins with a single extracellular C-type carbohydrate recognition domain in one subunit (*2, 3*). Human MGL (hMGL) has been termed ClecSF10A, macrophage asialoglycoprotein receptor, and CD301 (*7, 8*). MGL recognizes carbohydrate structures possessing terminal galactose or *N*-acetylgalactosamine residues, particularly clusters of truncated *O*-linked carbohydrate chains such as those occurring on the Tn antigen (*2*). Although these binding specificities were determined by the recombinant forms of human MGL and mouse MGL1 and 2, macrophages and dendritic cells derived from human monocytes and mouse bone marrow cells expressing these lectins have the capacity to recognize and incorporate mucin-like polymers with attached *N*-acetylgalacosamine residues (*9, 10*). These MGL+ cells may represent unique subsets within macrophages and dendritic cells.

To clarify the biological role of MGL and MGL+ cells in the immune system, it is essential to develop monoclonal antibodies (mAbs) specific for this molecule. mAbs specific for mouse MGL1 have been established and are widely used (*11, 12*). mAbs specific for mouse MGL2 has also been prepared (Denda *et al.*, manuscript in preparation). However, mAb specific for human MGL has not previously been established, although a preliminary report was published based on the use of an antibody described in this paper (*10, 13*). Some of the new mAbs were shown to be useful in detecting macrophages and dendritic cells in human tissues.

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MATERIALS AND METHODS

Reagents—3-(4-morpholino) propane sulfonic acid (MOPS), phenylmethylsulfonyl fluoride, pepstatin A, Triton X-100, polyethylene glycol (Hybrimax), oxaloacetate/pyruvate/bovine insulin-media supplement (OPI-Media supplement), and *N*-hydroxysuccinimide biotin were purchased from Sigma Chemical Co., (St. Louis, MO); 905 μM $CaCl_2$–490 μM $MgCl_2$-PBS (DPBS), DPBS 0.1% BSA 0.1% NaN_3 (FCM Buffer), EDTA, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), Tween 20, and HEPES was purchased from WAKO Pure chemical (Tokyo, Japan); Protein A Sepharose CL-4B was purchased from Pharmacia Biotech (Uppsala, Sweden); galactose-Sepharose 4B was prepared by conjugation of lactose to amino-derivatized Sepharose 4B beads by reductive amination. A soluble recombinant form of MGL was purified from *Escherichia coli* transformed with cDNA containing extracellular domains of hMGL as previously described (*2*). An enhanced chemiluminescence (ECL) kit was obtained from Amersham (Amersham, UK). An isotype Ab-STAT kit was obtained from Sang Stat Medical Corp. (Menlo Park, CA). Asialo bovine submaxillary mucin (asialo-BSM) was prepared by incubation of bovine submaxillary mucin in 0.1 N HCl at 80°C for 1 h. Immunoglobulin purification magnesium pyrophosphate column was obtained from Kanto Chemicals (Tokyo, Japan). The CRD of human MGL was obtained by trypsin digestion (treated by 0.05 mg/ml trypsin for 1 h at 37°C) and purification by affinity chromatography on galactose-Sepharose (*14*). Ascitic fluids were obtained by intraperitoneal injection of hybridoma cells into BALB/c mice. mAbs were purified from ascitic fluids by ammonium sulfate precipitation and affinity chromatography on protein G-Sepharose. Polyclonal anti-sera were prepared by immunizing Japanese white rabbits (Charles River Japan Inc., Tokyo, Japan) with 100 μg of purified MGL in Freund's complete adjuvant three times in 2-week intervals, then a final immunization with MGL (500 μg) was given two months after the third immunization. The sera were removed and purified as described earlier.

Cells—Myeloma cells SP2/0, COS-1 cells, and U937 myelocytic leukaemia cells were obtained from American type culture collection (Rockville, MD). COS-1 cells were transiently transfected with plasmid pRc/CMV containing cDNA corresponding to a splicing variant of hMGL (variant 6A) according to the condition previously described (*2*) and designated as COS-1-MGL cells. COS-1-mock was also obtained with pRc/CMV.

Preparation of Monoclonal Antibodies—Recombinant human MGL protein composed of the putative extracellular region was synthesized in an *E. coli* strain BL21(DE3) that had been transformed with the constructed plasmid pET-8c-HML. Soluble MGL was purified by affinity chromatography on a column of galactose–Sepharose 4B as previously described (*15*).

BALB/c mouse (Charles River Japan Inc., Tokyo, Japan) was subcutaneously immunized with 100 μg of purified MGL in complete adjuvant. Two weeks later, a second immunization with 100 μg of purified MGL in Freund's incomplete adjuvant was given, followed by one additional injection at two weekly intervals. Two months after the last immunization, the mouse was boosted by an i.v. injection of 250 μg of purified MGL 3 days before fusion. Splenocytes of the immunized mouse were fused with mouse myeloma cells (SP2/0) at a 5:1 cell ratio in the presence of 50% polyethylenglycol (Hybrimax, Sigma). Cell suspensions were distributed into wells of 96-well plates (2×10^5 spleen cell equivalents/well) in hypoxanthine aminopterin thymidine-containing Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum and OPI-Media supplement, and were cultured for 8 days. Cells in wells of interest were subjected to limiting dilution for cloning in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and OPI-media supplement. To scrutinize the hybridoma cells and clones they were screened using three methods: ELISA assay using MGL, Western blotting analysis and immunoprecipitation analysis with COS-1 cells transfected with MGL cDNA, and ELISA with COS-1 cells transfected with MGL cDNA fixed with 4% paraformaldehyde for 10 min or with 50% formalin for 2 h.

ELISA with Recombinant MGL—Recombinant MGL in 100 μl solution (2.5 μg/ml in buffer A (20 mM MOPS (pH 7.0), 0.15 M NaCl, 20 mM $CaCl_2$) containing 0.1 mM 2-mercaptoethanol) was adsorbed onto a 96-well ELISA plate (655061, Greiner, Kremsmünster, Austria) and incubated at 4°C overnight. After each well was blocked with 3% BSA in buffer A (containing glutathione, 100 μM reduced, 10 μM oxidized) for 2 h at room temperature, hybridoma cell culture supernatants or purified mAbs dissolved in buffer B (20 mM MOPS (pH 7.0), 0.15 M NaCl, 2 mM $CaCl_2$, 0.1% Tween 20) were added. After incubation for 1.5 h at 4°C, the wells were washed in buffer B to remove unbound antibodies, and then 100 μl of HRP-rabbit anti-mouse IgG (diluted 1/2000 in buffer B-1% BSA) was added to detect bound antibodies. After 1 h of incubation at room temperature, the wells were washed in buffer B and then received substrate solution (100 μl of 1 mM 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) dissolved in 0.1 M sodium citrate (pH 4.2) mixed with H_2O_2 (1/1000 final dilution of 34% stock solution) just before use. Absorbance readings were measured at 405 nm in a microplate reader (MTP-12, Corona Electric, Ibaragi, Japan).

Immunoprecipitation of Lysates of COS-1 Cells Transfected with MGL cDNA—COS-1 cells transiently transfected with MGL cDNA were used. Cells were washed three times with 10 mM HEPES, 0.15 M NaCl, and 1 mM $CaCl_2$ (pH 7.2) and extracted with the lysis buffer (20 mM MOPS [pH 7.0], 0.15 M NaCl, 20 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3, 1% Triton X-100, 3 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride) at a concentration of 5×10^7 cells/ml. The lysates were subjected to three cycles of preclearing with recombinant protein A-Sepharose 4B beads adsorbed with normal mouse IgG. Aliquots of the extract were incubated for 2 h at 4°C with 10 μl of recombinant protein A-Sepharose 4B beads that had been conjugated with mAbs (1 μl of ascitic fluids dissolved in 1 ml of lysis buffer). The beads were extensively washed with the same buffer, boiled in 20 μl of SDS-PAGE sample buffer, and analysed by SDS-PAGE on 12.5% polyacrylamide gels. The separated components were transblotted onto polyvinylidene

difluoride (PVDF) membranes. The membranes were blocked with 3% BSA dissolved in water at 4°C overnight and incubated with HRP-streptavidin for 30 min at room temperature and visualized with ECL.

Immunoblotting Analysis of Lysates of COS-1 Cells Transfected with MGL cDNA—Pellets of the cells were mixed with the same amount SDS-PAGE buffer (62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol), boiled at 90°C for 5 min, and then, electrophoretically separated on 12.5% polyacrylamide gels in the presence of 0.1% SDS. These proteins were transblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Boston, MA). The membranes were blocked with 3% BSA dissolved in water at 4°C overnight and incubated with hybridoma supernatants or polyclonal anti-serum diluted in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature. The membranes were washed with TBST three times and incubated for 1 h at room temperature with HRP-rabbit anti-mouse IgG in TBST. The membranes were washed again with TBST and the bound antibodies were visualized with ECL.

Detection of MGL on COS-1 Cells Transfected with MGL cDNA—COS-1 cells transfected with MGL cDNA in 24-well plates were gently washed with DPBS four or five times and fixed with 4% paraformaldehyde at room temperature for 10 min or with 50% formalin at room temperature for 2 h. The wells were washed with DPBS, incubated with 0.3% H_2O_2 solution for 30 min, blocked with 2% BSA in DPBS for 30 min, and reacted with hybridoma supernatants at room temperature for 2 h. After being washed three times with DPBS, the antibody bindings were detected by HRP-rabbit anti-mouse IgG using ABTS for 40 min.

COS-1 cells transfected with MGL cDNA or mock transfectant cells were washed with DPBS, suspended in DPBS, and blocked for non-specific antibody bindings with 5% normal goat serum in DPBS on ice for 30 min. The cells were incubated with ascitic fluids of the mAbs diluted 1:100 in DPBS containing 0.1% BSA and 0.1% sodium azide (FMC buffer) on ice for 30 min and with FITC-goat F(ab')**2** specific for mouse IgG Fc fragments. Fluorescence intensity was examined with a flow cytometer (Epics XL, Beckman Coulter, Miami, FL).

Calcium-Dependency of mAb Binding—In some experiments, the wells were washed with buffer B with or without 10 mM EDTA, and then various concentrations of purified mAbs diluted in buffer C (20 mM MOPS (pH 7.0), 0.15 M NaCl, 0.1% Tween 20) containing either 2 mM $CaCl_2$ or 10 mM EDTA were added to each well. For the blocking assays, ELISA plates were coated with asialo-BSM (250 ng/well) overnight at 4°C. After blocking of the wells with BSA, 100 μl of biotinylated recombinant MGL solution (1 μg/ml in buffer B) containing various concentrations of purified mAbs with or without 0.5 M lactose, which had been pre-incubated for 40 min at room temperature, was added to each well. After incubation for 30 min at room temperature, the wells were washed with buffer B to remove unbound biotinylated recombinant MGL, and then 100 μl of HRP-streptavidin (diluted in 1/2000 in buffer B-1% BSA) were added to detect bound MGL. After 30 min of incubation at room temperature, the wells were washed with buffer B and then received substrate solution containing H_2O_2. After this, absorbance was measured.

Using the ELISA, the effects of calcium ions on the mAb binding was investigated as previously described (*11*). Ten mAbs at various concentrations were tested in the presence of 2 mM calcium chloride or 10 mM EDTA in PBS at 4°C.

Blockade of MGL–ligand Interactions by mAbs—Microtiter wells (96-well plates) were incubated with asialo-BSM (250 ng/well) at 4°C overnight. After washing, the wells were incubated with 3% BSA at room temperature for 2 h to block nonspecific bindings. The mAbs at various concentrations were incubated with biotinylated recombinant MGL at room temperature for 40 min, then applied to the wells. After washing, inhibition of the binding of recombinant MGL with each mAb between asialo-BSM and biotinylated recombinant MGL was compared.

Epitope Mapping of mAbs—Recombinant proteins corresponding to the CRD of human MGL, mouse MGL1, and two human–mouse chimeras C-HM3 or C-MH9 were prepared. C-MH9 has the mouse MGL1 sequence from the amino terminus to Gly^{245} and the rest corresponds to the human MGL sequence. C-HM-3 has the human MGL sequence from the amino terminus to Gly^{229} corresponding to Gly^{245} of mouse MGL1. ELISA plates were coated with these proteins overnight at 4°C. After blocking of the wells with BSA, 100 μl of ascitic fluid solution (diluted 1/1000 in buffer B-1% BSA) was added to the wells, then we detected the bindings between mAbs and these chimera bodies using the ELISA method described previously.

Expression of Splicing Variants of Human MGL—The pET-15b encoding the extracellular region of human MGL splicing variants was introduced into *E. coli* BL21 (pLysS) cells. Cells were incubated at 37°C in 25 ml of 2 × YT medium, and treated with 1 mM isopropyl-β-D-thiogalactoside for 3 h. The cultured cells were washed with TBS, suspended in TBS containing 1 mM PMSF, and lysed by freezing and thawing. The lysates were added with DNase I (49.5 μg/ml) and lysozyme (0.4 mg/ml), incubated for 30 min at 37°C, and centrifuged at 15,000 rpm for 10 min at 4°C. The pellets were recovered and washed with TBS containing 0.5% TritonX-100 and 10 mM EDTA, and with then H_2O. The washed pellets were solubilized with 2 M NH_4OH (0.5 ml) and soluble His-human MGL splicing variants were refolded with a mixture (0.5 ml) of 2 mM glutathione reduced form and 0.2 mM glutathione oxidized form. After dialysis against buffer A with or without 20 mM $CaCl_2$ at 4°C, soluble proteins were diluted with phosphate buffer pH 8.0 (native binding buffer for Ni-NTA purification), precleared with Sepharose CL-2B (Pharmacia), and mixed with 40 μl of Ni-NTA agarose (Invitrogen) at 4°C overnight. After washing twice with 200 μl of phosphate buffer pH 8.0 containing 20 mM imidazole, the columns were eluted with 200 μl of phosphate buffer pH 8.0 containing 500 mM imidazole twice to collect active proteins.

Flow Cytometric Analysis of Differentiated Monocytic Cell Lines—U937 cells were cultured in the absence and presence of phorbol myristate acetate (PMA; 50 nM) and

IL-2 (200 U/ml) for 3 days. After gentle washing with PBS, the cells were treated with 10 mM EDTA in PBS for 10 min at room temperature. After centrifugation at 900 rpm for 5 min, the cells were washed with FCM buffer to remove EDTA and were suspended in FCM buffer at 10^6 cells/ml. After incubation with 2% normal goat serum in FCM buffer for 50 min on ice, first antibodies were allowed to react for 30 min on ice. Normal mouse serum was used as a control. After washing with FCM buffer, the cells were incubated with the second antibody, FITC-labeled goat anti-mouse immunoglobulins (1:100) for 30 min on ice. After washing with FCM buffer, each sample was re-suspended in 500 μl of FCM buffer. The cells were analysed by a flow cytometer after addition of propidium iodide (10 μl of 1 mg/ml in PBS).

RESULTS

Preparation of Hybridoma Cells Producing mAbs Specific for Anti-human MGL—Hybridomas were prepared by fusion between SP2/0 mouse myeloma cells and fresh spleen cells (3.85×10^8) from a mouse immunized with recombinant MGL purified from *E. coli*. Hybridoma culture supernatants in 187 wells with growing hybridomas out of 1776 wells were screened for antibody production as described in Materials and Methods. Positive cultures were processed for subcloning three or four times by limiting dilution, and 10 hybridoma clones were obtained. These clones were designated as MLD-1, 4, 6, 7, 8, 12, 13, 15, 16 and 17, respectively. The isotypes of mAbs was igG1 except mAb MLD-13 (IgG2a).

Binding of mAbs to MGL Expressed on COS-1 Cells Transfected with MGL cDNA—Hybridoma culture supernatants were tested for their capacity to immunoprecipitate MGL from lysates of COS-1 cells transiently transfected with MGL cDNA (splicing variant 6A). mAbs MLD-4, 7, 8 and 16 were found to be useful in immunoprecipitation because of their capacity to precipitate MGL (Fig. 1A). MGL was also precipitated with mAb MLD-1, 6 and 15 incompletely (Fig. 1A). By the use of the same lysates of mAb, MLD-13 was found useful for Western blotting analysis (Fig. 1B), whereas other mAbs were not.

The hybridoma culture supernatants were also tested for their cytological use. COS-1 cells transiently transfected with MGL cDNA were cultured on 24 well plates and the bindings of the mAbs were examined by ELISA unfixed and after fixation. Almost all monoclonal antibodies recognized the MGL protein on the surface of COS-1 cells transiently transfected with MGL when the cells were unfixed (Fig. 1C). The reactivity of all mAbs was maintained after fixation of the cells with 4% paraformaldehyde for 10 min at room temperature (Fig. 1D). However, when the cells were fixed with buffered 50% formalin, the cells lost their binding sites except those for mAb MLD-15 and 16 (Fig. 1E).

Flow cytometric analyses were performed with COS-1 cells transiently transfected with MGL cDNA (splicing variant 6A) (Fig. 2). mAb MLD-1, 4, 6, 7, 15 and 16 were found to be useful in detecting cell surface expression of MGL. The proportions of MGL-expressing cells were

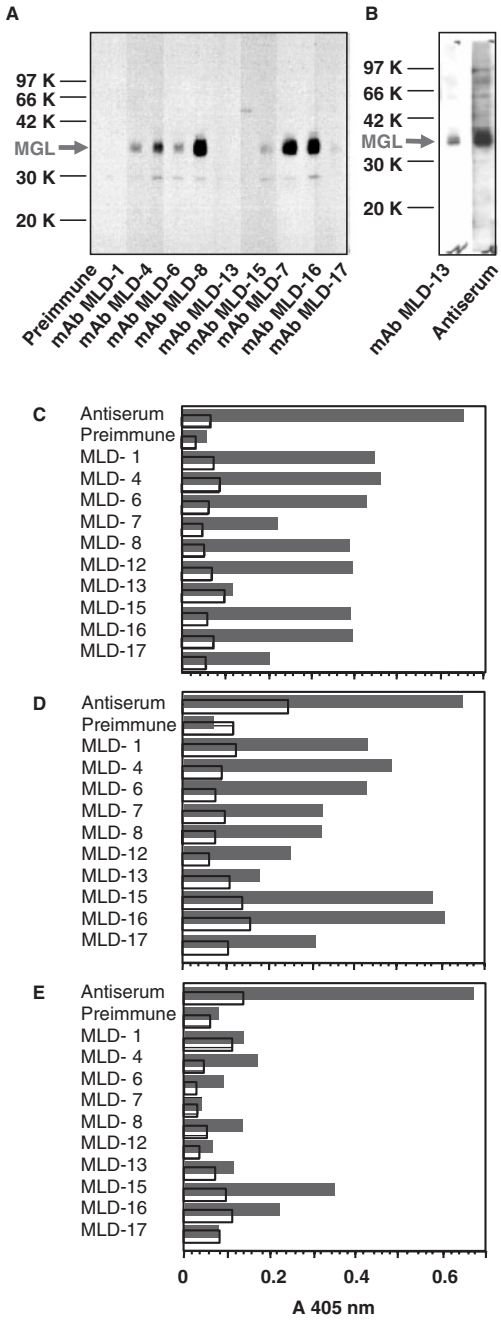

Fig. 1. **Immunoprecipitation, Western blotting and ELISA with ten mAbs by the use of lysates of COS-1 cells transfected with MGL cDNA (splicing variant 6A) and hybridoma culture supernatants.** (A) Electrophoretic analysis of precipitated materials detected by Western blotting analysis with polyclonal antiserum. Lane 1, pre-immune serum, lanes 2 to 11 mAb MLD-1, 4, 6, 8, 12, 13, 15, 7, 16 and 17. After immunoprecipitation by incubation of the lysates of COS-1 cells transfected with human MGL cDNA with the mAbs, dissolved samples were separated on 7.5% polyacrylamide gels, blotted onto PVDF membranes, incubated with polyclonal rabbit anti-human MGL. (B) Results of the Western blotting analysis of the lysates of COS-1 cells transfected with MGL cDNA. (C)–(E) Results of mAb binding to COS-1 cells expressing MGL (closed bars) or to mock transfectant cells (open bars) in the ELISA.

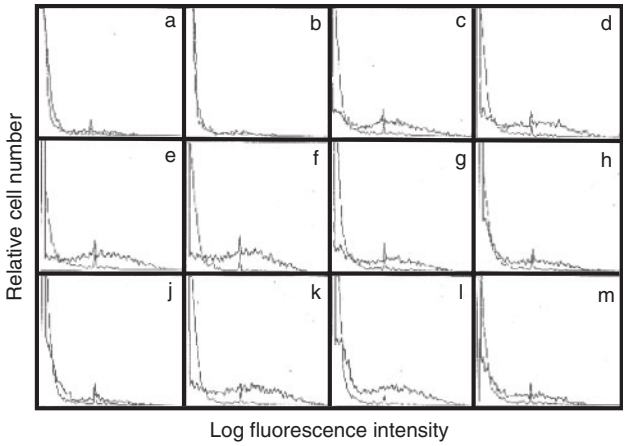

Fig. 2. **Flow cytometric analysis of COS-1 cells transfected with MGL cDNA (thick lines) or mock-transfected COS-1 cells with mAbs MLD-1, 4, 6, 8, 12, 13, 15, 7, 16 and 17 (panels c–m, respectively).** These cells and mock transfectant cells were suspended in the DPBS solution after separation from the culture plates and mAb bindings were tested by flow cytometry. Of the mAbs tested, all except mAb MLD-13 showed binding to the surface. Negative controls were made without addition of the first antibody (panel a) or usage of normal mouse serum in place of the first antibody (panel b).

not high, probably because these are transiently transfected cells.

mAb MLD-15 and 16 Recognize Calcium-independent Epitopes—We previously reported that the binding of a mAb specific for mouse MGLs depended on the presence of calcium ions (*11*). mAbs specific for human MGL were tested in ELISA using purified mAbs, to ascertain whether the bindings were influenced by the presence of EDTA. MLD-15 and 16 were shown to bind recombinant MGL at equal levels in the absence or presence of EDTA. In contrast, the bindings of all other mAbs were reduced by EDTA (Fig. 3). Thus, it is likely that the all mAbs except MLD-15 and 16 recognize the proper conformation maintained by calcium ions, a hallmark property of C-type lectins.

mAb MLD-1, 4 and 6 Block the Binding of MGL to its Carbohydrate Ligands—The inhibitory effects of purified mAbs against the binding of immobilized asialo-BSM to recombinant MGL were tested. With increasing concentration of mAb MLD-1, 4 and 6, inhibition of the binding of recombinant MGL to asialo-BSM was observed as the antibody binding to MGL increased. A complete inhibition of the MGL binding to its ligand was observed with these mAbs at 10 μg/ml. Inhibitory effects were not seen with the other mAbs (Fig. 4).

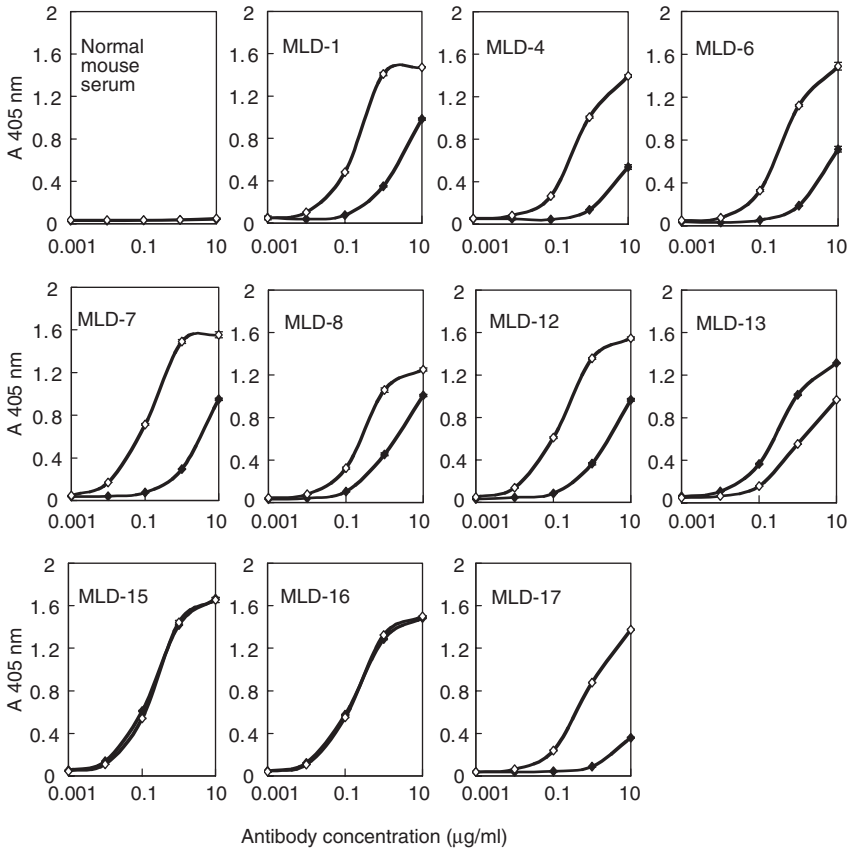

Fig. 3. **mAbs MLD-1, 4, 6, 8, 12, 13, 15, 7, 16, and 17 were tested for their binding to recombinant MGL in the presence (open diamond) or absence (closed diamond) of calcium ions.** Microtiter plates were coated with recombinant MGL and blocked with BSA. The binding of mAb was tested. In the presence of EDTA, which is supposed to change the conformation of CRD, seven mAbs (mAb MLD-17, 4, 6, 7, 8, 12 and 17) lost their binding capacity. MLD-15 and 16 showed very similar levels of bindings regardless of the presence of calcium ions.

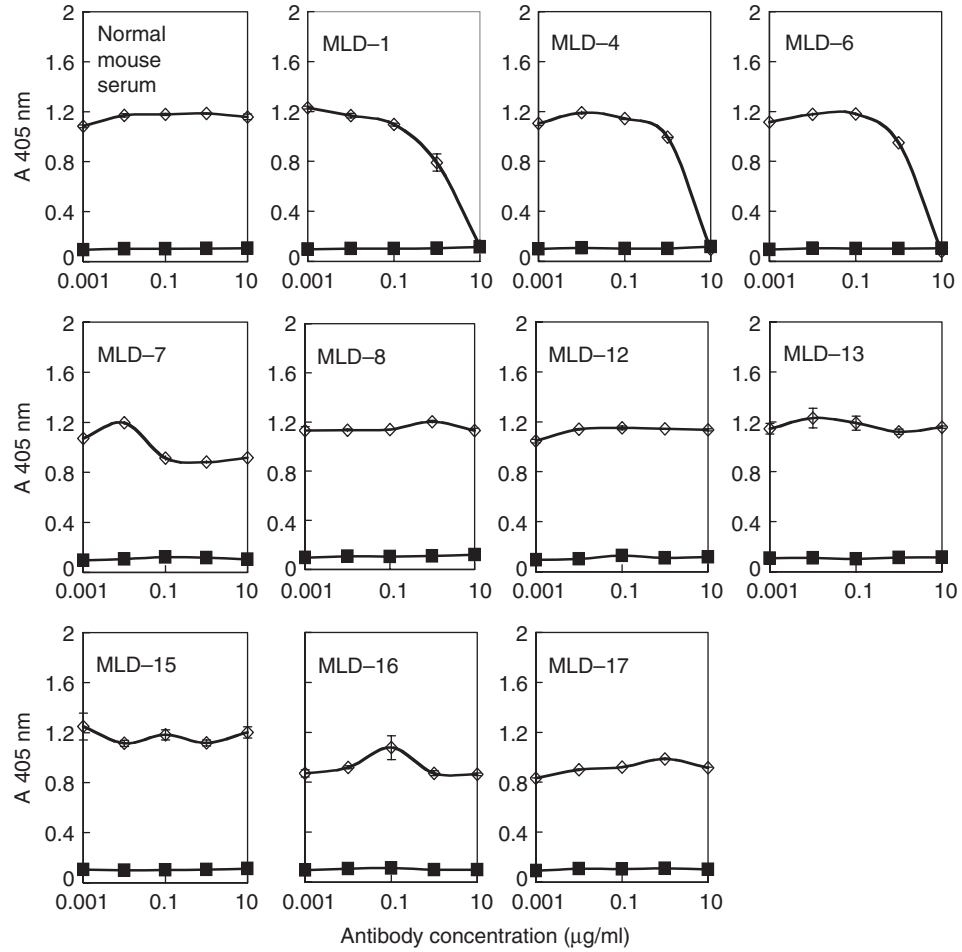

Fig. 4. **Blocking of MGL-ligand interactions by mAbs.** mAbs MLD-1, 4, 6, 8, 12, 13, 15, 7, 16 and 17 diluted at various concentrations were mixed with biotinylated recombinant MGL and incubated for 40 min. The mixtures were added to microtiter wells pre-coated with asialo-BSM overnight and blocked with BSA solutions (open diamond). After 30 min incubation, bound biotinylated recombinant MGL was detected with HRP-streptavidine. Controls (closed square) represent the results of incubation with an addition of 0.5M lactose. mAb MLD-1, 4 and 6 showed marked effects on the binding of biotinylated recombinant MGL, whereas the other seven mAbs (MLD-7, 8,12, 13, 15, 16 and 17) did not show any effects.

Epitope Mapping—To determine the location of epitopes along the MGL polypeptides for these mAbs, mAb bindings to recombinant human MGL, mouse MGL1, their chimera proteins, and purified CRD of human MGL were tested by means of ELISA. The design of the chimera proteins and their reactivity with the mAbs are shown in Fig. 5A. Interestingly, all mAbs were shown to recognize the CRD of MGL. According to the results of the binding experiments, the mAbs were categorized into three groups, as shown in Fig. 5B. The first group includes MLD-7, 12 and 17, the second group includes MLD-8, 13, 15 and 16, and the third group includes MLD-1, 4 and 6. A further point of interest was that mAbs in the third group, which did not have binding capacity to any of the chimera proteins, blocked ligand recognition by MGL and appeared to interact with residues on the amino terminal side and carboxyl terminal side of a consensus WVDGTD sequence. Two calcium-independent mAbs, MLD-15 and 16, belong to the group C. These antibodies were also reactive with formalin-fixed COS-1 cells transfected with MGL cDNA. Fig. 5B also illustrates that a sequence upstream of a consensus WVDGTD locates to the proximity of the QPD sequence known as the key residue to determine the carbohydrate binding specificity of C-type lectins.

mAb Reactivity with the Splicing Variants of Human MGL—Human MGL has mRNA splicing variants, though the distribution of the protein products and their biological roles were not fully understood (*10*). Ten mAbs were compared for their reactivity with the recombinant proteins of the CRD of the spliced forms as shown in Figs. 6A and B. All mAbs were reactive with the full-length MGL, splice forms 6A, 6B, and 6C to similar extents and were not reactive with splice forms 6B8A and 8A. mAbs MLD-1, 4, 6, 7, 8, 12, 13 and 17 did not bind splice forms 6A8A and 6C8A, whereas mAbs MLD-15 and 16 were reactive with the variant forms 6A8A and 6C8A.

Immunocytochemical Analysis of Differentiated U937 Cells—Monocytic cell line U937 differentiated by incubation with PMA and IL-2 were investigated for their reactivity with mAb MLD-1. A small fraction of U937

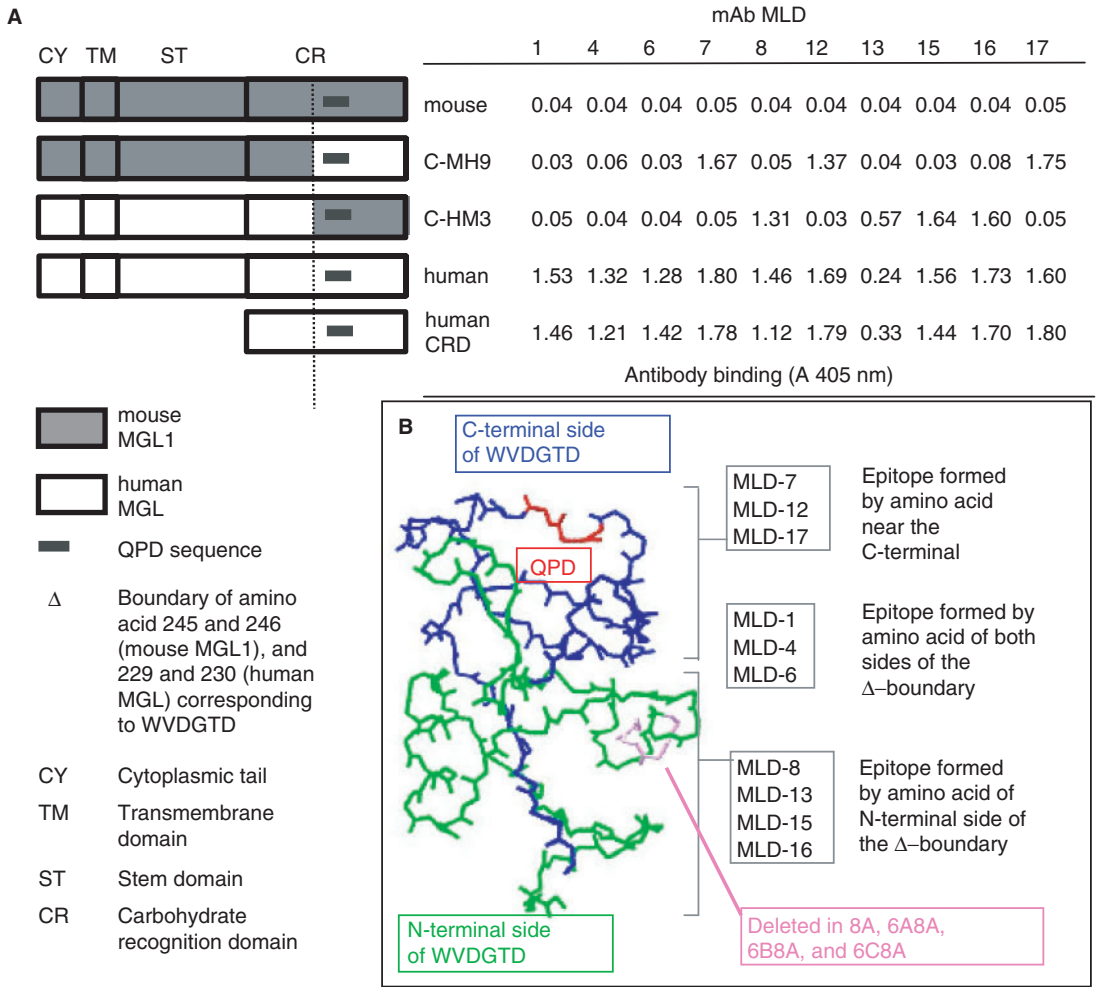

Fig. 5. **Binding of mAbs MLD-1, 4, 6, 8, 12, 13, 15, 7, 16 and 17 to recombinant human MGL, mouse MGL1 and chymeric proteins between human and mouse MGL.** The chimeric recombinant proteins (MH9 and HM3) were prepared as described in Materials and Methods. Based on the reactivity, mAbs were classified into three groups; those reactive with the amino terminal portion of CRD (mAbs MLD-7, 12 and 17), those reactive with the CRD (mAbs MLD-8, 13, 15 and 16); and those with epitopes involved with both the CRD and the stem domain (mAbs MLD-1, 4 and 6). Interestingly, all blocking mAb belonged to the third group.

cells were reactive with anti-CD14, anti-CD68 mAbs, and markers for monocytes and macrophages when the stimuli were not provided, and the cells did not show reactivity with mAb MLD-1. After incubation with PMA and IL-2, 17.8% of the cells showed reactivity with mAb MLD-1 (Fig. 7), whereas the same cells showed 44.7% and 12.3% positive staining with anti-CD14 and anti-CD68 respectively (Fig. 7). The results indicate that the antibody was useful for examining cells having macrophage-like cell surface properties after differentiation driven by a combination of PMA and IL-2.

DISCUSSION

MGL is a unique type-2 transmembrane C-type lectin because of its carbohydrate specificity to galactose and *N*-acetylgalactosamine. It was shown to be expressed exclusively with macrophages and dendritic cells (*9, 10, 13*). Humans and rats have a single gene whereas mice have two genes, *Mgl1* and *Mgl2*, apparently due to gene duplication (*16*). Besides the carbohydrate recognition domain, MGL has a stem domain, transmembrane domain, and cytoplasmic tail with a tyrosine uptake motif. Monoclonal antibodies were previously prepared in a rat system by the use of a mixture of MGL1 and MGL2 purified from cell line RAW264.7 as an immunogen and screened with recombinant MGL1 prepared in *E. coli* (*11*). One of these monoclonal antibodies was shown to be reactive with both MGL1 and MGL2, whereas four antibodies, including mAb LOM-8.7 that has a blocking capacity, were specific for MGL1 (*3*). However, comprehensive approaches to generate mAbs specific for human MGL have not previously been described.

The biological functions of MGL1 in mice have been clarified recently by the use of *Mgl1*-deficient mice. In *Mgl1*-deficient mice, granulation tissue formation induced by repeated antigen administration did not

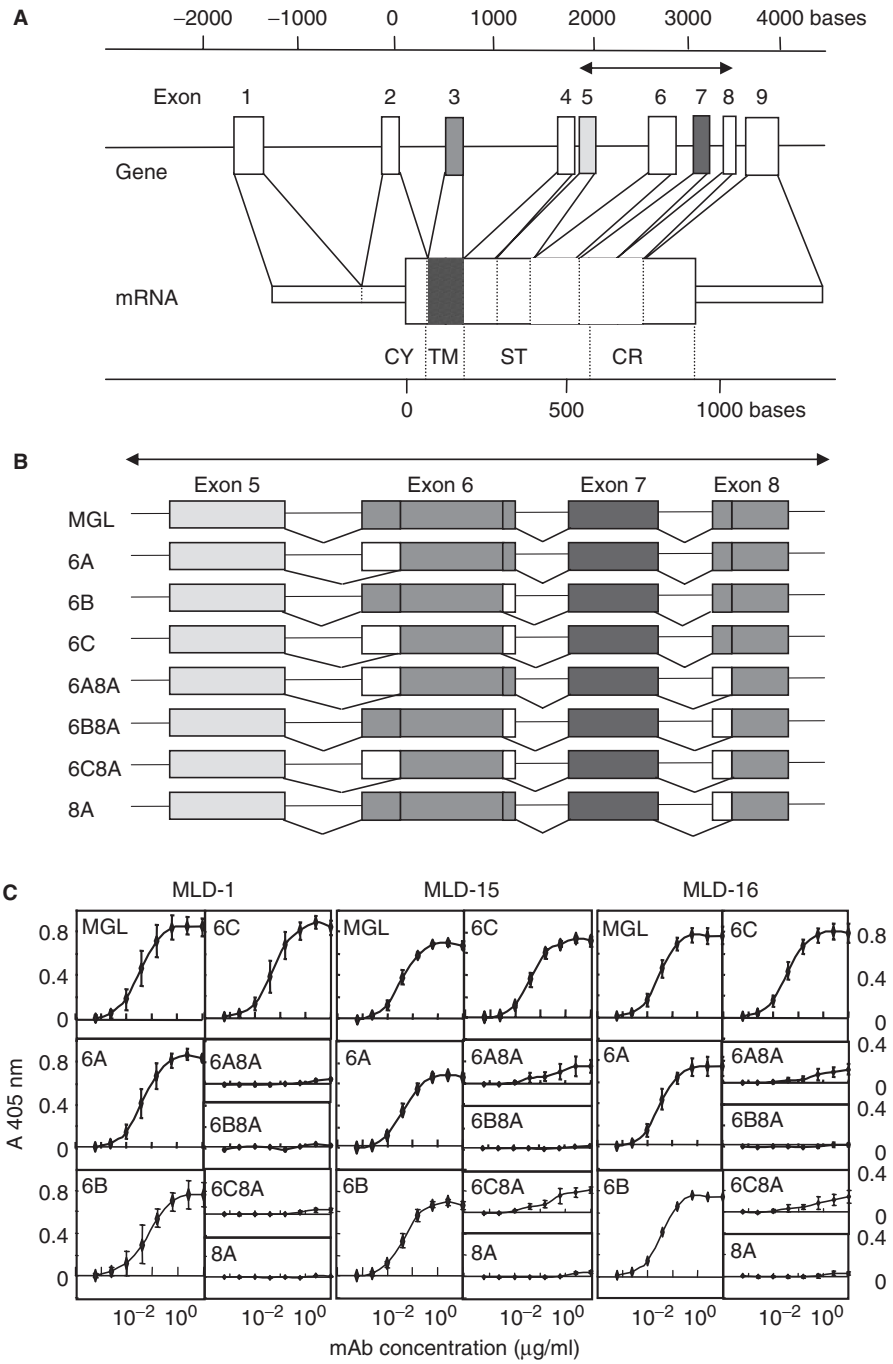

Fig. 6. **Bindings of mAbs MLD-1, 15 and, 16 to recombinant proteins corresponding to full length human MGL and seven splicing variants.** (A) Relationship between human genomic DNA structure of MGL and corresponding mRNA. CY, TM, ST and CR correspond to the cytoplasmic, transmembrane, stem, and carbohydrate recognition domains, respectively. (B) The RNA splicing variants of human MGL according to our previous report (*10*). All variants produced from splice forms apparently resulted from splicing between exons 5 and 6, 6 and 7, and/or 7 and 8. (C) Bindings to mAb MLD-1, 15, and 16 to recombinant CRD of full-length MGL and 7 splicing variants. mAb MLD-4, 6, 7, 8, 12, 13, and 17 showed very similar binding profiles to that of mAb MLD-1.

occur (*17*). Because the expression of MGL1 in wild-type mice was limited to macrophages and dendritic cells, MGL1 expressed on these cells is likely to be critical in inflammatory tissue formation. In another study, clearance of apoptotic cells caused by X-ray irradiation in 10.5 dpc was retarded and a significant decrease occurred in the number of newborn mice from X-ray irradiated pregnant mice of Mgl–/– genotype mated with male mice with Mgl1+/– genotype (*18*). MGL was also shown to act as an uptake site for glycosylated antigens and enveloped

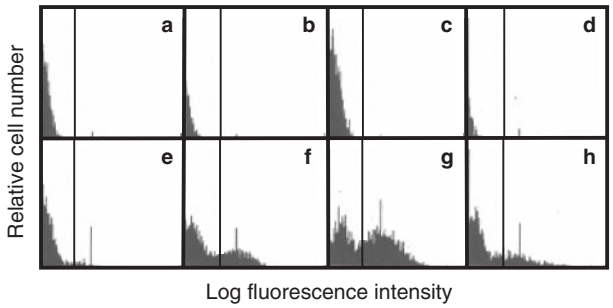

Fig. 7. Flow cytometric analysis of U937 cells before (a)-(d) and after (e)-(h) differentiation. U937 cells were cultured in the presence of 50 nM PMA and 200 U/mL IL-2 for 3 days. LPS at 100 ng/ml was added to the culture at day 3. Antibodies used were: control normal mouse serum (a) and (e); mAb MLD-1 (b) and (f); anti-CD14 mAb (c) and (g), or anti-CD68 mAb (d) and (h). FITC-labeled goat anti-mouse IgG was used as the second antibody.

viruses (*9, 19*). It acts as a mediator of trafficking of MGL-positive cells from skin to lymph nodes (*20, 21*), and the counter-receptor for MGL1 in this process was shown to be sialoadhesin (*22*). Expression of MGL1 and 2 were claimed to be associated with alternatively activated macrophages by parasitic protozoa (*23*). Human MGL was shown to modulate T cell functions through its interaction with CD45 (*24, 25*).

In the present paper, we established hybridomas, which produce mAbs specific for human MGL. mAb MLD-1 was successfully used to identify MGL-positive immature dendritic cells and macrophages at an early stage of differentiation from human peripheral blood monocytes by the use of flow cytometric analysis and by ELISA (*10, 13*). The gene products have splicing variants and four out of eight variants have a 4-amino-acid deletion in the CRD. Differential distributions and carbohydrate binding specificity of the protein products of these splicing variants is important for elucidating the diverse functions of MGL. Thus, mAb MLD-15 and 16 are potentially useful for identifying the protein products of variants having deletions in the CRD.

Tissue distribution of the binding sites of mAb LOM-14, a mAb reactive with both mouse MGL1 and 2, were extensively investigated in mice (*26*). MGL1/2-positive cells revealed by the mAb LOM-14 were distributed in the connective tissue of almost all organs (*26*). They are also detected in connective tissue within the metastatic foci in livers (*27*). Binding of mAb MLD-1 into cells having similar distributions to those of MGL1/2-positive cells was observed in human skin (*13*). Whether the tissue cells expressing mAb MLD-1 binding sites are macrophages, dendritic cells, or both remains to be elucidated. Our previous studies on double immunohistochemical staining of skin sections suggested that at least a portion of mAb MLD-1 binding cells corresponded to dermal DCs. These cells in the skin were considered to be monocyte-derived cells, as more than 90% of them also expressed CD68, but half of the mAb MLD-1 binding cells also expressed the dermal DC marker CD1c. It is likely that skins contain MGL-positive, CD68-positive, and CD1c-negative macrophage precursors and MGL-positive, CD68-positive, and CD1c-positive immature dermal DCs. MGL-positive cells might still have plasticity during their differentiation into macrophages and DCs. mAb MLD-1 and other mAbs developed in the present study should be useful in clarifying the complex lineages of macrophages and dendritic cells. As shown in Fig. 7, mAb MLD-1 was able to show that differentiated monocytic leukemia U937 cells express MGL on the cell surfaces. This is an example of the use of mAbs prepared in the present study.

Recombinant forms of mouse-human chimera MGL were prepared and used to investigate the epitopes for anti-MGL mAbs. Three blocking antibodies, MLD-1, 4 and 6, were shown to recognize epitopes formed by a combination of the amino terminal upstream and the carboxyl downstream of the consensus WVDGTD sequence immediately upstream of the QPD sequence (Fig. 5). Two mAbs, MLD-15 and 16, which had calcium-independent epitopes, bound to the amino terminal side of WVDGTD. These two mAbs were also shown to be non-blocking, and to react with MGL-transfectant cells even after formalin-fixation (Fig. 1E). These two antibodies were also shown to be reactive with two splicing variants, 6A8A and 6C8A, which have a common deletion within the carbohydrate recognition domain. In conclusion, the series of mAbs prepared in the present study should prove to be useful for studying the structural and functional diversity of MGL and related C-type lectins.

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