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Contribution of Complement Component C3 and Complement Receptor Type 3 to Carbohydrate-dependent Uptake of Oligomannose-coated Liposomes by Peritoneal Macrophages

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Peritoneal macrophages (PEMs) preferentially and rapidly take up oligomannose-coated liposomes (OMLs) and subsequently mature to induce a Th-1 immune response following administration of OMLs into the peritoneal cavity. Here, we examine the contributions of complement component C3 and complement receptor type 3 (CR3) to carbohydrate-dependent uptake of OMLs by PEMs. Effective uptake of OMLs into PEMs *in vitro* was observed only in the presence of peritoneal fluid (PF), and OMLs incubated with PF were incorporated by PEMs *in vitro* in the absence of PF. These phenomena were inhibited by methyl-α-mannoside, *N*-acetylglucosamine or EDTA, but not by galactose. Pull-down analysis followed by peptide mass fingerprinting of PF-treated OMLs indicated that the OMLs were opsonized with complement fragment iC3b. *In vivo* uptake of OMLs by PEMs was inhibited by intraperitoneal injection of an antibody against CR3, a receptor for iC3b, and OML uptake by PEMs in the peritoneal cavity was not observed in C3-deficient mice. Thus, our results indicate that OMLs are opsonized with iC3b in a mannose-dependent manner in the peritoneal cavity and then incorporated into PEMs via CR3.

Key words: complement C3, CR3, C-type lectin, macrophage, oligomannose.

Abbreviations: APC, antigen-presenting cell; OML, oligomannose-coated liposome; PEC, peritoneal cell; PEM, peritoneal macrophage; PF, peritoneal fluid.

Antigen-presenting cells (APCs) such as dendritic cells and macrophages play a key role in regulating antigen-specific immune responses. Thus, strategies for activation of APCs and delivery of antigens to APCs are required to induce such immune responses (*1*). Liposomes can encapsulate antigen and immunomodulatory factors, and thus may serve as potent delivery vehicles (*2*, *3*). Delivery of antigen-containing liposomes to APCs can be facilitated by agents that bind selectively to molecules on the surface of the cells, and lectin-like receptors expressed on APCs, including mannose receptors thought to be involved in phagocytosis of pathogens, can be utilized to direct delivery of liposome-encapsulated antigens to APCs (*4*, *5*).

In previous studies, we have investigated the properties of synthetic neoglycolipids constructed from oligosaccharides and dipalmitoylphosphatidylethanolamine (DPPE) (*6–9*). We have shown that liposomes coated with a neoglycolipid constructed from mannotriose and DPPE (Man3–DPPE) are preferentially and rapidly taken up by peritoneal macrophages (PEMs) following administration of the Man3–DPPE-coated liposomes (hereafter referred to as oligomannose-coated liposomes, OMLs) into the peritoneal cavity (*8–10*). The PEMs are activated and mature with up-regulation of MHC class II and co-stimulatory molecules and production of IL-12 in response to intraperitoneal uptake of OMLs (*8–10*), and such administration of OMLs leads to a T-helper type I (Th1) immune response specific for the antigen entrapped in the OMLs (*7*, *10*). Therefore, liposomes coated with synthetic Man3–DPPE have novel adjuvant and immunostimulatory activities following specific incorporation into PEMs.

In this process, the key event for subsequent induction of a strong immune response is OML uptake into PEMs. Carbohydrate-uncoated liposomes and liposomes coated with other types of neoglycolipids (e.g. a neoglycolipid constructed with lactoneotetraose and DPPE) are not taken up by PEMs following intraperitoneal administration (*8*), indicating that mannose residues on OMLs and mannose receptors on PEMs might be involved in uptake of OMLs. However, the receptors used by PEMs to internalize OMLs have not been identified. Therefore, the present study was conducted to investigate the mechanisms of preferential and rapid OML uptake by PEMs in the peritoneal cavity. Our results indicate that carbohydrate-dependent deposition of complement component C3 followed by recognition by the complement receptor type 3 (CR3, CD11b/CD18) are involved in the uptake of OMLs by PEMs.

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MATERIALS AND METHODS

Reagents and Antibodies—Cholesterol, DPPE, dipalmitoylphosphatidylcholine (DPPC) and methyl-α-mannoside were purchased from Sigma (St Louis, MO, USA). Man3 with the structure Manα1-6(Manα1-3)Man was purchased from Funakoshi (Tokyo, Japan). Man3–DPPE was prepared in our laboratory by conjugation of these oligosaccharides with DPPE, as described previously (*7*, *8*). The structure and purity of Man3–DPPE were confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS; AutoFlex, Bruker Daltonics, Bremen, Germany) and high-performance thin-layer chromatography, respectively. The purity of Man3–DPPE used in the study was at least 98%. A polyclonal antibody against C3, a monoclonal antibody (mAb) against CD11b (M1/70, rat IgG2b), and a mAb against mouse MBL-A (8G6, rat IgG) were obtained from GeneTex (San Antonio, TX, USA), Leinco Technologies (St Louis, MO, USA) and Hycult Biotechnology (Uden, The Netherlands), respectively. A rat IgG2b isotype control was obtained from R&D Systems. Peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)-labeled antibodies against mouse CD11b and an Fc-block (anti-mouse CD16/32) antibody were purchased from BD PharMingen (San Diego, CA, USA). Phycoerythrin (PE)-labelled anti-F4/80 antibody was purchased from Caltag Laboratories (Burlingame, CA, USA).

Animals—Six- to 8-week-old female C57BL/6 mice and ICR mice were purchased from Japan SLC (Hamamatsu, Japan) and used 2 days after arrival at the animal facility at Tokai University. C3-deficient ($C3^{-/-}$) mice or wild-type (WT) mice bred in a C57BL/6 genetic background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the specific pathogen-free animal facility at the National Research Center for Protozoan Diseases (*11*). Six- to 8-week-old female $C3^{-/-}$ or WT mice were used in the experiments. All animal experiments were conducted in accordance with protocols approved by Tokai University and by Obihiro University of Agriculture and Veterinary Medicine.

Preparation of Liposomes—Liposomes were prepared as described previously (*7*, *8*). Briefly, a chloroform–methanol (2:1, *v*/*v*) solution containing 1.5 μmol of DPPC and 1.5 μmol of cholesterol was added to a flask with or without 0.15 μmol of Man3–DPPE and evaporated to prepare a lipid film containing neoglycolipid. PBS (180 μl) containing 5 mg/ml of fluoroscein isothiocyanate (FTIC)-labelled BSA (Sigma-Aldrich, St Louis, MO, USA) or PBS was added to the dried lipid film and multilamellar vesicles were prepared by intense vortex dispersion. The vesicles were extruded 10 times through a 1 μm pore polycarbonate membrane (Nucleopore, Pleasanton, CA, USA). Liposomes with entrapped proteins were separated from free soluble antigen by three rounds of washing in PBS with centrifugation (20,000*g*, 30 min at 4°C).

Preparation of Peritoneal Fluid—Peritoneal fluid (PF) was recovered from the peritoneal cavities of ICR mice by lavage using 5 ml of ice-cold PBS. After cellular materials were removed by centrifugation, the PF was passed through a 0.22 μm filter and then concentrated by ~10-fold using a 10,000-MW cut-off concentrator (Vivaspin, Sartorius, Hannover, Germany). The protein concentration in the PF was measured using a modified Lowry protein assay reagent (Pierce, Rockford, IL, USA), and then the PF was stored at −80°C until use. To deplete mannose-binding molecules from PF, serum-free RPMI 1640 medium (5 ml) was added to 10 ml of PF, and then mannose–agarose (1 ml of resin, Sigma-Aldrich) equilibrated with serum-free RPMI 1640 was added to the PF sample and incubated at 4°C overnight with gentle rotation. After removing the resin by centrifugation, the supernatant was used as mannose-binding molecule-depleted PF. PF treated with GalNAc–agarose was used as control PF.

Incorporation of OMLs by PEMs In Vivo *and* In Vitro—Incorporation of liposomes into PEMs was evaluated by flow cytometry (FACSCalibur, BD Biosciences, San Diego, CA, USA) with data analysis using FlowJo software (Tree Star). OMLs with encased FITC-BSA (60 μg cholesterol) were injected into the peritoneal cavity of C57BL/6, $C3^{-/-}$ or WT mice. One hour after injection, peritoneal cells (PECs) were harvested by lavage using 5 ml of ice-cold PBS. For the *in vitro* assay, the harvested PECs were suspended in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 2 mM L-glutamine in a siliconized tube at a cell density of 5×10^5/ml. In some experiments, PF was added to the culture medium at a concentration of 4 mg/ml. For the inhibition assay, PECs were incubated with OMLs in the presence of different concentration of inhibitors (methyl-α-mannoside, GlcNAc or EDTA) for 30 min before addition of OMLs. OMLs suspended in PBS were then added to the culture medium and the PECs were incubated for 1 h at 37°C. The PECs were then washed twice with PBS and treated with Fc block to decrease non-specific binding of immunoglobulins. After this step, the PECs were stained with specific antibodies (PerCP-Cy5.5-labeled CD11b or PE-labeled F4/80) and analysed by FACS. CD11b-positive cells or F4/80-positive cells were identified as PEMs, and uptake of OMLs into PEMs was analysed based on the fluorescent intensity of FITC in CD11b-positive or F4/80-positive cells after gating.

Western Blots and Peptide Mass Fingerprinting—OMLs or bare liposomes (BLs) (60 μg of cholesterol) were incubated with 1.2 mg protein of PF in serum-free RPMI 1640 medium at 37°C for the indicated times. The liposomes were recovered by centrifugation (20,000*g*, 30 min at 4°C), washed with serum-free RPMI 1640 medium, and then the proteins bound to liposomes (10 μg of cholesterol) were separated by SDS–PAGE on 10% gels under reducing conditions. The proteins were stained using a Silver Stain MS Kit (Wako, Osaka, Japan). Alternatively, the separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membranes (BioRad, Hercules, CA, USA) and probed with an anti-C3 polyclonal antibody. An enhanced chemiluminescence (ECL) detection system (GE Healthcare Bio-Science, UK) was used to detect the specific protein bands. For peptide mass fingerprinting, the silver-stained protein

bands were excised from the SDS–PAGE gel. The gel pieces were de-stained with the Silver Stain MS Kit according to the manufacturer's instructions and dehydrated in 100 µl of 100% acetonitrile for 5 min. For reductive alkylation, the gel pieces were treated with 100 µl of 25 mM NH_4HCO_3 containing 10 mM DTT for 1 h at 56°C and then with 100 µl of 25 mM NH_4HCO_3 containing 55 mM iodoacetamide in the dark for 45 min at room temperature. The gel pieces were washed with 100 µl of 25 mM NH_4HCO_3 for 10 min at room temperature and then dried in a vacuum centrifuge for 15 min (*12*). Subsequently, the gel pieces were digested using sequencing grade modified trypsin (Promega, Madison, WI, USA). The tryptic digests were concentrated and desalted using C_{18} ZipTips (Millipore, Bedford, MA, USA). MALDI-TOF-MS was performed using an Autoflex MALDI-TOF mass spectrometer, and the resulting peptide mass fingerprinting and peptide fragment ion data were searched against the NCBI database using Mascot (Matrix Science, London, UK) software. To detect mannose-binding lectins (MBLs), proteins bound to liposomes (10 µg of cholesterol) were separated by SDS–PAGE on 10% gels under non-reducing conditions, electroblotted onto PVDF membranes and probed with an anti-mouse MBL-A mAb.

RESULTS

Effective OML Uptake by PEMs In Vitro *Requires the Presence of PF*—We have shown that OMLs are preferentially and rapidly ingested into PEMs following intraperitoneal administration (*8*). To confirm uptake of OMLs by PEMs *in vivo*, OMLs containing FITC-BSA (FITC-OMLs) were administrated into peritoneal cavity, and PECs including PEMs were isolated from peritoneal cavity one hour after the administration. The level of OML uptake into PEMs was determined by FACS analysis of the fluorescent intensity of FITC in the cells. As shown in Fig. 1, over 95% of CD11b-positive cells ingested OMLs and about 90% of CD11b-positive cells expressed F4/80, suggesting that CD11b and F4/80 double-positive cells take up OMLs preferentially (Fig. 1). Since both CD11b and F4/80 are markers for macrophages in mice, we used CD11b-positive cells as PEMs for evaluation of OML uptake, unless otherwise indicated.

PEMs took up significant amounts of OMLs, but did not take up uncoated BLs, when the liposomes were administered directly into the peritoneal cavity (Fig. 2A), as shown previously (*8*, *10*). In comparison with the *in vivo* uptake of OMLs, the fluorescent signal from OMLs in PEMs was reduced significantly (about 10% of *in vivo* uptake evaluated by mean fluorescence intensity) when PEMs were isolated from the peritoneal cavity and incubated *in vitro* with OMLs in serum-free RPMI 1640 medium, although the signals from OMLs in PEMs were still stronger than those for BLs (Fig. 2B). However, the fluorescent signals from OMLs in PEMs *in vitro* recovered to levels comparable to *in vivo* uptake (about 70% of *in vivo* uptake) when PF was added to the culture medium (Fig. 2C). In contrast, fluorescence from FITC-BLs in the presence of PF did not change compared with that in the absence of PF (Fig. 2B and C). Intense signals from FITC-OMLs were observed in the cytoplasm of PEMs (data not shown), indicating that OMLs were ingested into PEMs under the *in vitro* experimental conditions. These results indicate that humoral factors

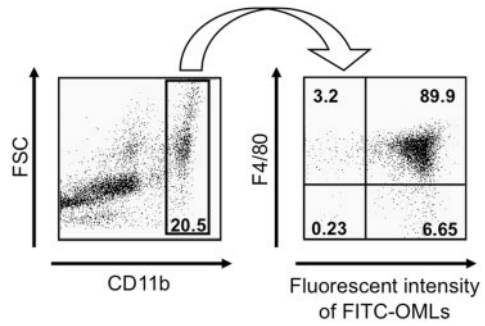

Fig. 1. **Identification of cells that ingested OMLs in the peritoneal cavity.** OMLs containing FITC-BSA (FITC-OMLs) were injected into the peritoneal cavity of mice and PECs were recovered 1 h after injection. The cells were then stained with PerCP-Cy5.5-labelled anti-CD11b and PE-labelled anti-F4/80 antibodies. Cells that were strongly positive for CD11b were gated (left panel) and ingestion of OMLs and expression of F4/80 was analysed in the gated cells (right panel). About 90% of the CD11b-positive cells expressed F4/80 and ingested OMLs preferentially.

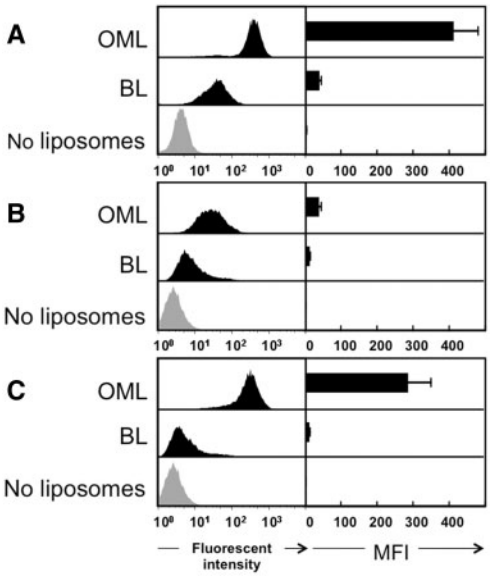

Fig. 2. **Effect of PF on uptake of OMLs into PEMs *in vitro*.** *In vivo* uptake of OMLs into PEMs (A) and *in vitro* uptake of OMLs into PEMs with (C) or without (B) PF were analysed. For analysis *in vivo* uptake of OMLs, OMLs or BLs with encased FITC-BSA were injected into the peritoneal cavity of C57BL/6 mice, and PECs were harvested 1 h after injection. For analysis of *in vitro* uptake of OMLs, PECs were harvested from the peritoneal cavity and then incubated with OMLs or BLs with encased FITC-BSA with or without PF (4 mg/ml) at 37°C for 1 h. The resulting PECs were then stained with PerCP-Cy5.5-labelled CD11b and analysed by FACS. CD11b-positive cells were identified as PEMs and the fluorescent intensity of FITC in these cells is shown. Shaded peaks indicate PEMs incubated without FITC-BSA-containing OMLs. Data are expressed as both histograms and mean fluorescent intensities (MFI) from three independent experiments. Each bar represents the mean ± SD.

contained in PF are involved in uptake of OMLs by PEMs.

Preferential uptake of OMLs by PEMs in the peritoneal cavity is inhibited in the presence of methyl-α-mannoside (*7*), and therefore the effects of carbohydrates on *in vitro* uptake of OMLs by PEMs were investigated. *In vitro* uptake of OMLs in the presence of PF was reduced by methyl-α-mannoside in a concentration-dependent manner (Fig. 3A). GlcNAc (50 mM) and EDTA (10 mM) also inhibited incorporation of OMLs into PEMs, but galactose (50 mM) had no effect (Fig. 3B). *In vitro* uptake of OMLs in the absence of PF was also inhibited by methyl-α-mannoside and EDTA (data not shown). These results are consistent with the *in vivo* uptake of OMLs by PEMs and indicate that C-type lectins that recognize mannose residues may be involved in uptake of OMLs by PEMs both in the presence and absence of PF.

OML is Opsonized with iC3b in the Presence of PF—OMLs that were incubated with PF, washed with serum-free medium, and then incubated with PEMs without PF were incorporated into PEMs (Fig. 4A), suggesting that the OMLs were modified with molecules from PF to allow recognition by PEMs. Therefore, pull-down analysis was performed to identify these molecules. OMLs or BLs were incubated with PF for 30 min, washed twice with serum-free RPMI 1640 medium and then subjected to SDS–PAGE. As shown in Fig. 4B, several protein bands (69, 67, 43 and 38 kDa) were observed for PF-treated OMLs that were not observed with BLs. Analysis by peptide mass fingerprinting indicated that the bands with molecular masses of 69, 67, 43 and 38 kDa were due to fragments of mouse C3. In addition, a polyclonal antibody against mouse C3 detected the 69 and 43 kDa protein bands in OMLs (Fig. 4B). Time-course analysis of C3 deposition on OMLs in PF indicated a decrease in a fragment of mass 104 kDa (C3b) and an increase of a fragment of 43 kDa (iC3b) during the incubation (data not shown), indicating that C3 is deposited and processed to iC3b on OMLs during incubation with PF.

CR3 and C3 are involved in OML Incorporation into PEMs in the Peritoneal Cavity—Complement receptor 3 (CR3, CD11b/CD18, Mac 1) is a receptor for iC3b, suggesting involvement of CR3 in uptake of OMLs by PEMs. To examine this possibility, PECs were treated with an anti-CD11b mAb (M1/70) or an isotype control (rat IgG2b) and then incubated with FITC-BSA-containing OMLs in the presence or absence of PF. In this experiment, PEMs were identified as F4/80-positive cells, since most of the CD11b-positive cells in the peritoneal cavity expressed F4/80 (Fig. 1). *In vitro* OML incorporation into PEMs in the presence of PF decreased in cells treated with the anti-CD11b antibody compared with untreated cells or cells treated with the isotype control (Fig. 5A), whereas OML incorporation in the absence of PF was unaffected by the antibody (Fig. 5B).

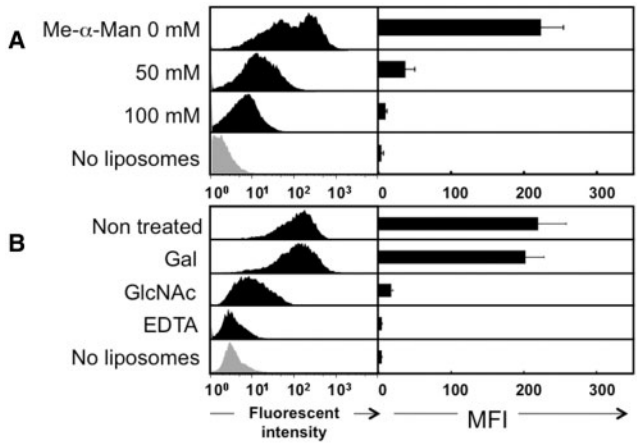

Fig. 3. **Effect of carbohydrates on *in vitro* uptake of OMLs into PEMs in the presence of PF.** (A) Isolated PECs were incubated with OMLs with encased FITC-BSA in the presence of PF and with different concentrations of methyl-α-mannoside. (B) PF-mediated uptake of FITC-BSA-containing OMLs into PEMs was determined in the presence of 50 mM *N*-acetylglucosamine (GlcNAc), 50 mM galactose (Gal) or 10 mM EDTA. Data are expressed as both histograms and MFI from three independent experiments. Each bar represents the mean ± SD.

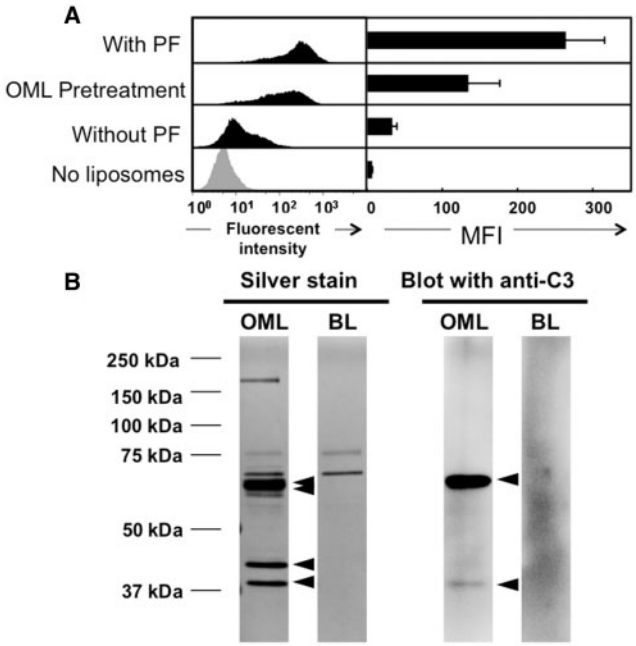

Fig. 4. **Uptake of PF-treated OMLs into PEMs *in vitro*.** (A) FITC-BSA-containing OMLs (60 μg of cholesterol) were incubated with PF (1.2 mg of protein) in serum-free RPMI 1640 medium for 30 min, and then recovered by centrifugation and washed with serum-free RPMI 1640 medium. The resulting PF-treated OMLs were incubated with isolated PECs in serum-free RPMI 1640 medium without PF for 1 h. PECs were harvested, washed and stained with PerCP-Cy5.5-labelled CD11b, as described in the legend to Fig. 1. Data are expressed as both histograms and MFI from three independent experiments. Each bar represents the mean ± SD. (B) OMLs or BLs (60 μg of cholesterol) were incubated with PF (1.2 mg of protein) in serum-free RPMI 1640 medium for 30 min, and then recovered by centrifugation and washed with serum-free RPMI 1640 medium. The resulting PF-treated OMLs or PF-treated BLs (each 10 μg of cholesterol) were subjected to SDS–PAGE on 10% gels under reducing conditions. The proteins were stained using a Silver Stain MS Kit. Alternatively, the separated proteins were probed with an anti-C3 polyclonal antibody. Arrowheads indicate protein bands corresponding to C3 fragments (69, 67, 43 and 38 kDa).

Involvement of CR3 in OML uptake by PEMs in the peritoneal cavity was investigated by injecting the anti-CD11b monoclonal antibody or the isotype control (5 μg) into the peritoneal cavity 10 min before administration of OMLs containing FITC-BSA. The PECs were recovered 30 min after OML administration, and OML ingestion into PEMs was analysed by FACS after the cells were stained with PE-labelled anti-F4/80 antibody. Intraperitoneal OML incorporation into PEMs was significantly reduced in the presence of the anti-CR3 antibody, but not with the isotype control (Fig. 5C), indicating that CR3 is directly involved in uptake of OMLs by PEMs *in vivo*. To investigate involvement of C3 in *in vivo* uptake of OMLs by PEMs, FITC-BSA-containing OMLs were administered into the peritoneal cavity of $C3^{-/-}$ mice or WT mice. OML uptake into PEMs was not observed in $C3^{-/-}$ mice, but was clearly apparent in WT mice (Fig. 5D), indicating that C3 is essential for effective uptake of OMLs by PEMs in the peritoneal cavity. Therefore, OMLs are incorporated into PEMs preferentially via CR3 after iC3b opsonization in the peritoneal cavity.

Soluble C-type Lectins are involved in Uptake of OMLs by PEMs—As shown in Fig. 4, OMLs were opsonized spontaneously with iC3b in PF, with this process depending on mannose residues on OMLs since opsonization in PF was inhibited by methyl-α-mannoside and EDTA, but not by galactose (data not shown). These results suggest that soluble C-type lectins that recognize mannose residues are involved in spontaneous deposition of C3 on OMLs. To clarify the involvement of lectins in opsonization of OMLs, mannose-binding molecules were depleted from PF by treating the fluid with a mannose–agarose resin at 4°C for overnight, and then the level of opsonization was determined based on the intensity of the iC3b fragments of 69, 67 and 43 kDa. The level of opsonization of OMLs by mannose–agarose-treated PF was significantly decreased compared with untreated OMLs or those treated with GalNAc–agarose-treated PF (Fig. 6A). Consistent with these results, the level of uptake of OMLs by PEMs in the presence of mannose–agarose-treated PF was significantly lower than that in the presence of untreated PF (Fig. 6B). On the other hand, treatment of PF with GalNAc–agarose did not affect opsonization or *in vivo* uptake of OMLs.

Serum MBLs bind to bacterial surfaces and activate complement to deposit C3 through the action of MBL-associated serine proteases (*13*, *14*), suggesting that spontaneous deposition of C3 on OMLs in PF may be due to the action of MBLs in PF. To examine this possibility, PF was incubated with OMLs or BLs for 30 min at 37°C, during which OMLs are opsonized by C3,

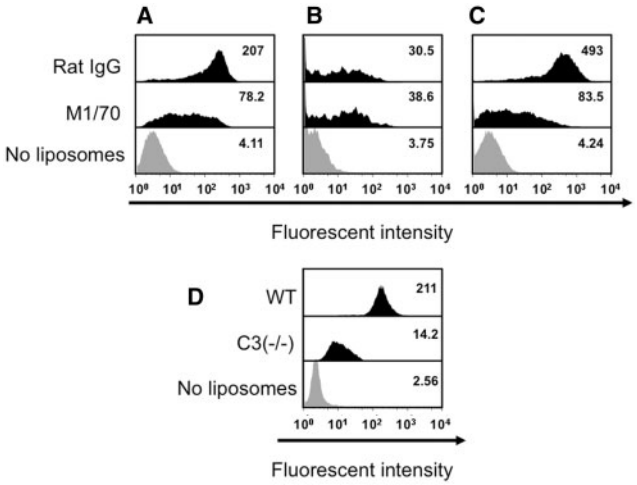

Fig. 5. **Contribution of CR3 and C3 to uptake of OMLs by PEMs *in vitro* and *in vivo*.** (A and B) Isolated PECs were treated with 10 μg/ml of an anti-CR3 monoclonal antibody M1/70 (rat IgG2b) or a rat IgG2b isotype control for 20 min at 4°C. After washing the cells with cold PBS, the PECs were incubated with OMLs containing FITC-BSA in the presence (A) or absence (B) of PF at 37°C for 1 h, as described in the legend to Fig. 2. (C) An anti-CR3 monoclonal antibody (5 μg) or an isotype control (5 μg) was injected into the peritoneal cavity 10 min before injection of FITC-BSA-containing OMLs into the peritoneal cavity. Thirty minutes after injection of OMLs, PECs were harvested and OML uptake into PEMs was analysed as described in the legend to Fig. 1. (D) OMLs with encased FITC-BSA (60 μg cholesterol) were injected into the peritoneal cavity of C3-deficient ($C3^{-/-}$) mice or WT mice bred in a C57BL/6 genetic background. One hour after intraperitoneal injection, PECs were harvested and OML uptake into PEMs was analysed. The MFI is indicated in each figure.

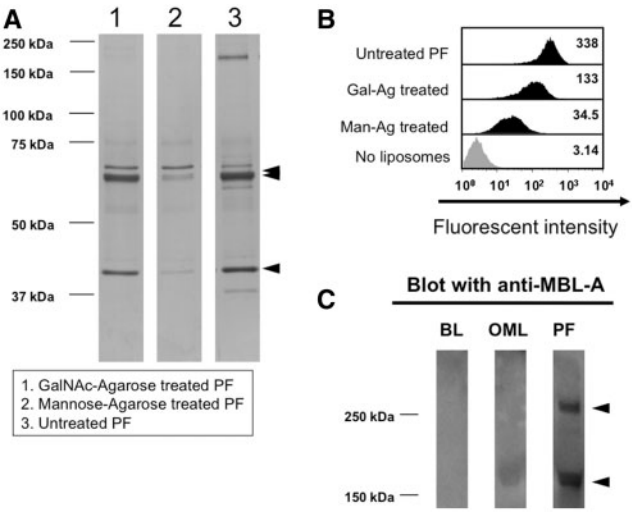

Fig. 6. **Contribution of soluble C-type lectins in PF to opsonization and uptake of OMLs *in vitro*.** PFs were treated with mannose–agarose (Man–Ag) or *N*-acetylgalactosamine–agarose (GalNAc–Ag) at 4°C overnight, as described in the MATERIALS AND METHODS section. (A) OMLs were then incubated with the same concentration of Man–Ag-treated PF, GalNAc–Ag-treated PF or untreated PF at 37°C for 30 min. After washing with PBS, the OML samples were subjected to SDS–PAGE. Arrowheads indicate protein bands corresponding to C3 fragments with molecular weights of 69, 67 and 43 kDa, respectively. (B) Isolated PEMs were incubated with FITC-BSA-containing OMLs (60 μg of cholesterol) for 1 h at 37°C in the presence of Man–Ag-treated PF, GalNAc–Ag-treated PF or untreated PF (4 mg/ml). (C) OMLs or BLs (60 μg of cholesterol) were incubated with PF (1.2 mg of protein) for 30 min at 37°C, as described in the legend to Fig. 4. The resulting PF-treated OMLs or PF-treated BLs (each 10 μg of cholesterol) and PF (30 μg of protein) were subjected to SDS–PAGE on 10% gels under non-reducing conditions and probed with an anti-MBL-A polyclonal antibody. Arrowheads indicate the MBL dimer (180 kDa) and trimer (270 kDa), respectively.

and MBLs bound to OMLs were analysed using an anti-MBL-A antibody. As shown in Fig. 6C, PF contained MBL-A, since this molecule was detected in the mannose–agarose resin used to prepare PF depleted of mannose-binding molecules (data not shown). However, MBLs were not detected on PF-treated OMLs or PF-treated BLs, showing that MBLs did not bind to OMLs following opsonization of the OMLs by iC3b in the presence of PF (37°C for 30 min).

DISCUSSION

The study shows that carbohydrate-dependent deposition and processing of C3 fragments on OMLs and subsequent recognition of iC3b-opsonized OMLs by CR3 (CD11b/CD18) are involved in the preferential and rapid incorporation of OMLs into PEMs in the peritoneal cavity, based on the following evidence: (i) preferential and effective uptake of OMLs by PEMs was observed in the presence of PF *in vitro*; (ii) OMLs were opsonized by iC3b in the presence of PF *in vitro*, and the opsonized OMLs were ingested by PEMs without PF; and (iii) *in vitro* uptake of OMLs by PEMs was inhibited by an anti-CR3 antibody. The contributions of C3 and CR3 in uptake of OMLs by PEMs *in vitro* were confirmed by *in vivo* evidence that an antibody directed against CR3 inhibited OML uptake into PEMs in the peritoneal cavity, and that OML uptake by PEMs in the peritoneal cavity was completely abolished in C3-deficient mice.

Uptake of OMLs by PEMs in the presence of PF depends on oligomannose residues exposed on the OMLs, since the *in vitro* uptake was inhibited by methyl-α-mannoside in a concentration-dependent manner, but not by galactose. The *in vitro* results are consistent with those observed in the peritoneal cavity, since it has been shown that co-administration of methyl-α-mannoside, but not galactose, with OMLs reduces uptake by PEMs (*7*). GlcNAc and EDTA also inhibited the OML uptake, and opsonization of OMLs in PF was also inhibited by methyl-α-mannoside, GlcNAc and EDTA. These results indicate that preferential uptake of OMLs by PEMs depends on oligomannose residues on OMLs, and that C-type lectins which recognize mannose are involved in opsonization with iC3b and subsequent uptake of OMLs via CR3.

It has been shown that CR3 acts as a β-glucan receptor (*15*) and participates in cellular recognition of non-opsonized zymosan and microorganisms via the lectin-like activity of CR3 (*16*, *17*). The lectin-like domain in CR3 is located in the C-terminal region of CD11b, distinct from the I-domain that is essential for recognition of iC3b (*18*), and binds to polysaccharides containing glucose, mannose and *N*-acetylglucosamine in a divalent cation-independent manner (*19*). These properties led us to assume that CR3 participates in recognition of non-opsonized OMLs as well as iC3b-opsonized OMLs. In fact, OMLs were clearly recognized and/or ingested by PEMs without PF (Fig. 2B), but treatment of PEMs with anti-CD11b mAb M1/70 did not affect OML uptake without PF (Fig. 5). In addition, uptake of non-opsonized OMLs by PEMs was inhibited by EDTA (data not shown), indicating that uptake of non-opsonized OMLs depends on the presence of divalent cations. The mAb M1/70 is directed to the I-domain and blocks binding and phagocytosis of iC3b-opsonized particles and inhibits fibrinogen binding (*20*, *21*), but does not bind to the recombinant I-domain of CD11b (*22*). In addition, M1/70 can block the lectin-like activity of CD11b (*15*). Therefore, our results with M1/70 suggest that the lectin-like domain of CD11b is not involved in recognition of non-opsonized OMLs, and that CR3 expressed on PEMs participates mainly in uptake of iC3b-opsonized OMLs.

Three pathways, the classical, alternative and lectin pathways, lead to cleavage of complement component C3, which is a central event in activation of the complement system (*23*). Since OMLs are spontaneously opsonized with iC3b in PF in a mannose-dependent manner, this process may involve soluble C-type lectins that recognize mannose. Indeed, OML opsonization by mannose-binding molecules-depleted PF was clearly lower than that with non-treated PF (Fig. 6). MBLs are candidates for this activity, since binding of MBLs is known to activate complement to deposit C3 through the action of MBL-associated serine proteases (*13*, *14*), and PF used in this experiment contained an MBL (Fig. 6C). However, we were unable to detect MBLs bound to OMLs after incubation with PF at 37°C for 30 min (Fig. 6D), although OMLs were opsonized by PF under the same conditions (Fig. 4B). These results may indicate that only weak association of MBLs with mannose residues of OMLs is required to trigger activation and subsequent deposition of C3 on OMLs. Alternatively, other C-type lectins that recognize mannose residues, distinct from MBLs, may participate in this process.

In addition to soluble C-type lectins, membrane bound C-type lectins expressed on PEMs might also be involved in opsonization and uptake of OMLs. Antigen-presenting cells such as macrophages and dendritic cells express several types of C-type lectins that recognize mannose residues, including macrophage mannose receptor (CD206), dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN, CD209) and SIGN-related 1 (SIGN-R1), and thus these membrane-bound lectins are thought to participate in phagocytosis of bacteria, parasites, viruses and mannose-exposed particles (*24–30*). Recently, it has been reported that C3 deposition on *Streptococcus pneumoniae* is initiated by SIGN-R1 expressed on APCs that interact with both pneumococcal polysaccharides and the complement C1 subcomponent, C1q, followed by activation of the classical complement pathway (*31*). Since SIGN-R1 is expressed on PEMs (*32*), it may also serve as a counter receptor for OMLs, in addition to CR3, for effective uptake of OMLs by PEMs. Further studies focused on membrane-bound C-type lectins, as well as soluble C-type lectins and complement receptors, are required to understand the mechanisms of induction of an immune response following OML uptake by PEMs.

The data presented in this article provide evidence that the complement system plays a role in internalization of OMLs by PEMs in the peritoneal cavity. As shown in previous papers, strong Th1 immune responses and CTLs specific for encased antigens in OMLs are induced following administration of OMLs in the peritoneal cavity (*7*, *9*), and PEMs are activated and mature in

response to incorporation of OMLs (*10*). These results suggest that OMLs have excellent properties as antigen-delivery vehicles and have a strong adjuvant effect, and that preferential uptake of OMLs by PEMs triggers induction of an immune response. Therefore, carbohydrate-dependent opsonization with complement components may be important to allow OMLs to serve as an antigen-delivery vehicle and a strong adjuvant.

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CONFLICT OF INTEREST

None declared.

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