Role of the Scavenger Receptor MARCO in Alveolar Macrophage Binding of Unopsonized Environmental Particles

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Summary

Alveolar macrophages (AMs) avidly bind and ingest unopsonized environmental particles and bacteria through scavenger-type receptors (SRs). AMs from mice with a genetic deletion of the major macrophage SR (types AI and AII; $SR^{-/-}$) showed no decrease in particle binding compared with $SR^{+/+}$ mice, suggesting that other SRs are involved. To identify these receptors, we generated a monoclonal antibody (mAb), PAL-1, that inhibits hamster AM binding of unopsonized particles (TiO_2, Fe_2O_3, and latex beads; 66 ± 5, 77 ± 2, and 85 ± 2% inhibition, respectively, measured by flow cytometry). This antibody identifies a protein of ~70 kD on the AM surface (immunoprecipitation) that is expressed by AMs and other macrophages in situ. A cDNA clone encoding the mAb PAL-1–reactive protein isolated by means of COS cell expression was found to be 84 and 77% homologous to mouse and human scavenger receptor MARCO mRNA, respectively. Transfection of COS cells with MARCO cDNA conferred mAb-inhibitable TiO_2 binding. Hamster MARCO also mediates AM binding of unopsonized bacteria (67 ± 5 and 47 ± 4% inhibition of *Escherichia coli* and *Staphylococcus aureus* binding by mAb PAL-1). A polyclonal antibody to human MARCO identified the expected ~70-kD band on Western blots of lysates of normal bronchoalveolar lavage (BAL) cells (>90% AMs) and showed strong immunolabeling of human AMs in BAL cytocentrifuge preparations and within lung tissue specimens. In normal mouse AMs, the anti-MARCO mAb ED31 also showed immunoreactivity and inhibited binding of unopsonized particles (e.g., TiO_2 ~40%) and bacteria. The novel function of binding unopsonized environmental dusts and pathogens suggests an important role for MARCO in the lungs' response to inhaled particles.

Key words: MARCO • alveolar • macrophage • unopsonized • environmental particle

Normal breathing inevitably results in deep penetration into the lungs of various environmental particles and microorganisms. The alveolar macrophage (AM)[1] is the cell that is primarily responsible for the binding, ingestion, and, ultimately, clearance of inhaled particulate matter (1, 2). If inhaled microorganisms are opsonized by antibodies and/or complement, AMs can bind and phagocytose them via Fc and C receptors (3, 4). AMs more frequently encounter various unopsonized environmental particles that they nevertheless bind and ingest with remarkable avidity (5, 6). The AM response to inhaled particles ranges from abundant production of inflammatory cytokines and reactive oxygen intermediates to simple ingestion and clearance with minimal inflammation (7–9). The latter capacity is especially useful for clearance of inert dusts and particles without risk of the potentially damaging effects of an unnecessary inflammatory response. Thus, receptor-mediated binding of inhaled particles and modulation of AM activation are important first steps in the maintenance of lung homeostasis.

Previous work in our laboratory identified a role for scavenger-type receptor(s) in this process (10). The scaven-

[1]*Abbreviations used in this paper:* AM, alveolar macrophage; BAL, bronchoalveolar lavage; BSS, balanced salt solution; CS, chondroitin sulfate; PI, polyinosinic acid; RAS, right angle scatter; SR, scavenger receptor; SRCR, scavenger receptor cysteine-rich domain.

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ger receptors (SRs) are characterized by high-affinity binding of a relatively broad array of ligands such as modified LDL, some (but not all) polynucleotides and polysaccharides, and bacterial endotoxin (11, 12). There are three classes of SRs, A, B, and C (13). The two class A SRs, type I and type II, are homotrimeric glycoproteins composed of three 77-kD monomers generated from alternative splicing of a message encoded by a single gene (14, 15). SR-As are abundant on monocytes and tissue macrophages as well as other cells (13, 16, 17). A recently identified SR-A, MARCO, also binds acetylated LDL (18, 19). MARCO, like SR-A, binds bacteria but not yeast and is expressed on peritoneal macrophages and splenic marginal zone macrophages but has not been found in the lung (18–21).

The SR ligand polyinosinic acid (PI) caused a marked inhibition of AM binding of oxide particles, latex beads, fly ash, and particles collected from ambient air (10, 22). PI also inhibited the uptake of quartz particles (SiO_2) by AMs in vivo (10). These data suggested that SR-like receptors mediate nonopsonic phagocytosis of pollutant particles. Although SR-A (I/II) seemed the most likely candidate, the use of PI precluded precise identification of receptor(s), as this reagent can inhibit more than one member of the SR family (11, 13).

In the studies reported here, we found that SR-A–deficient AMs exhibited normal (comparable to wild-type) ability to bind particles both in vitro and in vivo. To identify other scavenger-type receptors involved, we developed an mAb capable of blocking hamster AM particle binding. Using this mAb, we performed expression cloning for the receptor, which is similar to mouse and human MARCO in cDNA sequence homology and binding of bacteria. We also show that mouse and human AMs express MARCO and that mouse MARCO is involved in binding of unopsonized particles. The results indicate that MARCO is likely to play an important role in the initial responses to inhaled particles in the lung.

Materials and Methods

Reagents and Particles. TiO_2 and Fe_2O_3 were provided by Dr. J. Brain (Harvard School of Public Health, Boston, MA). These particles have been shown to be heterogeneous in size, with a median diameter of 1.3 μm (23). Latex beads (1.0 μm in diameter; sulfated polystyrene) that show green fluorescence after excitation at 488 nm were obtained from Interfacial Dynamics Co. All particles were suspended in balanced salt solution (BSS^- [124 mM NaCl, 5.8 mM KCl, 10 mM dextrose, and 20 mM Hepes]) as stock solutions and sonicated ~30 s before use. Anti-TNP (PharMingen), anti-heparan sulfate mAbs (Seikagaku Corp.), anti–human β2 microglobulin mAb (PharMingen), and a nonspecific rat IgG1 (PharMingen) were used as isotype-matched controls. The anti–human MARCO antibody was raised in rabbits as described (18). The anti–mouse MARCO mAb, ED31 (IgG1), was provided by Dr. G. Kraal (Vrije Universiteit, Amsterdam, The Netherlands) (21). All reagents not otherwise specified were obtained from Sigma Chemical Co.

Cell Isolation and Flow Cytometric Assay of Particulate Binding. SR-A–deficient mice were prepared as reported (24). Mice or hamsters were killed by intraperitoneal pentobarbital injection. AMs obtained by repeated lung lavage with BSS^- were centrifuged at 150 *g* and resuspended in BSS^+. AMs (2×10^5 in 100 μl BSS^+) were preincubated with mAbs (100 μl hybridoma supernatant or 10 μg/ml mAb) or inhibitors (10 μg/ml) and 2.5 μg/ml cytochalasin D for 5 min on ice in a 1-ml microfuge tube. After the addition of probe sonicated particles or beads, the tubes were rotated at 37°C for 30 min, placed on ice, and analyzed by flow cytometry. Flow cytometry was performed using an Ortho 2150 cytofluorograph as previously described (25). AM uptake of particles was measured using the increase in the mean right angle scatter (RAS) caused by these granular materials (25). Latex bead binding is expressed as relative fluorescence.

Assay of Bacteria Binding. Fluorescent-labeled, heat-killed bacteria (*Escherichia coli* and *Staphylococcus aureus*) and yeast (Zymosan) were purchased from PharMingen. The bacteria binding assay was performed exactly as described above, except that AMs were incubated with either bacteria (5×10^7) or yeast (2×10^5) instead of particles. Binding was measured by detecting AM-associated fluorescence by flow cytometry.

In Vivo Particle Uptake. Mice were anaesthetized with halothane (4%), and 125 μl TiO_2 (50 μg/ml) in BSS^+ was instilled into the lungs using a 22-gauge canula. Bronchoalveolar lavage (BAL) was performed 30 min after instillation, and aliquots of BAL were immediately analyzed by flow cytometry to quantitate cell-associated particles.

Production of mAb. BALB/c mice were immunized by intraperitoneal injection of 2×10^7 hamster AMs. After 3 wk, mice received another injection of 2×10^7 AMs i.p., and 3 d later, spleens of the mice were removed. The splenocytes were fused with a nonsecreting mouse myeloma, P3U1, using polyethylene glycol 4000 and cultured in DMEM containing hypoxanthine, aminopterin, and thymidine. After 2 wk, supernatants from hybridoma cultures were screened for their ability to inhibit the adhesion of TiO_2 to AMs.

Immunoprecipitation. Hamster AM cell surface proteins were labeled with sulfo-NHS-LC-biotin (Pierce Chemical Co.) as per the manufacturer's suggested protocol and resuspended at a concentration of 4×10^7 cells/ml in a 1% extraction buffer (1% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM iodoacetamide supplemented with 40 μg/ml PMSF, 2 μg/ml aprotinin, and 10 μg/ml phenanthroline as protease inhibitors). The lysates were precleared with rat anti–mouse IgM magnetic beads (Dynabeads®; Dynal Inc.). Aliquots of lysate were incubated with mAbs PAL-1 or RP-3 bound to rat anti–mouse IgM magnetic beads overnight at 4°C. The immunoprecipitates were washed in cold lysis buffer (without protease inhibitors), subjected to SDS-PAGE, electroblotted to membrane filters, probed with avidin–HRP conjugate (Pierce Chemical Co.), and developed using chemiluminence reagent (Supersignal®; Pierce Chemical Co.).

Immunohistochemistry. Cryostat sections of hamster tissues were fixed in buffered 2% paraformaldehyde for 10 min, followed by 10 min in 100% cold (−20°C) methanol. After rinsing, immunostaining was performed by sequential application of primary antibody (mAb PAL-1 [5 μg/ml] or anti-heparan sulfate mAb [5 μg/ml], goat anti–mouse IgG [1:50; Sternberger Monoclonals, Inc.], and mouse peroxidase–antiperoxidase complex [1:100; Sternberger Monoclonals, Inc.]), followed by labeling with chromogen diaminobenzidine and H_2O_2. To increase sensitivity, an additional cycle of secondary and peroxidase–antiperoxidase complex was applied to sections, followed by a second chromogen reaction. Paraffin sections of human lung tissue were immunostained using an avidin–biotin complex protocol as previously described (26). The slides were washed with water, counterstained with hematoxylin, dehydrated, and mounted for light microscopy.

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Expression Cloning. A hamster AM cDNA library prepared in the pcDM8 vector (provided by Dr. B. Seed, Harvard Medical School, Boston, MA; Paulauskis, J., unpublished data) was divided into small pools and transfected into COS cells. The transiently transfected COS cells were isolated by "panning" on mAb PAL-1–coated plates, and plasmid DNA was reisolated and amplified as described (27). A receptor-positive pool was identified by PAL-1 immunostaining of transfected COS cells after six rounds of screening. This pool was subdivided repeatedly until we obtained a single functional plasmid that upon transfection conferred mAb PAL-1 reactivity to COS cells. Both strands of the cDNA insert were sequenced at the Harvard University Biopolymers Laboratory.

Western Blot Analysis. Human AMs were collected by BAL from healthy adults under an institutionally reviewed and approved protocol. AMs were washed and lysates prepared as described above (see *Immunoprecipitation*). The lysates were mixed with 6× reducing or nonreducing SDS–solubilization buffer, subjected to SDS-PAGE, electroblotted to membrane filters, probed with either control rabbit IgG or purified rabbit anti–human MARCO antibody followed by avidin–HRP conjugate (Pierce Chemical Co.), and developed using chemiluminence reagent (Supersignal®; Pierce Chemical Co).

Statistics. Data were analyzed using ANOVA and paired *t* test components of a statistical software package (Statview; Abacus Concepts). Significance was accepted when $P < 0.05$.

Results

SR-A–deficient AMs Bind Unopsonized Particles. To determine whether SR-A (I/II) receptors mediate AM binding of unopsonized particles, the binding of TiO_2 by SR-A (I/II)–deficient AMs (SR-$A^{-/-}$) was tested and compared with the binding of TiO_2 by AMs from wild-type mice (SR-$A^{+/+}$). Microscopic evaluation of treated AMs showed similar robust binding of TiO_2 by both SR-$A^{-/-}$ and SR-$A^{+/+}$ AMs (Fig. 1 A). Quantitation by flow cytometric analysis of RAS increases showed that SR-$A^{-/-}$and SR-$A^{+/+}$ AMs demonstrated essentially identical particle binding (Fig. 1 B). SR-$A^{-/-}$ AMs also bound unopsonized ferric oxide and fluorescent latex beads with comparable avidity (data not shown). The SR ligand PI inhibited the adhesion of TiO_2 to both SR-$A^{-/-}$ and $SR^{+/+}$ AMs by 59 ± 1% and 58 ± 4%, respectively. The control polyanion, chondroitin sulfate (CS), had no effect on particle adhesion. To determine if the in vitro particle binding reflected in vivo events, we measured particle binding to AMs after intratracheal instillation of TiO_2. SR-A–deficient or wild-type mice were instilled with buffer alone or buffer containing TiO_2. After 30 min, mice were killed, BAL performed, and AM uptake of TiO_2 quantified by flow cytometry. As shown in Fig. 1 C, both SR-A–deficient AMs and wild-type AMs bound TiO_2 in vivo to a comparable degree. Thus, SR-A deficiency does not alter unopsonized particle binding by AMs. These results suggested that SRs other than SR-A are involved in unopsonized particle binding to AMs.

Effect of mAb PAL-1 on AM Binding of Particles. To develop an mAb to the receptor that mediates particle binding, mice were immunized with hamster AMs, and hybridomas were prepared and screened for mAbs that block AM

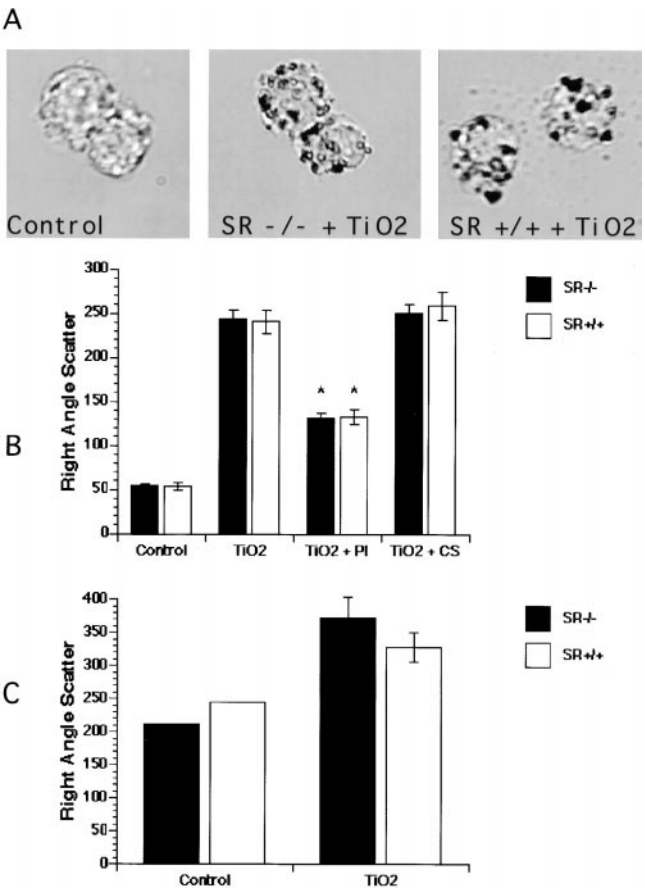

Figure 1. SR-A–deficient and –sufficient AMs bind TiO_2 equally. (A) Representative photomicrograph showing approximately similar binding of particles by SR-A–deficient ($SR^{-/-}$) and wild-type ($SR^{+/+}$) AMs incubated with unopsonized TiO_2 (original magnification 400). (B) $SR^{-/-}$ and $SR^{+/+}$ AMs were pretreated with the SR blocker PI or the control polyanion CS or left untreated, and their binding of TiO_2 was determined by flow cytometry. (C) $SR^{-/-}$ and $SR^{+/+}$ AMs show similar binding of TiO_2 in vivo as determined by intratracheal instillation of TiO_2 followed by BAL and flow cytometric analysis. TiO_2 binding is expressed as increase in flow cytometric RAS. The data represent the mean (± SEM) of three separate experiments (B) and three mice per treated group compared with one control (C). *Significantly different from TiO_2 alone, $P < 0.006$.

binding of TiO_2. As shown in Fig. 2 and reported previously (10), the scavenger receptor ligand, PI, blocked AM binding of TiO_2 and served as a positive control for these assays. A new mAb, PAL-1, inhibited AM binding of TiO_2 by 67 ± 5% ($n = 10$). An isotype-matched control mAb (anti-TNP) had no effect on AM binding of TiO_2. We next examined the effect of mAb PAL-1 on AM binding of other environmental particles such as Fe_2O_3 or quartz (SiO_2) and the surrogate particle, latex beads. As shown in Table I, PAL-1 inhibited AM binding of Fe_2O_3, SiO_2, and latex beads by 78 ± 2, 52 ± 24, and 85 ± 4%, respectively. Thus, mAb PAL-1 substantially inhibits AM binding of a broad range of particles.

Immunoprecipitation of a Cell Surface Protein by mAb PAL-1. To identify the protein(s) recognized by mAb PAL-1, immunoprecipitation experiments were carried out. AMs were surface-labeled by sulfo-NHS-biotin (Pierce Chemical

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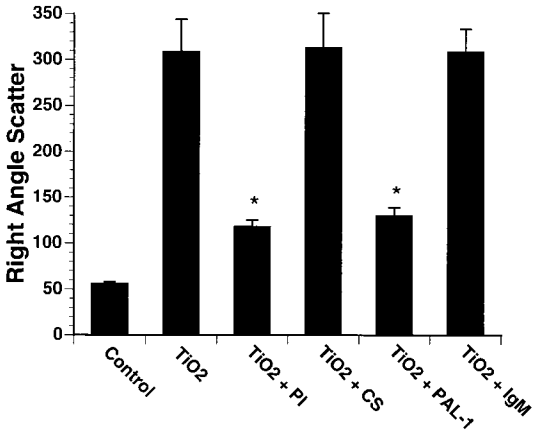

Figure 2. The mAb PAL-1 blocks AM binding of TiO_2. Hamster AMs were untreated or pretreated with PI, CS, mAb PAL-1, and an isotype-matched control mAb (IgM), and their binding of TiO_2 was determined by flow cytometry as described in Materials and Methods. TiO_2 binding is expressed as increase in flow cytometric RAS. The data represent mean (± SEM) of 10 separate experiments. *Significantly different from TiO_2 alone, $P < 0.001$.

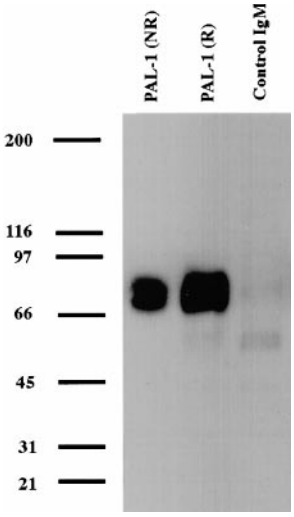

Figure 3. Immunoprecipitation of the AM surface protein recognized by mAb PAL-1. Hamster AMs were surface-labeled with sulfo-NHS-biotin, extracted with Triton X-100, precleared with anti–mouse IgM magnetic beads, and immunoprecipitated with mAb PAL-1 or a control IgM bound to anti–mouse magnetic beads. Immunoprecipitates were analyzed using reducing (R) or nonreducing (NR) SDS-PAGE as described in Materials and Methods. Relative molecular mass is indicated (kD).

Co.), and the cell surface molecules bound by PAL-1 were analyzed by SDS-PAGE (Fig. 3). In both reducing and nonreducing conditions, a single band with an apparent molecular mass of 70 kD was detected in lysates of normal AMs (Fig. 3). This band was absent from cells precipitated with an isotype-matched (IgM) negative control antibody.

Tissue Localization of the PAL-1 Antigen. Immunostaining of frozen sections from a range of normal hamster tissues with PAL-1 showed that it reacted strongly with most or all AMs, macrophages of lymph node sinuses, and von Kupffer cells of the liver (Fig. 4 A). Macrophages in other sites were also positive (e.g., splenic red pulp, intestinal Peyer's patch, thymus). Cross-reactions with other tissue structures such as endothelial cells were not observed; numerous other nonlymphoid tissues were negative (skin, brain, heart, stomach, prostate, and kidney; data not shown). Flow cytometric analysis showed that PAL-1 staining is detected on AMs. Lymphocytes isolated from different lymph nodes and thymocytes were negative (Fig. 4 B). Peritoneal macrophages, neutrophils, and monocytes were predominantly negative (data not shown).

Table I. *mAb PAL-1 Inhibits AM Binding of a Panel of Unopsonized Particles*

Particle	Percent inhibition
TiO_2 ($n = 10$)	67 ± 5
Fe_2O_3 ($n = 2$)	78 ± 2
Sulfated latex beads ($n = 2$)	85 ± 3
SiO_2 ($n = 3$)	52 ± 24

Hamster AMs were left untreated or pretreated with mAb PAL-1 or an isotype-matched control mAb and their binding of particles determined as described in Materials and Methods. Particle binding is expressed as percent inhibition of TiO_2 ($n = 10$), Fe_2O_3 ($n = 2$), latex bead ($n = 2$), and SiO_2 ($n = 3$) binding by mAb PAL-1. No significant inhibition of particle binding was observed with a control IgM (range is from −18 to 7% inhibition).

Expression Cloning of the Particle Adhesion Receptor. An expression cloning protocol was used to identify the receptor mediating AM binding of particles. When transfected into COS cells, a cDNA clone conferred mAb PAL-1 reactivity (Fig. 5, A and B). Both strands of the 1.6-kb insert were sequenced. The sequence, including part of the 5′- and 3′-untranslated regions, is shown in Fig. 6 A. From the first ATG, we predict an open reading frame of 1452 bp, which yields a protein of 483 amino acid residues with a predicted molecular mass of 49.5 kD. GenBank searches of the nucleotide and protein sequences revealed significant homology to murine and human MARCO (84 and 77% nucleotide identity with mouse and human MARCO cDNA, respectively; 18, 19). We refer to this clone as hamster MARCO. Amino acid identity between hamster, murine, and human MARCO was 77 and 65%, respectively (Fig. 6 B). As shown in Table II, amino acid identity between hamster, mouse, and human MARCO was highest in the collagenous domain. The intracellular domain had 80% identity between hamster and mouse MARCO, as compared with 50% identity between hamster and human MARCO. Hamster MARCO, like human MARCO, does not have a cysteine residue in the spacer domain, but like mouse MARCO, it retains two cysteines in the cytoplasmic domain. The two potential carbohydrate attachment sites in the spacer domain, the interruption of Gly-Xaa-Yaa repeats in the collagenous domain, and all six cysteine residues in the scavenger receptor cysteine-rich domain (SRCR) are conserved between all three species (Fig. 6 B). Hamster MARCO differs from both murine and human MARCO in containing a shorter collagenous domain (237 amino acids).

MARCO Expression Confers mAb PAL-1–inhibitable TiO_2 Binding to COS Cells. To confirm the role of MARCO in particle binding, we transfected COS cells with hamster MARCO cDNA. As shown in Fig. 5 C, COS cells transfected with MARCO cDNA bound TiO_2 (4.6-fold increase in RAS). TiO_2 binding by MARCO-transfected

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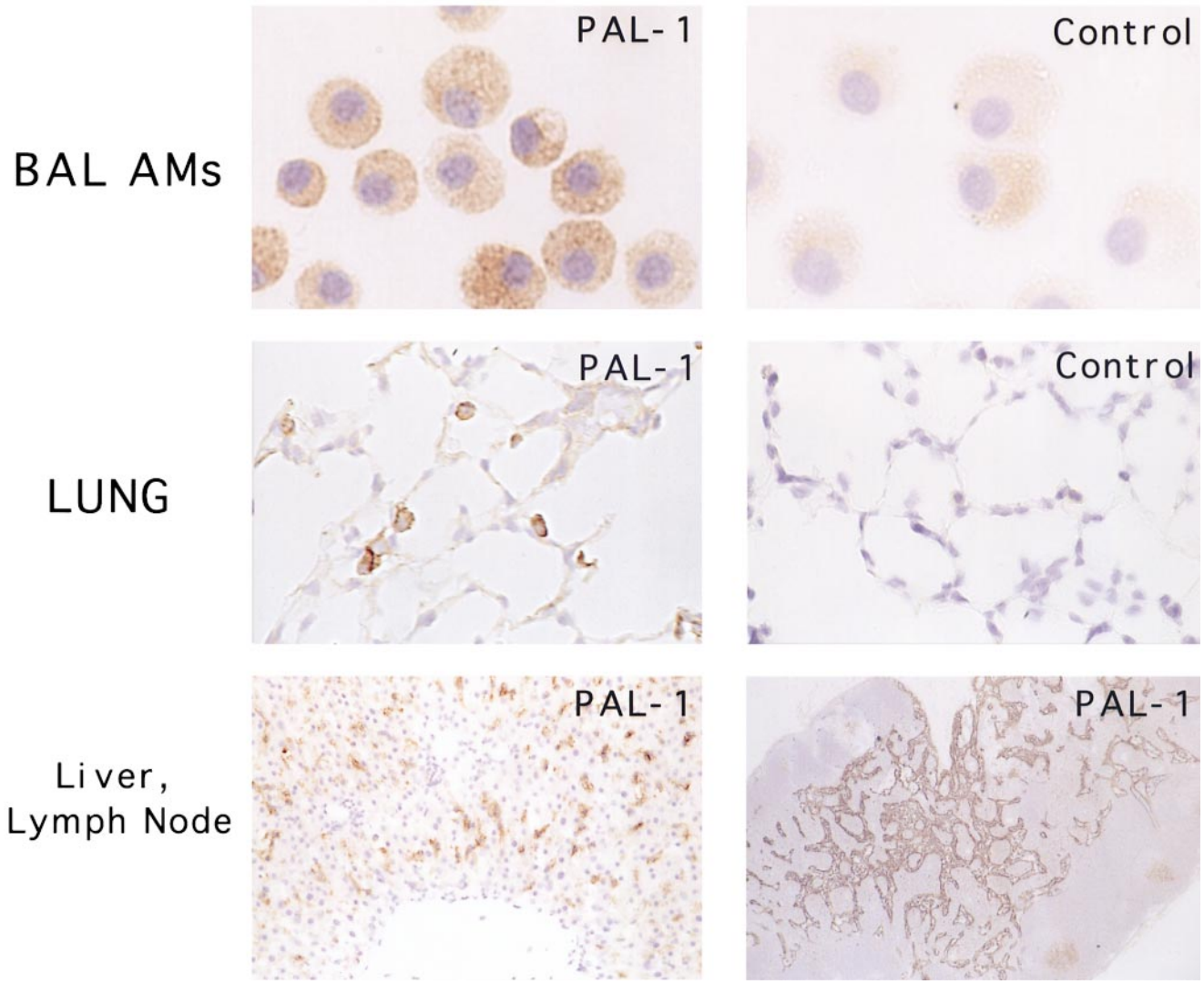

Figure 4. Immunohistochemical detection of the molecule recognized by PAL-1. (A) Photomicrographs of cytospins of hamster BAL cells. Frozen sections of lung, liver, and lymph nodes were stained with mAb PAL-1 or an irrelevant control mAb. Original magnification: 400 for BAL cells, 200 for tissue sections. (B) The expression of the molecule recognized by mAb PAL-1 was analyzed by flow cytometry. Single-cell suspensions prepared from the indicated organs (CLN, cervical lymph node; MLN, mesenteric lymph node; PP, Peyer's patch; PMN, neutrophils) or hamster BAL (AM) were incubated with either mAb PAL-1 or an isotype-matched control mAb followed by goat anti–mouse IgM–FITC and analyzed by flow cytometry. The data are presented as relative fluorescence intensity (mean channel number from flow cytometric analysis). White bar, control; hatched bar, IgM; black bar, PAL-1.

COS cells but not untransfected COS cells was significantly inhibited by the anti-MARCO mAb PAL-1 (47 ± 6% inhibition; Fig. 5 C). Controls, including untransfected COS cells and COS cells transfected with a plasmid encoding the cDNA for hamster CD44, exhibited binding of TiO_2 that was inhibited by both PI and heparin (an agent that does not inhibit AM binding of TiO_2 [10]) but not mAb PAL-1 (data not shown). The constitutive heparin-sensitive particle-binding receptor on COS cells is different from MARCO and remains to be identified (see Discussion).

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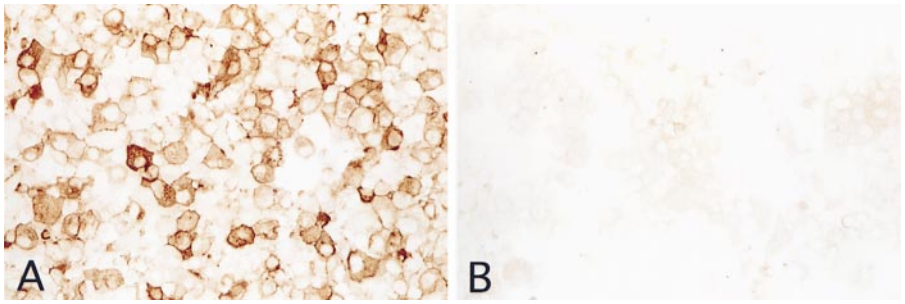

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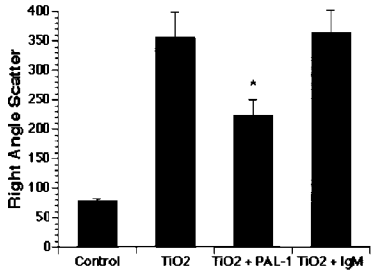

Figure 5. (A and B) Transfection of COS cells with a cDNA clone (isolated by an expression cloning strategy) confers PAL-1 reactivity. COS cells were transfected with the isolated cDNA clone (A) or the empty vector (B); cytospins prepared 48 h later, and they were immunostained with mAb PAL-1. Control IgM antibody showed no labeling (not shown). (C) Transfection of COS cells with MARCO cDNA (for sequence identification, see Fig. 6) confers mAb PAL-1 inhibitable TiO_2 binding. COS cells, transfected with MARCO cDNA, were pretreated with mAb PAL-1 and a control mAb and their binding of TiO_2 determined using a flow cytometric assay. The data represent mean (± SEM) RAS increase of five separate experiments. *Significantly different from TiO_2 alone, $P < 0.01$.

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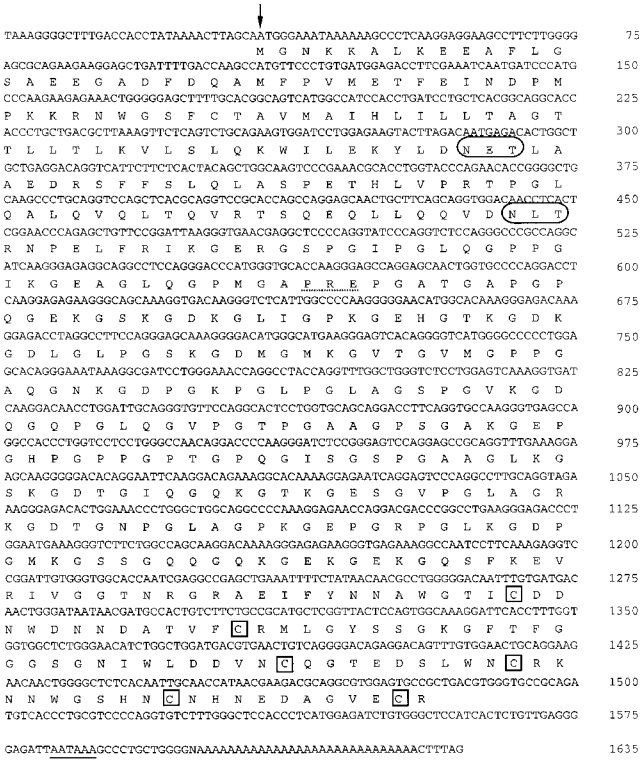

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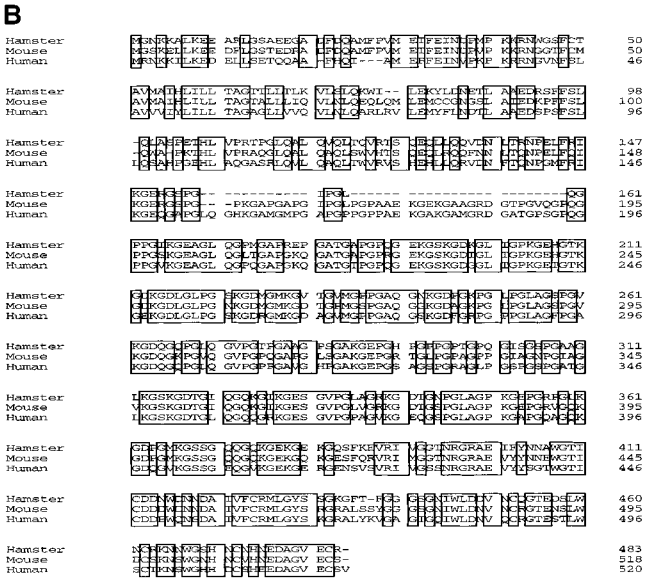

Figure 6. (A) Nucleotide and deduced protein sequences of hamster MARCO. Conserved cysteine residues in the SRCRs are boxed. Putative carbohydrate attachment sites are circled. The consensus polyadenylation signal is underlined. The start ATG is indicated with an arrow. A kink in the collagenous domain is indicated by dotted underline. (B) Comparison of hamster, mouse, and human MARCO. Amino acids were aligned using the Align program (GeneWorks package; Intelligenetics). Regions of identity are boxed. These sequence data are available from GenBank/EMBL/DDBJ under accession number AF125191.

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Table II. *Amino Acid Identity between Hamster, Mouse, and Human MARCO*

Hamster MARCO domains	Percent amino acid identity	
	Human MARCO	Mouse MARCO
Cytoplasmic	48	77
Transmembrane	61	78
Spacer	58	65
Collagenous	76	77
SRCR	65	77
Across all domains	65	77

Amino acid identity was determined by comparing the indicated putative domains with each other using the Align program in the Biology Workbench (http://biology.ncsa.uiuc.edu).

Hamster MARCO Mediates AM Binding of Bacteria. To further investigate the range of ligands for hamster MARCO, we tested the effect of mAb PAL-1 on AM binding of unopsonized microorganisms. As shown in Fig. 7, mAb PAL-1 inhibited AM binding of *E. coli* and *S. aureus* by 67 ± 5% and 47 ± 4%, respectively. PAL-1 had no effect on AM binding of Zymosan. An isotype-matched control antibody did not inhibit bacteria and yeast binding. Thus hamster MARCO, like human and mouse MARCO (18, 19), mediates AM binding of bacteria but not yeast.

Expression of MARCO on Human Cells. The original report of human MARCO did not detect MARCO expression in the lungs of a limited number of perinatal autopsy specimens (18). To evaluate whether adult human AMs express MARCO, we performed immunologic analysis using a polyclonal antibody to a peptide from domain V of human MARCO (18). Histochemical analysis of human BAL cells (>90% AMs) and lung tissue showed that the anti-MARCO antibody detects antigen on AMs but not on other cells or surrounding tissue structures (Fig. 8, A–D). We prepared lysates from human AMs ($n = 5$) and performed SDS/Western blot analysis. The anti–human MARCO antibody reacted specifically with a discrete band in all AM lysates, which ran at a relative molecular mass of ~70 kD (results from two samples are shown in Fig. 8 E). No reactivity was seen with an irrelevant control antibody. The same pattern was repeated when the analysis was performed in nonreducing conditions (data not shown). Thus, adult human AMs express MARCO.

SR-A–deficient AM Binding of TiO_2 Is Inhibited by an Antibody to MARCO. An mAb to mouse MARCO has been shown to block bacteria binding by mouse macrophages (19). Although expression of MARCO was not detected in mouse lungs in the original report, we found significant immunostaining (albeit weaker than that seen in human and hamster AMs) of normal mouse AMs by the anti-MARCO mAb ED31 (data not shown; see Discussion) (19). We therefore examined whether ED31 would block TiO_2 binding by mouse AMs. In these experiments, the SR inhibitor, PI, reduced TiO_2 binding by 44 ± 12 and 52 ± 11% in $SR^{-/-}$ and $SR^{+/+}$ mice, respectively; the control polyanion CS had no significant effect ($n \geq 3$). Treatment of $SR^{-/-}$ AMs with ED31 blocked binding of TiO_2 by 40 ± 11% ($n = 4$). TiO_2 binding by $SR^{+/+}$ was also inhibited by anti-MARCO mAb treatment by 25 ± 4% ($n = 3$). Control IgG1 had no effect on binding. Similar inhibition of mouse AM binding of unopsonized fluorescent latex beads and bacteria was seen (data not shown). Thus, MARCO also functions to bind environmental particles and bacteria in mouse AMs.

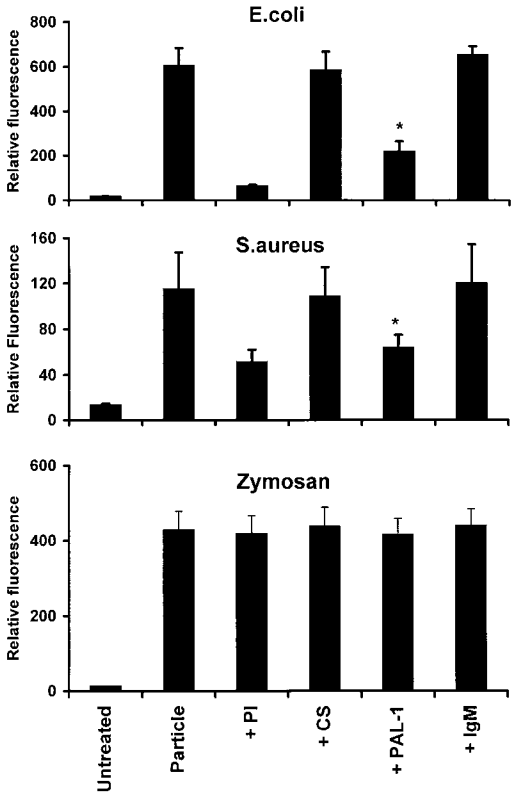

Figure 7. Effect of mAb PAL-1 on bacteria and yeast binding by AMs. Hamster AMs were left untreated or pretreated with PI, CS, mAb PAL-1, and a control mAb (IgM). Following pretreatment, AM binding of *E. coli*, *S. aureus*, and the yeast particle *Saccharomyces cerevisiae* (Zymosan) was determined by flow cytometry. The data represent mean (± SEM) green fluorescence of four separate experiments. *Significantly different from particle alone, $P < 0.05$.

Discussion

In this study, we have identified MARCO as a major receptor on AMs for binding of unopsonized environmental particles and certain microorganisms. The lung is constantly exposed to environmental substances such as microbes, pollutant particles, and allergens. The levels of ambient air particles in the environment have been correlated with increased morbidity and mortality (28–31). The clearance of inhaled particulate matter is primarily mediated by AMs through the process of phagocytosis (1, 2). AMs have been previously shown to avidly phagocytose unopsonized particles (5, 6). However, the receptors on the AMs that recognize and bind particles are not known. Here we have gen-

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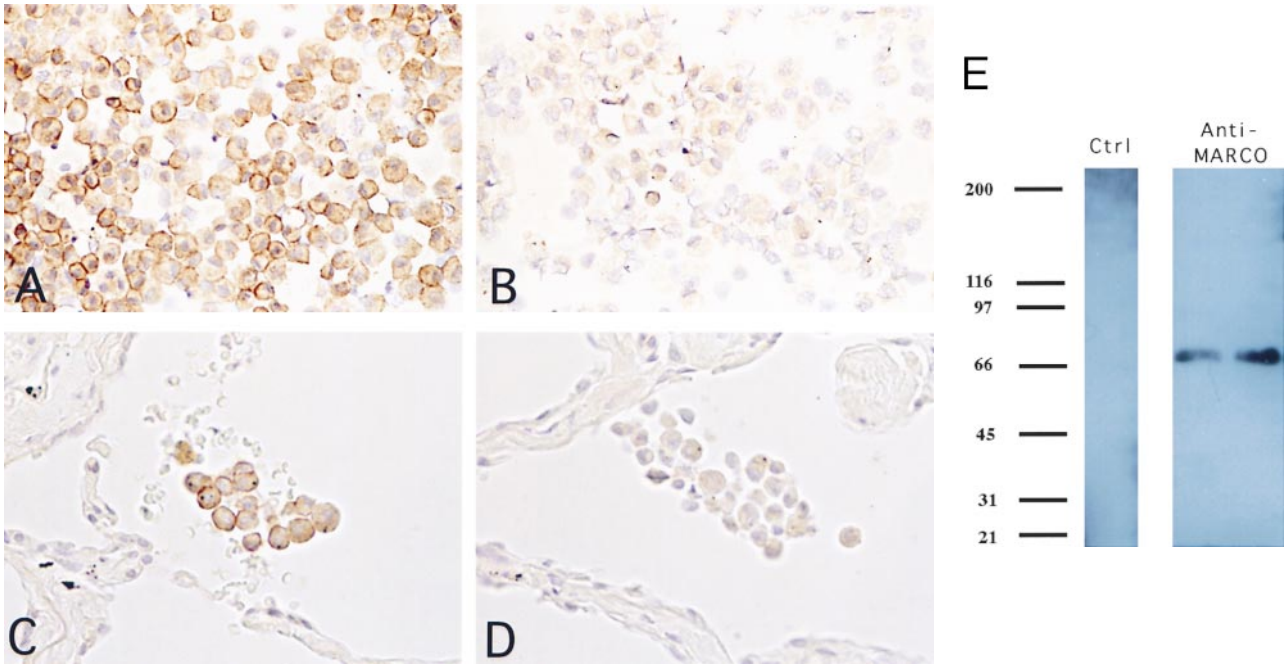

Figure 8. (A–D) Human AMs express MARCO. Immunostaining of normal volunteer BAL cytocentrifuge preparations (A) and surgical lung tissue specimens (C) showed strong labeling by a rabbit anti–human MARCO antibody; control rabbit IgG showed only minimal nonspecific background staining (B, D) (original magnification 400). (E) Lysates prepared from BAL cells collected from adult volunteers (2/5 similar results shown) were analyzed by reducing SDS-PAGE and immunoblotted with a rabbit anti–human MARCO antibody or control rabbit IgG (Ctrl). Relative molecular mass standards are indicated (kD).

erated an mAb, PAL-1, that blocked particle and bacteria binding by AMs. Using mAb PAL-1 as a probe, we have cloned the cDNA encoding for the receptor from an AM cDNA library. Transfection of COS cells conferred mAb PAL-1 reactivity and mAb PAL-1–inhibitable binding of TiO_2. By sequence homology and functional similarity, we conclude that the receptor is the hamster homologue of MARCO.

The recognition of phagocytic targets is mediated by specific receptors on the phagocytes that either recognize serum components (opsonins) bound to the particle or directly recognize molecular determinants on the target. Thus, based on the mechanism of particle recognition, phagocytosis is either opsonin-dependent or opsonin-independent. The best characterized opsonin-dependent phagocytosis receptors are the Fcγ receptor and the complement receptor CR3 (3, 32). Recent advances have highlighted the significance of other receptors, such as the collectin receptor, C1q, in opsonin-dependent phagocytosis (33, 34). The opsonin-independent recognition of microorganisms and apoptotic cells is mediated by receptors such as the scavenger receptor, mannose receptor, vitronectin receptor, asialoglycoprotein receptor, and the β2 integrins (35–37). Based on the high expression of SRs on macrophages and their broad specificity, we considered this class of receptors as likely suspects for interaction with inhaled particles. Indeed, we found that AM phagocytosis of unopsonized environmental dusts (TiO_2, Fe_2O_3, SiO_2) or fluorescent latex beads is strongly inhibited by the SR blocker, PI (10). To more precisely determine the role of SR-A in this interaction, we examined the ability of AMs from SR-A (I/II)–deficient mice (24) to bind particles. We showed that both wild-type and SR-A–deficient macrophages bound unopsonized TiO_2 (Fig. 1), Fe_2O_3, and latex beads essentially identically (data not shown). The scavenger receptor ligand PI inhibited the binding of particles to SR-A–deficient macrophages, suggesting that AMs express additional SR-like molecules that mediate opsonin-independent phagocytosis of particulate matter. To identify this receptor, we generated a monoclonal antibody, PAL-1, which blocked AM binding of particles (Fig. 2 and Table I). Using this antibody, we have cloned the cDNA encoding this receptor. Transfection of COS cells with this cDNA clone confers mAb PAL-1 reactivity (Fig. 5 A). Importantly, transfection confers PAL-1–inhibitable binding of TiO_2 by COS cells (Fig. 5 C).

Interpretation of the COS cell transfection experiments was complicated by the observation that COS cells constitutively bind particles to a substantial degree. This indicated the presence of endogenous particle adhesion receptor(s). However, transfection of COS cells with MARCO but not control plasmid or cDNA resulted in mAb PAL-1–inhibitable particle binding (Fig. 5 C). Importantly, the constitutive COS cell receptor(s) for particles is sensitive to heparin inhibition, whereas AM receptor–mediated particle binding is not heparin sensitive (10). Interestingly, transfection with MARCO, in addition to conferring mAb PAL-1–inhibitable particle binding, substantially diminished the component that was heparin inhibitable (data not shown). We speculate that the transfected cDNA competes with the endoge-

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nous COS cell particle receptor(s) for either surface expression or binding function. Existence of a particle-binding receptor on nonphagocytes such as COS cells is not unprecedented. The lung epithelial cell line A549 binds and ingests unopsonized TiO_2, and this binding can also be inhibited by PI and heparin (38).

The particle-binding receptor on hamster AMs is similar to murine and human MARCO (Fig. 6). Murine MARCO was originally identified by screening a murine macrophage cDNA library with a human type XIII collagen probe. Murine MARCO is a 210-kD trimer made up of three disulfide-linked, 52-kD monomers and is expressed only on macrophages in spleen and lymph nodes (19). Human MARCO was recently cloned by screening human liver and spleen cDNA libraries with a murine MARCO probe (18). Human MARCO shares 68% sequence identity with mouse MARCO. Hamster MARCO had higher sequence identity to mouse than to human MARCO across all domains (Table II). Interestingly, hamster MARCO has a shorter collagenous domain (less 34 amino acids) than both human and mouse MARCO. Also, like human MARCO, hamster MARCO does not have a cysteine residue in the spacer domain. The six cysteines in the cysteine-rich domain are conserved in all three species (Fig. 6). The cysteine-rich domain defines a recently identified family of proteins (39). The true function of the SRCR is not clear. Hamster MARCO, like mouse and human MARCO, binds bacteria but not yeast (Fig. 7; references 19, 21).

The expression of MARCO on normal AMs merits discussion. Initially, both human and mouse MARCO were not detected in normal lung (18, 19). Using Western blot and immunolabeling techniques, we detected MARCO expression in normal lavaged AMs and AMs presented in diseased lungs (surgical specimens) (Fig. 8). The use of adult AMs may explain the difference between this finding and the absence of mRNA in neonatal lungs (18). In mouse AMs, we have detected faint but reproducible immunoperoxidase labeling, using mAb ED31, of normal BAL AMs. This antibody also partially blocks mouse AM binding of particles, indicating that low levels of ED31 (MARCO) are present and functional on these cells. In these studies, we used normal, healthy looking mice housed in conventional facilities, not a "clean room" barrier facility. Expression of MARCO is induced on lung and liver macrophages in mice infected with *Klebsiella pneumoniae* (21). It is possible that our mice expressed MARCO because of low-level activation from their less clean environmental surroundings, a possibility yet to be formally tested. It is also noteworthy that both PI and anti-MARCO (ED31) cause lower levels of inhibition of unopsonized particle binding by mouse AMs compared with that seen with hamster AMs. Whether this reflects a significant difference (e.g., the presence of other receptor[s]), minor species, or technical variables remains to be determined.

Our finding that MARCO mediates AM binding of particles defines a novel and immunologically important function for MARCO. As MARCO mediates binding of both inert particles and potentially pathogenic microorganisms, it will be interesting to determine how binding by MARCO modulates AM bactericidal functions. Specifically, the laudable absence of AM activation that accompanies binding (and ingestion) of inert environmental dusts may prove harmful for encounters with viable pathogens. Additional studies of the function of MARCO may provide more insight into the role of AMs in the initial, innate response of the lung to inhaled particles and pathogens.

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