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MARCO Is the Major Binding Receptor for Unopsonized Particles and Bacteria on Human Alveolar Macrophages

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Alveolar macrophages (AMs) avidly bind and ingest inhaled environmental particles and bacteria. To identify the particle binding receptor(s) on human AMs, we used functional screening of anti-human AM hybridomas and isolated a mAb, PLK-1, which inhibits AM binding of unopsonized particles (e.g., TiO2, latex beads; 63 ± 5 and 67 ± 4% inhibition, respectively, measured by flow cytometry; n = 11) and unopsonized bacteria (~84 and 41% inhibition of Escherichia coli and Staphylococcus aureus binding by mAb PLK-1, respectively). The PLK-1 Ag was identified as the human class A scavenger receptor (SR) MARCO (macrophage receptor with collagenous structure) by observing specific immunolabeling of COS cells transfected with human MARCO (but not SR-AI/II) cDNA and by immunoprecipitation by PLK-1 of a protein of appropriate molecular mass (~70 kDa) from both normal human bronchoalveolar lavage cells (>90% AMs) and human MARCO-transfected COS cells. PLK-1 also specifically inhibited particle binding by COS cells, only after transfection with human MARCO cDNA. Immunostaining showed specific labeling of AMs within human lung tissue, bronchoalveolar lavage samples, as well as macrophages in other sites (e.g., lymph node and liver). Using COS transfectants with different truncated forms of MARCO, allowed epitope mapping for the PLK-1 Ab to MARCO AMs within human lung tissue, bronchoalveolar lavage samples, as well as macrophages in other sites (e.g., lymph node and liver). When used to transfect COS cells, only after transfection with human MARCO cDNA. Immunostaining showed specific labeling of AMs within human lung tissue, bronchoalveolar lavage samples, as well as macrophages in other sites (e.g., lymph node and liver). Using COS transfectants with different truncated forms of MARCO, allowed epitope mapping for the PLK-1 Ab to MARCO AMs within human lung tissue, bronchoalveolar lavage samples, as well as macrophages in other sites (e.g., lymph node and liver).
Materials and Methods

Reagents and particles

TiO$_2$ was generously 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 $\mu$m (20). Latex beads (1.0 $\mu$m in diameter, sulfated, polystyrene), which show green fluorescence after excitation at 488 nm, were obtained from Interfacial Dynamics. 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 at $\sim$30 s before use. Anti-CD23 (IgG3), anti-CD44, and a nonspecific mouse IgG3 (Southern Biotechnology Associates) were used as controls. All reagents not otherwise specified were obtained from Sigma-Aldrich. A panel of Abs for known or potential scavenger-type receptors on human macrophages was used and is detailed in Table I.

Cell isolation and flow cytometric assay of particle binding

Human AMs were collected by bronchoalveolar lavage (BAL) from healthy adults under an institutionally reviewed and approved protocol. AMs obtained by lung lavage were centrifuged at 150 x $g$ and resuspended in BSS+ (124 mM NaCl, 5.8 mM KCl, 10 mM dextrose, 20 mM HEPES, Ca (0.3 mM), and Mg (1 mM)). AMs (2 x 10$^5$ in 400 $\mu$l of BSS+) were preincubated with mAbs (25 $\mu$g/ml mAb) or inhibitors (10 $\mu$g/ml and 2.5 $\mu$g/ml cytocalasin D for 5 min on ice in a 1-ml microfuge tube. Following the addition of probe-sonicated particles (25 $\mu$m/ml or beads (10:1 particle:cell ratio) the tubes were rotated at 37°C for 30 min, placed on ice, and analyzed by flow cytometry. Flow cytometry was performed using the Coulter Epics Elite flow cytometer (Beckman Coulter) as described previously (21). AM uptake of particles was measured using the increase in cell ratio) the tubes were rotated at 37°C for 30 min, placed on ice, and analyzed by flow cytometry. Therefore, results of particle binding studies are pooled data from smoker, nonsmoker, and either fresh or cultured BALs.

Assay of bacteria binding

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

Production of mAb

BALB/c mice were immunized by i.p. injection of 2 x 10$^7$ human AMs. After 3 wk, mice received another injection of 2 x 10$^7$ AMs i.p., and 3 days later spleens of the mice were removed. The splenocytes were fused with a nonsecreting mouse myeloma, P3U1, using PEG 4000 and cultured in DMEM (BioWhittaker) containing 40% 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. The clone PLK-1 was isolated and characterized as an IgG3. The Ab was produced and purified on a protein A affinity column by BioExpress Cell Culture Services (BioExpress).

Immunoprecipitation

Human AM cell surface proteins were labeled with Sulfo-NHS-LC-Biotin (Pierce), as per the manufacturer’s suggested protocol, and resuspended at a concentration of 4 x 10$^7$ cells/ml in 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 $\mu$g/ml PMSF, 2 $\mu$g/ml apro tinin, and 10 $\mu$g/ml phenanthroline as protease inhibitors). The lysates were precleared with pan-mouse-Ig magnetic beads (Dynabeads; Dyanal Biotech). Aliquots of lysate were incubated with mAbs PLK-1 or IgG3 bound to pan-mouse-Ig 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 and probed with avidin-HRP conjugate (Pierce), and developed using a chemiluminescence reagent (Supersignal; Pierce).

Plasmids and cell transfections

Full-length and truncation mutants of human MARCO cDNA in pcDNA3 expression vectors were prepared as described previously (23). The human SR-AI cDNA (24) was provided by Dr. T. Kodama (University of Tokyo, Tokyo, Japan).

COS cells were grown in DMEM (BioWhittaker) with 10% FBS plus 100 IU/ml penicillin and 100 $\mu$g/ml streptomycin. For transfection, COS cells were plated at 5 x 10$^5$ cells/100-mm tissue culture dish overnight and transfected with 4 $\mu$g of cDNA using the LipofectAMINE PLUS reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The cells were used after 48 h. COS cell expression of human MARCO was confirmed by immunohistochemical staining with a polyclonal rabbit anti-human MARCO Ab (16). COS cell TiO$_2$ adhesion assays were performed as described for AMs above.

Immunohistochemistry

Tissue samples were snap-frozen on OCT (Miles Laboratory). Cryostat sections were fixed in buffered 2% paraformaldehyde for 10 min. Cyto centrifuge preparations of human BAL samples were air-dried before similar fixation. After rinsing, immunostaining was performed by sequential application of primary Ab (mAb PLK-1) 5 $\mu$g/ml or IgG3 mAb (5 $\mu$g/ml), goat anti-mouse IgG (1:50; Steinberg Monoclonals), and rabbit anti-human MARCO Ab (16). COS cell TiO$_2$ adhesion assays were performed as described for AMs above.
Statistics

Data were analyzed using ANOVA (StatView; Abacus Concepts). Significance was accepted when $p < 0.05$.

Results

**Effect of mAb PLK-1 on AM binding of particles**

To develop a mAb to AM receptor(s) that mediate particle binding, mice were immunized with human AMs, and hybridomas were prepared and screened for mAbs that block human AM binding of TiO$_2$. As shown in Fig. 1, and reported earlier (7, 25), the SR ligand, polyinosinic acid (PI), blocked the majority of AM binding of TiO$_2$ and served as a positive control for these assays. The functional screening led to the identification of a new IgG3 mAb, PLK-1, which inhibits AM binding of TiO$_2$ by 63 ± 5% ($n = 11; p < 0.001$; Fig. 1, A and C). Chondroitin sulfate (CS; a control polyanion), an isotype-matched control mAb (IgG3) and anti-CD23 mAb, a control Ab that binds to AMs and is also an IgG3, had no effect on AM binding of TiO$_2$. We next examined the effect of mAb PLK-1 on AM binding of another inert particle, fluorescent latex beads. As shown in Fig. 1, B and D, PLK-1, but not other control agents, inhibited AM binding of latex beads by 67 ± 5% ($n = 11; p < 0.001$). Thus, mAb PLK-1 substantially inhibits human AM binding of unopsonized inert particles.

The SR ligand, PI, consistently caused a slightly greater inhibition than mAb PLK-1 for both particles (79 ± 2; 63 ± 5% inhibition of TiO$_2$ binding by PI and PLK-1, respectively ($p < 0.05$); 83 ± 3; 67 ± 4% inhibition of latex beads binding by PI and PLK-1, respectively ($p < 0.05$)). These data suggest a role, albeit minor, for a non-PLK-1 inhibitable SR(s) in this function or alternatively greater affinity of PI than PLK-1 for MARCO.

**Identification of the mAb PLK-1-reactive particle adhesion receptor**

We used COS cell transfection and surface labeling to identify the receptor-mediating AM binding of particles. A human MARCO cDNA clone transfected into COS cells conferred mAb PLK-1 reactivity (Fig. 2A). mAb PLK-1 did not react with untransfected COS cells or COS cells transfected with a cDNA encoding for human SR-AI. Immunoprecipitation analysis was used to corroborate the surface labeling studies. Human MARCO and human
SR-AI-transfected COS cells were surface-labeled with sulfo-NHS-biotin (Pierce), and the cell surface molecules bound by PLK-1 were analyzed by SDS-PAGE (Fig. 2B). PLK-1 immunoprecipitated bands of 60 kDa and 50 kDa from MARCO, but not untransfected or SR-AI-transfected COS cell lysates (Fig. 2B). This molecular mass pattern is consistent with previous results regarding human MARCO expressed in COS cells (16). To characterize the AM protein(s) recognized by mAb PLK-1, immunoprecipitation experiments (as described for COS cells above) were conducted on human AMs. A nonspecific background band of ~50 kDa was observed in both control IgG3 and PLK-1 immunoprecipitates (Fig. 2B). A major band with an apparent molecular mass of 70 kDa was detected in lysates of normal AMs (Fig. 2B). This band was absent from cells precipitated with an isotype-matched (IgG3) negative control Ab (Fig. 2B). This 70-kDa molecular mass size band is consistent with the molecular mass pattern determined by Western blot analysis with a polyclonal anti-human MARCO Ab (7). These data indicate that mAb PLK-1 recognizes the SR MARCO.

**MARCO expression confers mAb PLK-1-inhibitable TiO2 binding to COS cells**

To further test the role of MARCO in particle binding, we transfected COS cells with either human MARCO or human SR-AI cDNA and tested the effect of mAb PLK-1 on TiO2 binding. In line with our previous findings with primary hamster (7) and mouse (8) AMs, TiO2 binding by MARCO-transfected COS cells but not untransfected COS or SR-AI-transfected cells was significantly inhibited by the anti-MARCO mAb PLK-1 (Fig. 3B). Controls, including untransfected COS cells and COS cells transfected with a plasmid encoding the cDNA for human SR-AI, exhibited binding of TiO2 that was inhibited by PI and heparin, but not mAb PLK-1 (Fig. 3A and C, and data not shown). The constitutive heparin-sensitive particle binding receptor on COS cells is distinct from MARCO and remains to be identified. Thus, mAb PLK-1 recognizes human MARCO and inhibits MARCO-mediated particle binding.

**PLK-1 Ab binds to the SR cysteine-rich (SRCR) domain of MARCO**

To map the epitope of the PLK-1 Ab on MARCO receptor, and therefore the ligand binding site, we performed immunohistochemistry on COS cells transfected with either the cDNA encoding the full-length human MARCO or with truncated forms lacking different peptide sequences of the SRCR domain. PLK-1 labels the full-length MARCO, as well as the h442 (MARCO extending 22 residues into the SRCR domain) and h431 (MARCO extending 11 residues into the SRCR domain) mutants, whereas it failed to label the h420 variant (MARCO lacking the SRCR domain) (Fig. 4). This indicates that the epitope is located between residues 420 and 431, consistent with a previous report mapping the ligand binding site on domain V of the MARCO receptor in the human (23) and mouse (16).

**Tissue localization of human MARCO**

Immunostaining of cryostat sections of a panel of normal human tissues (lung, lymph node, liver, spleen, kidney) and cytocentrifuge preparations of human BAL cells (n = 10) with PLK-1 showed that it reacted strongly with most or all AMs, macrophages of lymph node sinuses, and Kupffer cells of the liver (Fig. 5A). Ag expression was also detected on macrophages within intestinal mucosa and to a lesser extent on macrophages in the splenic red pulp (data not shown). Cross-reactions with other cells or tissue components were not observed. Flow cytometric analysis showed that PLK-1 specifically, but weakly, labels AMs (MFI CD44, PLK-1, and IgG3: 503 ± 84, 61 ± 9, and 39 ± 7, respectively; n = 8; p < 0.001) (Fig. 5B) (see Discussion). Thus, human MARCO is predominantly expressed on AMs and tissue macrophages.

**MARCO mediates human AM binding of bacteria**

To further investigate the range of ligands for human AM MARCO, we tested the effect of mAb PLK-1 on AM binding of unopsonized microorganisms. As shown in Fig. 6, A and B, mAb PLK-1-inhibited AM binding of fluorescent heat killed *E. coli* by 84 ± 4% (n = 7) and binding of the Gram-positive bacteria *S. aureus* by 41 ± 9% (n = 9). PLK-1 inhibition of AM binding of *S. aureus* was consistently lower than inhibition of *E. coli* binding, suggesting differences in affinity and/or mechanism in MARCO binding of unopsonized bacteria. PLK-1 had no effect on binding of yeast (Zymosan; Fig. 6C) by normal AMs. An isotype-matched control Ab did not inhibit bacteria and yeast binding. Thus, MARCO mediates human AM binding of unopsonized bacteria in vitro, as reported previously with hamster (7) and mouse (8) AMs.

**MARCO is the major particle and bacteria-scavenging receptor on AMs**

Additional, unidentified receptor(s) likely mediate the portion of AM binding of unopsonized particles that is not inhibited by...
anti-MARCO PLK-1. To evaluate the potential contribution of
other SRs to AM binding of unopsonized TiO2 and S. aureus, we
tested a panel of Abs raised against various SRs known or likely
to be expressed on human AMs including several with known func-
tional blocking activity (see Table I). Irrelevant isotype-matched
mAbs or normal polyclonal antisera or IgG were used as controls
(Table I). Although some variability was observed, expression of
all receptors was identified on human AM samples (Fig. 7A).
However, none of these Abs caused inhibition of binding of either
unopsonized TiO2 and S. aureus (Fig. 7, B and C). The data indicate a major role for MARCO in human AM binding of
unopsonized particles.

Discussion
This study has identified the class A SR MARCO as a dominant
receptor for unopsonized TiO2 and S. aureus, we tested a panel of Abs raised against various SRs known or likely
to be expressed on human AMs including several with known func-
tional blocking activity (see Table I). Irrelevant isotype-matched
mAbs or normal polyclonal antisera or IgG were used as controls
(Table I). Although some variability was observed, expression of
all receptors was identified on human AM samples (Fig. 7A).
However, none of these Abs caused inhibition of binding of either
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unopsonized particles.
studied, and several opsonin-receptors have been well characterized, such as FcγR, complement receptor CR3, and collectin receptor, C1q (4, 40–43). However, the recent studies on opsonin-independent recognition of microorganisms and apoptotic cells have implicated receptors such as the SR SR-AI/II, mannose receptor, vitronectin receptor, asialoglycoprotein receptor, and the β2 integrins (44, 45). In this study, we have identified MARCO as a major receptor on AMs for binding of unopsonized inert particles and certain microorganisms.

Although human MARCO is the major receptor on AMs for unopsonized particles, the SR ligand, PI, caused a slightly higher inhibition of particle binding, suggesting a role for other SR(s) on AMs. We initially considered this receptor to most likely be SR-AI/II, because we have found that an Ab to SR-AI/II partially blocks TiO2 binding by mouse macrophages (our unpublished observation). However, analysis of a panel of Abs to other SRs on human AMs failed to detect inhibition of particle binding, including monoclonal and polyclonal Abs reported to have functional blocking ability against SR-AI/SR-AII (K. Nakamura, personal communication and Ref. 28, respectively). One limitation to these data is that we did not independently confirm the blocking function reported by other investigators, although in some cases we were able to use two or three different Abs with the same (negative) result. Another unanswered question is the structure(s) responsible for the ~20% of particle binding and the up to 60% of bacteria (S. aureus) that is not blocked by either PI or PLK-1.

There is noteworthy discordance between the rather low expression level of MARCO on human AMs detected by immunofluorescence labeling and flow cytometry and the high capacity for particle binding by these cells via surface structures blocked with the PLK-1 anti-MARCO. We have observed similar findings in mouse AMs and another anti-MARCO mAb, ED31 (data not shown). Immunohistochemical differences in blocking vs labeling capacities may be a relatively trivial cause for this discordance. Another speculative possibility is that the unopsonized particles studied (dusts, bacteria) are large enough to engage a complex of multiple surface receptors that require MARCO (present in small amounts) to function optimally. It is worth noting that whereas our analysis focused on the cardinal property of SRs, namely the binding of particles and pathogens, the role, if any, of these receptors in the subsequent internalization phase of phagocytosis remains to be characterized.
Our finding that MARCO mediates human AM binding of un-opsonized particles and bacteria indicates an important function for MARCO in human lung host defense. Additional investigation is needed to address the potential role of other receptors involved in particle binding and to identify the signaling events (if any) initiated by SRs like MARCO.

Disclosures
The authors have no financial conflict of interest.

References