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*J Immunol* 2008; 181:7902-7908; doi: 10.4049/jimmunol.181.11.7902

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Complement Receptor of the Ig Superfamily Enhances Complement-Mediated Phagocytosis in a Subpopulation of Tissue Resident Macrophages

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An important function of the complement cascade is to coat self and foreign particles with C3-proteins that serve as ligands for phagocytic receptors. Although tissue resident macrophages play an important role in complement-mediated clearance, the receptors coordinating this process have not been well characterized. In the present study, we identified a subpopulation of resident peritoneal macrophages characterized by high expression of complement receptor of the Ig superfamily (CRIg), a recently discovered complement C3 receptor. Macrophages expressing CRIg showed significantly increased binding and subsequent internalization of complement-opsonized particles compared with CRIg negative macrophages. CRIg internalized monovalent ligands and was able to bind complement-opsonized targets in the absence of Ca2+ and Mg2+, which differs from the β2-integrin CR3 that requires divalent cations and polyvalent ligands for activation of the receptor. Although CRIg dominated in immediate binding of complement-coated particles, CRIg and CR3 contributed independently to subsequent particle phagocytosis. CRIg thus identifies a subset of tissue resident macrophages capable of increased phagocytosis of complement C3-coated particles, a function critical for immune clearance.

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Received for publication December 24, 2007. Accepted for publication September 30, 2008.


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Phagocytosis, an important component of host defense and a primary function of macrophages, is facilitated by opsonization, a process by which serum components tag pathogens and immune complexes for recognition by neutrophils and macrophages (1). Triggering of the classical, lectin, and alternative pathways of complement activates the central component C3 resulting in covalent binding of C3b to a particle with the release of C3a. C3b is rapidly degraded to iC3b by the action of factor I and cofactors and further degraded to C3c, C3d, and C3dg (2, 3). Binding of the C3dg and C3d fragments to CR2 on B cells leads to enhanced Ab production (4), whereas binding of C3 proteins to complement receptors (CRs);3 on macrophages results in phagocytosis and clearance of the particle (5–8).

CR3, a transmembrane heterodimer composed of two integrin subunits (CD11b or αM and CD18 or β2), is a well established CR for the dominant C3 cleavage product, iC3b, and in mice is present on all myeloid cells and a subset of NK cells (7). CR3-mediated phagocytosis is dependent on receptor redistribution and conformational changes, two processes that require activating stimuli (9, 10). Upon activation of phagocytes with phorbol esters (11) or β-glucan (12, 13), cell surface expression and activation state of CR3 are increased, enabling CR3-mediated phagocytosis. Similarly, inflammatory macrophages freshly recruited from the circulation to peritoneal cavity use CR3 for phagocytosis (14, 15). Although tissue resident macrophages are thought to play an important role in both initiation and resolution of inflammation (16–18), CR3 does not act as a potent phagocytic CR on these cells (19).

Recently, we identified a novel CR of the Ig superfamily (CRIg) that is highly expressed on Kupffer cells (20, 21). CRIg is required for rapid sequestration of blood-borne Listeria monocytogenes and Staphylococcus aureus in a C3-dependent manner, thereby limiting systemic bacteremia and promoting survival of the host. In this study, we show that CRIg is required for efficient complement-mediated phagocytosis by non-activated as well as activated resident peritoneal macrophages (RPMs). Thus, CRIg identifies a subset of tissue macrophages that is capable of integrin-independent rapid recognition and phagocytosis of iC3b-opsonized particles in the absence or presence of inflammatory stimuli, a feature of importance for tissue homeostasis and host defense.

Materials and Methods

Abs, proteins, and dyes

Non-blocking mAbs against CRIg were generated as described (20). Blocking Abs (clone 2H1 and 14G8; Genentech) were generated by immunizing CRIg-deficient mice with CRIg-Fc fusion protein generated as described (20). Phycoerythrin-labeled anti-F4/80 Ab (F4/80-PE) was purchased from Caltag Laboratories. Unless indicated, all other Abs were purchased from BD Biosciences. C4b was purchased from Complement Technologies. Human C3, C3b, and iC3b (used in Fig. 2) were obtained from fresh serum and purified as described (20). Murine C3 was purified from fresh mouse serum using the same method. Murine C3b was generated by cleavage of purified C3 by trypsin as described elsewhere (22). Murine iC3b was purified from pooled murine ascites obtained from the
i.p. cavity following implantation of various hybridomas. Ascites was buffered with 25 mM Tris (pH 7.5), 5 mM EDTA, and 100 mM NaCl. PEG 6000 was added to the ascites to a final concentration of 10% and continuously mixed for 1 h. This solution was centrifuged at 20,000 × g for 15 min. The supernatant was decanted, and the pellet was re-suspended in 50 mM Tris (pH 8.6) and 5 mM EDTA and loaded over a protein G column to deplete the IgG from the solution. The flow through was applied directly onto a mono Q (10 × 100; GE Healthcare), which was eluted with a gradient of 0 to 0.7 M NaCl over 20-column volumes. The murine iC3 was isolated and loaded separately over a Superdex 200 (16/60; GE Healthcare) at 7 cm/h in 25 mM Tris (pH 8.0), 150 mM NaCl, and 5 mM EDTA. The resulting fractions were analyzed by SDS-PAGE, and identity was confirmed by Edman degradation.

Abs and proteins were labeled with Alexa Fluor 555 (A555), Alexa Fluor 488 (A488), or Alexa Fluor 647 (A647) according to manufacturer’s instruction (Invitrogen). CRIg-ECD was generated by expression of the extracellular domain of murine CRIg in Chinese hamster ovary cells and purified as described (20). All other reagents are from Sigma-Aldrich unless noted.

**Animals and flow cytometry analysis of resident peritoneal macrophages**

All animals were held under sterile pathogen free conditions and animal experiments were approved by the Institutional Animal Care and Use Committee of Genentech. Six to 7 wk old C57BL/6J mice and AKR mice were purchased from Jackson ImmunoResearch Laboratories. C3-deficient mice have been described (23). Resident peritoneal cells were lavaged with PBS containing 2 mM EDTA, resuspended in PBS containing 1% BSA, and incubated on ice with anti-mouse CD16/32 and purified rat IgG2a and IgG2b to block Fc receptors followed by A647-conjugated anti-CRIg Ab (clone 1C79), PE-conjugated Ab to F4/80, anti-CR1/CR2 (clone 7G6), anti-CRIg (CD11b, clone M1/70), anti-CD18, anti-CR3 (CD11c, clone 2F4), or anti-CR-related gene Y (Crry; clone 1F2). After incubation with 7-amino-actinomycin D (7-AAD) (Molecular Probes), cells were analyzed by a FACSCalibur (BD Biosciences). For immunohistochemistry of CRIg, CRIg+ F4/80+ and CRIg− F4/80− nonadherent RPMs (naRPMs) were sorted (FACSaria 1; BD Biosciences) and cultured in Lab-Tek II chamber slides (Nunc) in DMEM (High glucose DMEM (HDMEM); Cellgro) containing 10% heat inactivated FBS (HyClone), 4.5 mg/ml glucose, 10 mM HEPES, 10 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) (HDMEM-10). Cells were fixed with acetone/ethanol solution (75/25, v/v) at −20°C for 10 min, blocked for FcR-mediated binding as indicated above, and stained for CRIg as described (20).

**Binding and internalization of complement proteins by adherent RPMs (adrRPMs)**

For microscopic evaluation of iC3b, C3b, and C3 internalization, cells were incubated in Lab-Tech II chamber slides (Nunc) for 1 h at 37°C in HGD-MEM, nonadherent cells were removed and, after overnight, culture cells were stained with an A555-labeled anti-CR1g Ab (clone 14G6), incubated with A488-labeled complement proteins, and internalization visualized by microscopy.

**Binding and phagocytosis of sheep RBC (SRBC)**

SRBCs (5 × 10⁹/ml; Colorado Serum) were labeled with rat IgM-A488 anti-Forsman Ab (clone TIB-123, 5 μg/ml; ATCC) for 30 min at 22°C (IgM-coated erythrocyte; E-IgM). 2.5 × 10⁴ E-IgM were incubated with 1 ml Veronal Buffer (BioWhittaker) containing 10% serum from C5-deficient mice (The Jackson Laboratory), 0.15 mM CaCl₂, 0.1% gelatin for 30 min at 37°C to generate complement-opsinized E-IgM (E-EAC). Serum from AKR/J mice was used to prevent lysis of the SRBCs. For analysis of C3 fragments present on the EACs, EACs were then lysed in 1% NP40 at 1E8/ml, an equal volume of 2× gel loading buffer plus 10% β-mercaptoethanol was added to yield a final cell lysate concentration of 5E7/ml. 5E5 EACs in a volume of 10 μl were pelleted well (37°C). PAGE, C3 proteins were detected using a 1 μg/ml goat anti-mouse C3 (MP Biomedicals) and rabbit anti-goat Ab (Thermo Fisher Scientific).

Binding experiments were performed on freshly lavaged, naRPMs resuspended in PBS/0.5% BSA. Cells were placed on ice and FCRs were blocked as described above plus 25 μg/ml anti-CRIg (clone 14G8), anti-CD11b (clone M1/70), or isotype control (anti-ragweed; Genentech) blocking Abs. EACs were added to the naRPMs in a 20:1 ratio for 30 min at 37°C and the amount of EACs bound by flow cytometry as described below. To determine the effect of divalent cations on binding, naRPMs were resuspended in PBS/0.5% BSA with either 10 mM EDTA or 0.15 mM Ca²⁺/1 mM Mg²⁺ and incubated with EACs for 30 min at 37°C. To determine the binding and phagocytosis of EACs to adrRPMs, 1×10⁵ peritoneal cells were placed in 6-well tissue culture plates (Costar) or in chamber slides in HGD-MEM-10, nonadherent cells were removed after 1 h incubation at 37°C, and adherent cells were cultured overnight. Where indicated, adrRPMs were activated with 1 μg/ml LPS (Escherichia coli strain O26:B6; Sigma-Aldrich) for 24 h. Cells were placed on ice, washed 2× with cold PBS, and FCRs were blocked with cold HGD-MEM/5 μg/ml anti-CD16/32 (BD Biosciences), 20 μg/ml anti-ragweed mlgG2a and mlgG2b (Genentech) for 10 min on ice. FCR blocking solution was then aspirated and 50 μg/ml anti-CRIg (14G8), anti-CD11b (clone M1/70), or isotype control Abs was added for 10 min on ice to block CRs. A total of 2 × 10⁷ EACs in cold HGD-MEM was added and binding synchronized by spinning at 500 rpm for 1 min at 4°C. Cells were placed at 37°C for indicated times and phagocytosis was stopped by placing cells on ice. To monitor phagocytosis by flow cytometry, cells were washed in PBS/10 mM EDTA, scraped off, transferred to chilled FACS tubes, washed 1× with PBS/0.5% BSA, and stained with F4/80-PE and anti-CRIg A647 (clone 25C5) for 25 min. Extracellular SRBCs were lysed with 1 ml of ACK lysis buffer (82 mM NH₄Cl, 5 mM KHCO₃, and 50 μM EDTA) for 2 min on ice, washed 2× with PBS, and then incubated with 30 μg/ml anti-A488 Ab (Invitrogen) to quench fluorescence of A488-conjugated EACs bound to the adrRPM cell surface. Cells were analyzed on FACScalibur, 1 μg/ml propidium iodide was added to exclude dead cells, and data analyzed with FlowJo software (TreeStar).

**FIGURE 1.** CRIg and CR3 are the only phagocytic complement C3 receptors expressed on RPMs. A, Flow cytometry analysis of CRIg expression on F4/80+ RPMs. B and C, CRIg+ and CRIg− RPMs express similar levels of CD11b, CD18, and Crry, but lack expression of CR1, CR2, and CD11c. Shaded areas represent specific staining, solid lines represent isotype Ab staining. Histograms are representative of three independent experiments. D, CRIg+ (red) is expressed on RPMs. CRIg+ and CRIg− RPMs were FACS sorted and cultured for 7 days before immunostaining. Blue indicates nuclear staining with DAPI. Scale bar (D) = 10 μm.
For immunofluorescence, adRPMs with EACs were washed with PBS/0.15 mM CaCl₂/1 mM MgCl₂, FcRs were blocked for 10 min and the blocking solution was then removed, and, finally, cells were stained with 3g/ml A555-conjugated CRIg (clone 14G6) Abs for 15 min on ice. In some experiments, nonfluorescent EACs and 3g/ml anti-CD11b-FITC was used. Cells were washed and extracellular EACs lysed with ACK buffer for 2 min. In experiments where A488-conjugated EACs were used, the non-internalized EAC membrane was quenched with an anti-A488 Ab. adRPMs were fixed with 2% paraformaldehyde (EM Sciences) in PBS for 20 min and cover-slipped with VectaShield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Fluorescence and deconvolution microscopy
Images were acquired on a Nikon Eclipse TE300 inverted microscope using a ×60 objective, a Zeiss LSM 510 confocal microscope using a 60× objective, or an Olympus BX61 upright microscope using a ×60 objective.

Statistical analysis
Statistical analysis was performed using JMP software (JMP Release: 5.1.2; SAS Institute). All p values are calculated with an unpaired Student’s t test assuming unequal variance.

Results
CRIg is expressed on a subpopulation of RPMs
Both CRIg and CR3 (or α₃β₃) are receptors for iC3b present on serum-opsonized pathogens. To determine the relative contribution of CRIg and CR3 to complement-mediated phagocytosis, we focused our studies on RPMs that express high levels of CR3. CRIg was coexpressed with CR3 on 30% of the total population of RPMs (Fig. 1A) in line with a previous report (24). Next to expression on the cell surface, CRIg was also localized on an intracellular pool of recycling endosomes, as described in our previous study (25).

Sequential images were acquired with a spacing of 0.2 microns along the z-axis and then deconvolved using softWoRx software (Applied Precision).

FIGURE 3. CRIg is sufficient for binding of iC3b-opsonized particles to naRPMs. A, left panel, Flow cytometry of complement C3 proteins found on EACs opsonized with serum from C3-deficient mice or C5-deficient AKR mice. Solid lines represent isotype control Ab staining, shaded lines represent staining for C3. Right panel, Western blot analysis of EACs opsonized with serum from AKR mice or with serum from C3-deficient mice. Purified mouse C3 proteins (right lanes) are used as reference. B, naRPMs were mixed with A488-labeled EACs on ice or incubated for various times at 37°C and then stained with a non-blocking Ab to CRIg. The MFI of A488 was determined by flow cytometry. C, left panel, CRIg⁺ and CRIg⁻ naRPMs were incubated for 30 min at 37°C with control or CR3 and/or CRIg blocking Abs and A488-labeled EACs. The amount of EACs bound to CRIg⁺ and CRIg⁻ naRPMs was then determined by staining the cells with a nonblocking Ab to CRIg and measuring the MFI of A488 using flow cytometry. Right panel, naRPMs were treated as described above but incubated with A488-labeled EACs in the presence of 20 mM EDTA. Results are expressed as mean ± SD, n = 3; * p < 0.05; ** p < 0.001.

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Fluorescence and deconvolution microscopy
Images were acquired on a Nikon Eclipse TE300 inverted microscope using a ×60 objective, a Zeiss LSM 510 confocal microscope using a 60× objective, or an Olympus BX61 upright microscope using a ×60 objective.

Research support
This study was supported by National Institutes of Health grant AI-56644 (to S.J.).
and CR3 in the phagosomes surrounding the EACs. Scale bar indicates nuclear staining with DAPI. Arrows indicate accumulation of CRIg bind to either CRIg/H11001 or CR2, or CD11c, the α-chain of CR4 in accordance with a previous report (25). Neither CRIg nor CR3 RPMs expressed CR1, CR2, or CD11c, but in CR4 RPMs, the α-chain of CR4 was detected. This indicates that CRIg was able to bind to complement-opsonized particles in the absence of integrin activation. Thus, CRIg is stably expressed on a subpopulation of tissue resident macrophages that also express CR3.

**CRIg is required for binding and internalization of monomeric C3-derived proteins**

Since iC3b is the predominant C3 fragment found on complement-opsonized particles (27), we determined the contribution of CRIg vs CR3 in binding and internalization of soluble iC3b. For binding studies, freshly lavaged, naRPM were used. When incubated at 4°C, iC3b bound only to CRIg+ naRPMs (Fig. 2A), C3 did not bind to either CRIg+ or CR3- macrophages in line with CRIg’s selective recognition of a neoepitope exposed on cleaved, but not native, C3 (21). CRIg blocking Ab or a soluble CRIg protein significantly reduced iC3b binding to RPMs while CR3 blocking Ab or CRIg isotype control Ab had no effect on binding activity (Fig. 2B), confirming the requirement of CRIg, and not CR3, for binding to monomeric iC3b. For studying internalization of C3 proteins, freshly lavaged RPMs were cultured overnight to generate adRPMs. When incubated with cultured adRPMs at 37°C, the bound ligands C3b and iC3b were internalized and colocalized with CRIg+ vesicles in the areas surrounding the nucleus as well as at the tips of macrophage pseudopodia (inset, Fig. 2C). Residual uptake of C3 in CRIg+ adRPMs was observed and likely indicates that some of the C3 was hydrolyzed and subsequently internalized through CRIg. CRIg+ adRPMs, identified by nuclear staining only (Fig. 2C, arrows), did not internalize any of the C3 proteins. These results illustrate that CRIg is able to bind and internalize monomeric ligands while CR3 is not, confirming the requirement of a multivalent ligand for CR3-mediated binding and internalization of target particles (9, 10, 28).

**CRIg and CR3 differ in their requirement for binding to complement-opsonized particles**

To distinguish the contribution of CRIg and CR3 to binding and phagocytosis of particles coated with multivalent C3 proteins, IgM-opsonized SRBC were incubated with C5-deficient serum, obtained from AKR/J mice, to generate complement C3-coated SRBC (EACs). Flow cytometry analysis indicated deposition of C3 proteins on the surface of EACs that were incubated with C5-deficient serum but not on EACs incubated with C5-deficient serum (Fig. 3A, left panel). The major C3 protein coated on the surface of these particles was in the form of iC3b as illustrated by Western blot analysis using purified C3 proteins as a reference (Fig. 3A, right panel). Binding experiments were conducted using naRPMs. Under the conditions used and in line with a previous report (29), all A488-conjugated EACs bound to the surface of the naRPMs were not internalized as shown by the reduction of fluorescence to baseline values following lysis of these EACs and quenching of the extracellular fluorescence with anti-A488 Abs (results not shown). EACs incubated with C5-deficient serum did not bind to naRPMs (results not shown) indicating that all binding was mediated through complement C3 receptors. At 4°C, CRIg+ naRPMs showed significantly increased binding of EACs compared with CRIg- naRPMs (Fig. 3B). Although both CRIg+ and CR3+ naRPMs increased their binding to EACs with time at 37°C, the amount of complement-opsonized particles bound to CRIg+ naRPMs remained significantly greater than the number of particles bound to CRIg- naRPMs. CRIg and CR3 blocking Abs were subsequently used to determine the relative contribution of CRIg vs CR3 on particle binding after incubation for 30 min at 37°C. In CRIg+ naRPMs, blocking of CRIg or CR3 reduced the binding of EACs by 30% and 50%, respectively, whereas blocking both receptors simultaneously reduced particle binding to baseline levels (Fig. 3C, left panel). In the CRIg+ population of naRPMs, binding was fully mediated through CR3. Next, binding studies were performed in the absence of divalent cations to exclude binding through CR3 which is Mg2+-dependent (9). When chelating both Ca2+ and Mg2+ with EDTA, binding of EACs to CRIg+ naRPMs was reduced by 60% while negligible binding to CRIg- naRPMs was detected. In CRIg+ naRPMs, Ca2+/Mg2+-independent binding activity was entirely mediated through CRIg as CRIg blocking Abs completely abolished EACs binding to naRPMs (Fig. 3C, right panel). This indicates that CRIg was able to bind to complement-opsonized particles in the absence of integrin activation. Thus, increased binding activity of CRIg+ naRPMs to EACs is mediated through both CRIg and CR3 following incubation at 37°C. Finally, in contrast to CRIg, CR3 is dependent on divalent cations to efficiently bind to opsonized targets, indicating different requirements for CRIg and CR3 receptor function.

**CRIg localizes to phagosomes and enhances phagocytosis in adRPMs**

To further elucidate the subcellular localization and contribution of CRIg and CR3 to particle phagocytosis, adRPMs, capable of phagocytosing complement-opsonized particles (27), were used.
CRlg enhances phagocytosis in adRPMs. A, adRPMs, cultured overnight, were incubated with A488-conjugated EACs for the indicated time points at 37°C. adRPMs were then dissociated from the tissue culture plates and stained with a non-blocking Ab to CRlg. EACs bound to the cell surface were lysed and the extracellular green fluorescence was quenched using polyclonal anti-A488 Abs. The amount of phagocytosed EACs was then determined by the MFI of A488 using flow cytometry. B, adRPMs were incubated with A488-conjugated EACs for 30 min in the presence of isotype or CRlg and/or CR3 blocking Abs. The amount of phagocytosed EACs in CRlg⁺ and CRlg⁻ adRPMs was then determined by staining the cells with a non-blocking Ab to CRlg and measuring the MFI of A488 using flow cytometry. Results are expressed as mean ± SD, n = 3, *p < 0.05; **p < 0.01. C, adRPMs were incubated with EACs (green) for 30 min in the presence of isotype (+ Control Ab) or CRlg blocking Abs (+ CRlg Ab) followed by staining with a non-blocking Ab to CRlg (red). Blue indicates nuclear staining with DAPI. In the presence of control Ab, CRlg accumulates at sites where the EACs contact the macrophage plasma membrane (arrows). In the presence of CRlg blocking Abs (+ CRlg Ab), CRlg accumulation at the sites of EAC contact is prevented. Scale bar = 10 μm.

CR3 was distributed equally on all adRPMs while CRlg, as expected, localized to a subset of adRPMs (Fig. 4A). To participate in all steps of the phagocytic process, the receptors involved have to redistribute to the site of particle contact (30). Upon incubation with EACs, CRlg and CR3 redistributed to localize in the phagosome surrounding the EAC (Fig. 4B). To further determine whether CRlg and CR3 colocalization with phagosomes translates into more efficient phagocytosis, adRPMs were incubated with A488-fluorescently labeled EACs (Fig. 5A) followed by dissociation of the phagocytes from the culture dish. Phagocytosis was determined by FACS analysis after lysis of cell surface-bound EACs. Remaining fluorescence resulting from lysed EAC bound to the cell surface was quenched with a polyclonal anti-A488 Ab. As a result, internalized EACs were the primary source of fluorescence. Uptake of EACs was significantly increased in CRlg⁺ and CRlg⁻ adRPMs compared with CRlg⁻ adRPMs (Fig. 5A). In CRlg⁺ adRPMs incubated with EACs for 30 min, CRlg and CR3 each contributed to ~50% of total phagocytosis which was reduced to background levels when both receptors were simultaneously blocked (Fig. 5B, left panel). In CRlg⁻ adRPMs, as expected, phagocytosis was entirely mediated through CR3 (Fig. 5B, right panel). These results indicate that CRlg and CR3 act independently and additively to enhance binding and phagocytosis of complement C3-opsonized particles. To determine whether CRlg blocking Ab disrupts trafficking of CRlg to the forming phagosome, cultured adRPMs incubated with EACs were treated with isotype or CRlg-blocking Abs and then stained with a nonblocking Ab to CRlg. In the presence of isotype control Ab, CRlg accumulated in the forming phagosome (Fig. 5C, upper panels). Addition of CRlg-blocking Ab prevented CRlg accumulation in the phagosome and resulted in even distribution of CRlg on the cell surface (Fig. 5C, lower panels). This indicates that blocking CRlg binding to its ligand iC3b prevents CRlg accumulation in the phagosome and inhibits phagocytosis of the complement-opsonized particles.

In vivo, tissue macrophages often encounter various activating stimuli, including bacterial cell wall products and cytokines (31). To determine whether CRlg could act as a phagocytic receptor once the macrophages were activated, adRPMs were incubated overnight with LPS. This stimulation resulted in increased expression of the activation marker CD86, while CRlg expression was slightly reduced (Fig. 6A). In the activated macrophages, particle internalization was evident only after 20 min (Fig. 6B). Similar to nonactivated macrophages, LPS-activated CRlg⁺ adRPMs showed significantly increased phagocytosis at 30 min, contributed to by both CRlg and CR3 (Fig. 6C). Thus, CRlg significantly contributes to increased complement-mediated phagocytosis in both nonactivated and LPS-activated adRPMs.

Discussion

CRlg was initially described as a Kupffer cell-expressed CR required for clearance of pathogens from the circulation (20). This study shows that, besides Kupffer cells, CRlg is expressed on a
subpopulation of RPMs. These mononuclear phagocytes are capable of local synthesis of complement components (2, 32), enabling opsonization of target particles in the absence of direct exposure to serum. In the current study, we demonstrate that the two CRs co-expressed on these macrophages, CRIg and CR3, independently contribute to complement-mediated phagocytosis of C3b-opsonized particles. We further illustrate that the requirement for binding and ingestion of target particles differ between CRIg+ and CRIg− RPMs.

CRIg accelerates complement-mediated phagocytosis by rapidly binding and internalizing opsonized particles independent of receptor crosslinking, activating stimuli or divalent cations. Thus, CRIg functions differently from CR3/iC3b in regards to requirements for binding and macroparticle ingestion. In our studies adRPMs were cultured overnight to assure firm adhesion, a procedure that may have induced activation of the adRPMs sufficient to enable phagocytosis. Finally, in support of our observations, adRPMs can internalize constituents of C3b-opsonized sheep erythrocyte membranes (38), indicating that non-stimulated adRPMs are capable of phagocytosis of complement C3-opsonized particles.

Different from other CRs, CRIg is present on recycling endosomes in adRPMs as well as in monocyte-derived macrophages (20). Endosomes are important in trafficking of plasma membrane and for cytokeskeleton assembly required for phagosome formation (39, 40). Their recruitment to the site of particle contact may mediate incorporation of CRIg in the forming phagosome ensuring close contact of the macrophage plasma membrane with the opsonized particle. Indeed, Abs that blocked CRIg binding to C3b prevented recruitment of CRIg to the phagosomes, resulting in reduced phagocytosis of the opsonized target particles (Fig. 5).

Whether the recruitment of CRIg+ endosomes to the forming phagosome is an active process is unknown. CRIg has a relative large cytoplasmic tail containing several tyrosine residues that can potentially serve as docking sites for signaling components or cytoskeletal elements. The participation of CRIg in signaling pathways linking cytokeskeleton reorganization to phagocytosis is an area of current investigation.

Since in vivo tissue resident macrophages are continuously exposed to activating stimuli, including bacterial components from enteric microflora (41), we determined whether CRIg expression or phagocytic function changes in vitro following activation of adRPMs with LPS. Although cell surface expression of CRIg was slightly reduced under these circumstances, CRIg remained functional as a phagocytic receptor as shown by a similar contribution of CRIg to EAC phagocytosis in nonstimulated as well as stimulated adRPMs. Thus, while CRIg is absent from inflammatory macrophages, it remains expressed and functional when tissue resident macrophages are exposed to activating stimuli.

In a recent study (42), it was demonstrated that CRIg clears complement-opsonized platelets lacking DAF and Crry, for the first time illustrating a role of CRIg in clearance of self cells.
Unexpectedly, complement C3-coated erythrocytes were not cleared through a CR1g-dependent mechanism. A likely explanation for this finding is that complement opsonization of the DAF and Cry double-deficient erythrocytes was much less efficient compared with platelet opsonization, resulting in a significantly reduced interaction with CR1g. In the present study, IgM anti-Forssmann Ag was used to provide optimal complement activation on the erythrocyte surface leading to high levels of C3 deposition (Fig. 3A), comparable to what was found on the platelets (42). Thus, next to a potential influence of the nature of the particle, the degree of C3 opsonization is an important determinant for clearance by CR1g.

Together, CR1g identifies a novel subpopulation of macrophages with a high capacity for complement-mediated phagocytosis in the absence and presence of activating stimuli. These studies illustrate the functional diversity of tissue resident macrophages and will further guide research to understand the role of CR1g in tissue homeostasis and host defense.

Acknowledgments

We thank Drs. Rick Brown, Wouter Hazenbos, Nico Ghilardi, and Paul Godowski for insightful discussions. We are grateful to Im Cupp, Michael Hamilton, Susan Palmieri, Laszlo Komives, and Meredith Sagolla for their contributions.

Disclosures

All authors are current or former employees of Genentech.

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