Response Gene to Complement 32 Protein Promotes Macrophage Phagocytosis via Activation of Protein Kinase C Pathway*

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Background: Response gene to complement 32 (RGC-32) was initially identified in oligodendrocytes, suggesting an immune-related function.

Results: RGC-32 deficiency did not affect macrophage differentiation but attenuated macrophage phagocytosis by reducing protein kinase C activity and F-actin formation.

Conclusion: RGC-32 is a novel regulator for macrophage phagocytosis.

Significance: RGC-32 may serve as a novel target for modulating immune function.

Phagocytosis is a major mechanism used to remove pathogens (1, 2) and cell debris (3) in the immune system. Phagocytosis is also crucial for tissue remodeling (4), wound repair (5, 6), and tumorigenesis (7). Although phagocytosis is mainly achieved by the dedicated phagocytes such as macrophages and dendritic cells, other cells such as fibroblasts (8), epithelial cells (9), and endothelial cells (10) also exhibit phagocytic activity. During phagocytosis, exogenous particles or cells are first recognized by cell surface receptors such as Fcy receptors (11), dectin-1 (12), or complement receptor 3 (CR3) (13). Then signals from cell surface receptors will induce actin filament-guided internalization of foreign particles to form nascent phagosomes (14, 15). Finally, nascent phagosomes will undergo several maturation progresses to digest these foreign materials (16).

Response gene to complement 32 (RGC-32)2 is a cell cycle regulator expressed in many adult human tissues including heart, brain, liver, skeletal muscle, placenta, kidney, and pancreas (17, 18). It is also overexpressed in many tumors (19). Our previous studies have shown that RGC-32 is an important transcriptional co-activator involved in smooth muscle cell differentiation (17). In addition to its nuclear and cytoplasm expression, we found that RGC-32 is also expressed on cell membrane of macrophages. Therefore, we hypothesized that RGC-32 may play a role in the innate immune system.

Indeed, we have identified RGC-32 as an immune regulator in macrophage phagocytosis. RGC-32 is expressed in macrophage but not monocyte. Knock-out of RGC-32 (RGC-32−/−) does not affect macrophage differentiation. However, RGC-32−/− in macrophage significantly impairs its phagocytic activity. It appears that membrane-associated RGC-32 directly binds to protein kinase C (PKC) and induces F-actin assembly and formation of phagosomal cups, which facilitates the internalization of foreign particles and the phagocytosis.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—Human THP-1 cell lines were maintained at 37 °C in a humidified 5% CO2 incubator in RPMI 1640 medium containing 10% FBS (Life Technologies) and 0.05 mM 2-mercaptoethanol. Phorbol 12-myristate 13-acetate (PMA), LPS, zymosan-A, and PKH26 staining kit were purchased from Sigma-Aldrich. FITC-labeled zymosan-A and

2 The abbreviations used are: RGC-32, response gene to complement 32; PMA, phorbol 12-myristate 13-acetate; MARCKS, myristoylated alanine-rich protein kinase C substrate; iNOS, inducible nitric-oxide synthase; BMHPC, bone marrow hematopoietic progenitor cells; BMDM, bone marrow-derived macrophage; Ad, adeno virus.
Texas red-labeled zymosan-A particles were purchased from Life Technologies. IFNγ, G-CSF, IL-4, and M-CSF were purchased from R&D Systems.

**Animals**—Wild type and RGC-32 deficiency (RGC-32<sup>−/−</sup>) mice in C57BL/6 background were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal surgical procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Animal care was conducted in compliance with the Principles of Laboratory Animal Care and Use in the United States, and the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the National Academy Press, Washington, DC.

**Peritoneal Macrophage Purification and Counting**—3 ml of PBS were injected to the peritoneal cavity of C57BL/6 mice and left for 5 min with gentle massage. Resident macrophages were harvested using a syringe and centrifuged at 500 × g for 5 min. The cell pellet was washed with PBS and centrifuged again. The cell pellet was suspended in 10% FBS-contained RPMI 1640 medium and cultured in a culture dish. 2 h later, unattached cells were removed by aspirating the culture medium. 1 day after culture, the cells were collected, and cell numbers were counted using a hemocytometer under a microscope (Nikon).

**Macrophage Differentiation and Polarization**—For THP-1 to macrophage differentiation, THP-1 cells were treated with 100 ng/ml PMA in 10% FBS-RPMI 1640 for 48 h. For bone marrow-derived macrophage differentiation, bone marrow was obtained from mice by flushing pelvies, femurs, and tibiae with 15% FBS-RPMI 1640. Cells were harvested and centrifuged at 500 × g for 5 min. Cell pellets were suspended in RPMI 1640 medium containing 10% FBS and 15% L929 cell-conditioned medium and cultured for 7 days. Cells were then harvested, and cell numbers were counted. For M1 macrophage polarization, bone marrow cells were cultured in 10 ng/ml G-CSF for 7 days followed by treatment with 100 ng/ml LPS and 20 ng/ml IFN-γ for 24 h. For M2 macrophage polarization, bone marrow cells were cultured in 10 ng/ml M-CSF for 7 days followed by treatment with 20 ng/ml IL-4 for 24 h.

**Phagocytosis Assay**—Macrophage phagocytosis was determined by cellular uptake of FITC- or Texas Red-labeled zymosan-A particles. 1 mg/ml zymosan-A particles was added into macrophage culture medium and incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 10 min. Cells were then washed with ice-cold PBS and fixed with 1% paraformaldehyde for 5 min. Uptake of zymosan-A particles was analyzed via flow cytometry.

**Tumor Cell Clearance Assay**—THP-1-derived macrophages were transduced with control (Ad-GFP)-, RGC-32 (Ad-RGC32)-, or RGC-32 shRNA (Ad-shRGC32)-expressing adenovirus (20), seeded on glass coverslips in 24-well cell culture plates at 100% confluent, and incubated overnight under normal cell growth conditions. Human Hep3D tumor cells labeled with PKH26 were seeded onto the macrophage monolayer. 24 h later, cells were washed with PBS three times, fixed in 1% paraformaldehyde for 5 min, and stained with DAPI. Phagocytosis of tumor cells was detected using a fluorescent microscope (Nikon).

**Co-immunoprecipitation Assay**—Macrophages were treated with or without 1 mg/ml zymosan-A particles for 2 h. Cells were washed with ice-cold lysis buffer containing protease inhibitor mix (Sigma). The lysates were incubated with RGC-32, PKCa, or PKCe antibody (Abcam) for 1 h followed by incubation with protein G/G beads at 4 °C for 12 h. The immunoprecipitates were pelleted, washed, and subjected to immunoblotting.

**Western Blot**—Western blot was performed as described previously (20). Antibodies used were: anti-CD68 (Santa Cruz Biotechnology); anti-α-tubulin and anti-GAPDH (Sigma); anti-F4/80, anti-dectin-1, anti-iNOS, anti-arginase-1, anti-F-actin, anti-PKCα, and anti-PKCε (Abcam); and anti-Rab5, anti-Rab7, anti-phospho-MARCKS, and anti-MARCKS (Cell signaling).

**Quantitative PCR**—Quantitative PCR was performed as described previously (21). RGC-32 primers were: CGC CAC TTC CAC TAC GAG G (forward) and CAG CAA TGA AGG CTT CTA GCT C (reverse).

**Immunostaining**—Spleen tissue segments were cut by serial sectioning (5 μm). Immunostaining was performed as described previously using RGC-32 or MOMA-2 antibodies (Abcam) (20). For cultured cells, cells were seeded on cover glass slips in 24-well cell culture plates and incubated overnight. Immunostaining was performed using RGC-32 or F-actin antibody (20).

**Statistical Analysis**—Each experiment was repeated at least three times. All values are presented as means ± S.E. Comparisons between two groups were made by Student’s t test. Comparisons among multiple groups were made by one-way analysis of variance, and comparisons of different parameters between each group were made by a post hoc analysis using a Bonferroni’s test. p values <0.05 were considered to be statistically significant.

**RESULTS**

**RGC-32 Was Predominately Expressed in Differentiated Macrophages**—RGC-32 was initially reported as a complement-activated gene in oligodendrocytes (22), suggesting an immune-related function. However, its expression and function in macrophage lineage have not yet been reported. Thus, we first analyzed RGC-32 mRNA expression in THP-1 monocytes and PMA-induced macrophages from THP-1 cells. As shown in Fig. 1, A and B, RGC-32 mRNA was dramatically increased in PMA-induced macrophage. PMA also induced RGC-32 protein expression in THP-1 cells (Fig. 1, C and D). Interestingly, RGC-32 protein was expressed mainly in macrophages but undetectable in monocytes (Fig. 1, C and D). The PMA induction of macrophage differentiation was confirmed by the expression of macrophage marker CD68. In vivo, macrophages are differentiated from bone marrow hematopoietic progenitor cells (BMHPC). To test whether bone marrow-derived macrophage (BMDM) also expresses RGC-32, we isolated BMHPCs from 8-week-old male C56BL/6 mice and incubated the cells in RPMI 1640 medium containing 10% FBS and 15% L929 cell-conditioned medium for 7 days to allow macrophage differentiation. BMDM is confirmed by the expression of macrophage markers CD68 and F4/80. As shown in Fig. 1, E and F, RGC32 is mainly expressed in macrophage lineage but undetectable in the progenitor cells. To further test whether RGC-32 is expressed in mature macrophages in vivo, we detected the RGC-32-positive cells in mouse spleen via immunofluorescence staining. Spleen macrophages were stained with monocyte/macrophage marker MOMA-2 antibody. As shown in Fig. 1G, 90% of RGC-
32-expressing cells were MOMA-2-positive, suggesting that RGC-32 is strongly expressed in tissue macrophages.

RGC-32 Did Not Affect Monocyte-Macrophage Differentiation—Because RGC-32 is mainly expressed in macrophage, we supposed that RGC-32 regulates macrophage differentiation. However, by analyzing the peritoneal macrophage, we found that the numbers of peritoneal macrophages in RGC-32−/− mice were similar as compared with the wild type littermates (Fig. 2A), suggesting that RGC-32 may not be important for monocyte to macrophage differentiation. To confirm these
results, we induced the BMHPC isolated from both wild type and RGC-32 \(^{-/-}\) mice to differentiate to macrophage by culturing them in L929 cell-conditioned medium and found that RGC-32 \(^{-/-}\) did not alter the numbers of differentiated BMDM (Fig. 2B) or affect the expression of macrophage surface marker CD68 and dectin-1 (Fig. 2, C–E). Moreover, when RGC-32 was overexpressed or knocked down in PMA-treated THP-1 cells, it did not alter the expression of macrophage marker CD68 or dectin-1 in THP-1-derived macrophages (Fig. 2, F and G).

**Macrophages with RGC-32 Deficiency Exhibited an Attenuated M2 Polarization**—Depending on the local tissue environment, macrophages can undergo special differentiation and give rise to different subtypes, namely classically activated macrophage (M1 macrophage) and alternatively activated macrophage (M2 macrophage) (23, 24). Because RGC-32 did not impact monocyte differentiation to macrophage, we tested whether RGC-32 affects macrophage subtype polarization. GM-CSF and M-CSF are involved in the differentiation of monocytes to macrophages (24). GM-CSF can polarize monocytes toward the M1 macrophage subtype when treated with LPS and IFN-\(\gamma\) (25), whereas treatment with M-CSF and IL-4 will produce a macrophage profile similar to M2 macrophages (23, 24). To determine whether RGC-32 plays a role in macrophage polarization, we induced BMHPC from wild type or RGC-32 \(^{-/-}\) mice to M1 or M2 macrophage under different culture conditions. As shown in Fig. 3, A and B, RGC-32 \(^{-/-}\) increased the protein expression of iNOS, the hallmark marker of M1 macrophage. Mean-

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**FIGURE 3.** RGC-32 deficiency inhibited M2 macrophage polarization. A, RGC-32 deficiency (KO) promoted M1 macrophage marker iNOS expression in LPS/IFN-\(\gamma\)/G-CSF-treated bone marrow hematopoietic progenitor cells. B, quantification of iNOS expression shown in A by normalizing to \(\alpha\)-tubulin. C, RGC32 deficiency suppressed M2 macrophage marker arginase-1 expression in M-CSF- and IL-4-treated bone marrow hematopoietic progenitor cells. D, quantification of arginase-1 expression shown in C by normalizing to \(\alpha\)-tubulin. *, \(p < 0.01\) (\(n = 3\)).

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**FIGURE 4.** RGC-32 was essential for macrophage phagocytosis. A, RGC-32 deficiency (KO) in peritoneal macrophage (PM) attenuated phagocytosis of FITC-labeled zymosan-A particles as shown by flow cytometry analysis. B, overexpression of RGC-32 (Ad-RGC) increased while knockdown of RGC-32 (Ad-shRGC) decreased the phagocytosis of THP-1-derived macrophages as shown by flow cytometry analysis. C, RGC-32 overexpression increased THP-1-derived macrophage phagocytosis of PKH26-labeled Hep3D tumor cells. D, RGC-32 deficiency suppressed expression of phagosome markers Rab5 and Rab7 in bone marrow-derived macrophages. E, quantification of protein expression shown in D by normalizing to GAPDH. F, overexpression of RGC-32 increased while knockdown of RGC-32 decreased Rab5 expression in THP-1-derived macrophage. G, quantification of Rab5 protein expression shown in F by normalizing to GAPDH. *, \(p < 0.01\) (\(n = 3\)).
while, RGC-32$^{-/-}$ decreased the expression of arginase-1, the hallmark marker for M2 macrophage (Fig. 3, C and D). These results suggest that RGC-32 may inhibit macrophage M1 phenotype and promote M2 polarization in vitro.

RGC-32 Played an Important Role in Macrophage Phagocytosis—M1 macrophage is mainly involved in inflammatory response, whereas M2 macrophage is important for tissue repair including clearance of early apoptotic cells (7, 26–28). More importantly, M2 macrophage plays a major role in the clearance of exogenous pathogens and tumor cells. Because RGC-32$^{-/-}$ inhibited BMHPC-derived macrophages to express M2 phenotype marker (Fig. 3, C and D), RGC-32 may be critical for M2 macrophage-related functions. Therefore, we tested whether RGC-32 is involved in macrophage phagocytosis by adding FITC-labeled zymosan particles to peritoneal macrophage culture medium. Flow cytometry analyses showed that RGC-32$^{-/-}$ significantly reduced macrophage phagocytosis (Fig. 4A). To further determine its role in phagocytosis, we overexpressed or knocked down RGC-32 in THP-1-derived macrophages and used Texas Red-labeled zymosan particle to monitor the phagocytosis. As shown in Fig. 4B, knockdown of RGC-32 significantly decreased macrophage phagocytosis, whereas overexpression of RGC-32 increased the phagocytosis. Because macrophage phagocytosis is very important for tumor cell clearance, we co-cultured GFP-labeled THP-1-derived macrophage with PKH26-labeled human Hep3D liver carcinoma cells and found that overexpression of RGC-32 significantly increased macrophage phagocytosis and clearance of tumor cells (Fig. 4C). Because internalization of foreign particles initiates the formation and maturation of endosomes (16, 29), we monitored the formation of early and late endosomes by detecting the expression of early and late endosome marker Rab5 and Rab7 in macrophage after the phagocytosis, respectively. As shown in Fig. 4, D and E, RGC-32$^{-/-}$ dramatically suppressed both Rab5 and Rab7 expression after zymosan stimulation. Similarly, overexpression of RGC-32 increased while knockdown of RGC-32 suppressed Rab5 expression in THP-1-derived macrophages (Fig. 4, F and G). These data indicate that RGC-32 may also participate in endosome formation and maturation. Interestingly, RGC-32 altered Rab7 expression in primary macrophages (Fig. 4, D and E) but not in THP-1-derived macrophages (data not shown). This is probably due to the lack of factor(s) critical for Rab7 expression in THP-1 cells.

RGC-32 Co-localized with F-actin and Was Essential for F-actin Assembly during Macrophage Phagocytosis—Because RGC-32 did not impact phagocytosis receptor dectin-1 expression (Fig. 2, C and F), we sought to identify other mechanism underlying RGC-32 function in phagocytosis. Phagocytic cup formation is considered as the first step toward phagocytosis (30, 31), in which the F-actin assembly is the key regulatory event (32, 33). We found that F-actin assembly occurred on cell membrane 10 min after zymosan stimulation (Fig. 5). Interestingly, RGC-32 was recruited onto the cell membrane as early as 5 min after the zymosan stimulation, a time when F-actin assembly was not yet formed. Importantly, RGC-32 co-localized with F-actin on cell membrane (Fig. 5). These data suggest that RGC-32 may mediate the F-actin assembly on the cell membrane.

To test whether RGC-32 is indeed required for F-actin assembly during phagocytosis, we assessed F-actin formation/expression in THP-1 derived macrophages. As shown in Fig. 6, A and B, overexpression of RGC-32 increased while knocking...
down RGC-32 decreased F-actin expression in THP-1-derived macrophage. We also isolated peritoneal macrophage from RGC-32/H11002/H11002 mice and found that F-actin formation/expression was dramatically decreased in RGC-32/H11002/H11002 macrophage as compared with the wild type macrophage (Fig. 6, C and D). The decreased F-actin assembly occurred on the cell membrane of RGC-32/H11002/H11002 macrophage as shown by the phalloidin staining (Fig. 6E). These data demonstrate that RGC-32 plays a critical role in F-actin formation/assembling.

PKC Interacted with RGC-32 and Mediated RGC-32 Function in Phagocytosis—F-actin assembly during phagocytosis is regulated at different levels (32, 34, 35). PKC pathway has been reported to regulate F-actin assembly (36–38). Because the PKC activator PMA dramatically induced RGC-32 expression (Fig. 1, A–D), we hypothesized that RGC-32-mediated F-actin assembly involves PKC pathway. Indeed, we found that RGC-32 directly interacted with PKC. Zymosan stimulation dramatically increased their interaction (Fig. 7, A–C). RGC-32 appears to regulate PKC activity because RGC-32 deficiency (KO) significantly attenuated the phosphorylation of a PKC downstream target, myristoylated alanine-rich protein kinase C substrate (MARCKS), in both control and zymosan-treated macrophages (Fig. 7, D and E). MARCKS is an actin cross-linking protein that promotes F-actin assembly when it is phosphorylated by PKC (36, 39). To determine whether PKC activation is important for RGC-32-mediated phagocytosis, we used the PKC inhibitor Go6976 to block PKC activity and found that blockade of PKC activity suppressed RGC-32-induced F-actin expression in both THP-1-derived macrophages and BMDM (Fig. 7, F–I). These results clearly demonstrate that RGC-32 regulates macrophage phagocytosis by modulating PKC activity, which in turn promotes F-actin assembly.

DISCUSSION

We have found for the first time that RGC-32 is a novel regulator for the innate immune functions. RGC-32 was highly
induced and expressed in macrophage but not in monocyte. Although RGC-32 did not affect macrophage differentiation, it inhibited macrophage M1 phenotype and promoted M2 polarization. More importantly, RGC-32 significantly enhanced macrophage phagocytosis of both zymosan particles and tumor cells. During phagocytosis, RGC-32 appeared to be firstly recruited onto cell membrane where phagosomal cups were formed. Membrane RGC-32 then activated PKC and further stimulated F-actin assembly and facilitated foreign particle internalization (Fig. 8). Moreover, RGC-32 regulated phagosome/endosome formation as indicated by the expression of early and late endosome markers Rab5 and Rab7.

RGC-32 function in macrophage phagocytosis may be attributed to its role in regulating macrophage polarization. Because RGC-32$^{-/-}$ inhibited macrophages to express M2 phenotype marker, RGC-32 may be critical for M2 macrophage polarization. It has been shown that NF-κB signaling, a PKC downstream pathway, regulates M2 polarization (40, 41). RGC-32 may regulate M2 polarization through PKC-mediated NF-κB activation, which will be an interesting subject for future study.

Our study also uncovered a novel functional characteristic of RGC-32. The previously reported RGC-32 functions are pertinent to its cellular location in the cytoplasm or nuclei (21, 42, 43). However, we found in this study that RGC-32 also functions as a membrane-associated factor regulating signaling event in macrophage. Because RGC-32 is expressed in cytoplasm and nuclei of the macrophages as well (Fig. 5), it probably has other functions such as macrophage proliferation or cytokine production/secretion. In addition, the membrane-free RGC-32 may serve as an intercellular pool for membrane-associated RGC-32.

In summary, our study provided compelling evidences supporting that RGC-32 is a novel regulator for macrophage phagocytosis. RGC-32 regulates particle internalization through activation of PKC-induced F-actin assembly. Because M2 macrophage is critical for host defense of pathogen invasion and tumor cell recognition and cleavage during tumorigenesis, RGC-32 may be a potential novel target for modulating immune response.

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