Rac2-Deficient Murine Macrophages Have Selective Defects in Superoxide Production and Phagocytosis of Opsonized Particles

Akira Yamauchi, Chaekyun Kim, Shijun Li, Christophe C. Marchal, Jason Towe, Simon J. Atkinson and Mary C. Dinauer

J Immunol 2004; 173:5971-5979; doi: 10.4049/jimmunol.173.10.5971
http://www.jimmunol.org/content/173/10/5971

References This article cites 56 articles, 42 of which you can access for free at: http://www.jimmunol.org/content/173/10/5971.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Rac2-Deficient Murine Macrophages Have Selective Defects in Superoxide Production and Phagocytosis of Opsonized Particles

Akira Yamauchi, Chaeyun Kim, Shijun Li, Christophe C. Marchal, Jason Towe, Simon J. Atkinson, and Mary C. Dinauer

The Rho family GTPase Rac is a crucial participant in numerous cellular functions and acts as a molecular switch for signal transduction. Mice deficient in hematopoietic-specific Rac2 exhibited agonist-specific defects in neutrophil functions including chemotactic-stimulated filamentous actin polymerization and chemotaxis, and superoxide production elicited by phorbol ester, fMLP, or IgG-coated particles, despite expression of the highly homologous Rac1 isoform. In this study, functional responses of Rac2-null murine macrophages were characterized to examine whether Rac2 also has nonredundant functions in this phagocytic lineage. In contrast to murine neutrophils, in which Rac1 and Rac2 are present in similar amounts, Rac1 was ∼4-fold more abundant than Rac2 in both bone marrow-derived and peritoneal exudate macrophages, and macrophage Rac1 levels were unchanged by the absence of Rac2. Accumulation of exudate macrophages during peritoneal inflammation was reduced in rac2−/− mice. FcγR-mediated phagocytosis of IgG-coated SRBC was also significantly decreased in Rac2-null macrophages, as was NADPH oxidase activity in response to phorbol ester or FcγR stimulation. However, phagocytosis and oxidant production stimulated by serum-opsonized zymosan was normal in rac2−/− macrophages. Macrophage morphology was also similar in wild-type and Rac2-null cells, as was actin polymerization induced by FcγR-mediated phagocytosis or M-CSF. Hence, Rac2-null macrophages have selective defects paralleling many of the observed functional defects in Rac2-null neutrophils. These results provide genetic evidence that although Rac2 is a relatively minor isoform in murine macrophages, it plays a nonoverlapping role with Rac1 to regulate host defense functions in this phagocyte lineage.

assembly and migration, as do Rac2-null neutrophils; however, superoxide production is normal in contrast to Rac2-null counterparts (13). The above findings demonstrate that Rac1 and Rac2 play distinct roles in neutrophil functions.

The relative roles of the Rac1 and Rac2 isoforms in regulating macrophage responses are currently not well characterized. The majority of studies examining Rac in this phagocyte lineage have used activated or dominant-negative Rac1 derivatives (12, 14, 15, 23–26). However, because dominant-negative Rac1 derivatives act by sequestering exchange factors that could act on other Rac isoforms, their effects may not be specific to Rac1. In addition, almost all of these studies have been performed in cell lines rather than primary cells.

In our current study, we analyzed Rac2-null murine macrophages as a genetic approach to examine whether Rac2 has non-redundant functions in this phagocyte lineage. We found that Rac2 was ~4-fold more abundant than Rac2 and hence the predominant isoform in murine macrophages. However, Rac2-null macrophages exhibited defects in NADPH oxidase activation and phagocytosis in response to phorbol ester or IgG-opsinized particles and in accumulation of macrophages during peritoneal inflammation.

In contrast, phagocytosis and oxidant production elicited by serum-opsonized zymosan was similar in wild-type (WT) and Rac2-null macrophages, as was cell morphology and actin responses. These data suggest that although Rac2 is a relatively minor isoform, it plays a nonoverlapping and agonist-specific role to regulate selected functions in murine macrophages.

Materials and Methods

Abs, reagents, and buffers

A polyclonal Rac2 Ab raised in rabbits was a kind gift from G. Bokoch and U. Knaus (The Scripps Research Institute, La Jolla, CA). A mouse mAb against Rac1 was purchased from Upstate Biotechnology (Lake Placid, NY). Highly purified recombinant isoprenylated human Rac1 and Rac2 were kindly provided by E. Pick (Tel Aviv University, Tel Aviv, Israel) and R. Erickson and I. Cummuta (Genentech, South San Francisco, CA), respectively. PE-conjugated monoclonal rat anti-murine Mac-1 (CD11b), anti-murine FcγRII/III (CD16/CD32), anti-murine MHC class II (I-A/I-E), anti-murine Gr-1 rabbit and the isotype rat IgG2a were from BD Pharmingen (San Diego, CA). Anti-SRBC IgG was from ICN Pharmaceuticals (Irvine, CA). PBS (pH 7.2), dH2O, glycerol, cell dissociation buffer, RPMI 1640 medium, penicillin, streptomycin, and HEPES (125 mM, pH 7.5) were from Invitrogen Life Technologies (Grand Island, NY). FCS was from Hyclone (Logan, UT). Polyproprepe was purchased from Accurate Chemical and Scientific (Westbury, NY). Human M-CSF and murine IFN-γ were from PeproTech (Rocky Hill, NJ). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Buffers used in this study included PBS with 0.9 mM CaCl2, 0.5 mM MgCl2, and 7.5 mM glucose (PBS-G), 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100, 20 μg/ml chymostain, 2 mM PMSF, 10 μM leupeptin, and 1 mM 4-(2-aminoethyl benzenesulfonyl fluoride), and 5× MLB lysis buffer (125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 25 mM MgCl2, 25 mM EDTA, and 50% glycerol, 100 μg/ml chymostain, 10 mM PMSF). All reagents are endotoxin-free; Dishes (10 cm), 12-well plates and 96-well plates were from BD Labware, (Franklin Lakes, NJ), and eight-well chamber slides (Permanox) were from LAB-TEK (Nalge Nunc International, Rochester, NY).

Animals

C57BL/6d mice purchased from The Jackson Laboratory (Bar Harbor, ME) were used for WT controls. Rac2−/− mice had previously been generated by targeted disruption of the rac2 gene (16). Strains of rac2−/− mice used in this study had been backcrossed into C57BL/6d mice for over 10 generations. Mice were housed in microisolator cages under specific pathogen-free conditions and were fed autoclaved food and acidified water ad libitum. Both male and female mice of 8–12 wk of age were used in these experiments.

Isolation of macrophages

Murine peritoneal exudate macrophages (PEM) were harvested as described 96 h after i.p. injection of 5 mM sodium periodate (27). An aliquot was taken for cell counting and morphology by Diff-Quik staining of cytopsin. Peritoneal exudate cells were washed with chilled PBS twice and resuspended in RPMI 1640 containing 10% FCS, and plated. After 2 h incubation at 37°C, 5% CO2, nonadherent cells were removed and the remaining adherent cells were cultured in RPMI 1640 containing 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin for 48 h before functional assays. In some experiments, IFN-γ (final concentration, 20 ng/ml) and purified Escherichia coli LPS (to 10 ng/ml) were also added 18 h before harvest for immunoblot analysis of NO synthase 2 (NOS2) induction. In studies examining F-actin formation in response to M-CSF, serum was removed for 16 h before the assay.

To obtain murine bone marrow-derived macrophages (BMDM), a protocol for human BMDM was adapted to murine cells (28). Following lysis of RBC, murine bone marrow cells were resuspended in 10 ml of macrophage medium (IMDM containing 20% FCS, 50 ng/ml recombinant human M-CSF, 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) and cultured on 10-cm diameter dishes in 37°C, 5% CO2 atmosphere. Adherent cells were discarded twice after 4 and 16 h by transferring suspension cells to new dishes. At 7 days after harvesting, the medium was replaced with fresh. Cells that were still nonadherent were transferred to new dishes containing fresh media. At 12 days after harvesting bone marrow cells, adherent cells from all dishes were removed with cell dissociation buffer and pooled, spun down at 150 × g, resuspended in macrophage medium without M-CSF, and plated for 48 h before functional assays. In some experiments, IFN-γ (20 ng/ml) with or without LPS (10 ng/ml) were added 18–48 h before harvest.

Immunoblotting of macrophage cell lysates

Immunoblotting and Rac protein quantification using recombinant isoprenylated Rac1 and Rac2 were performed as previously described (18). For analysis of induction of NOS2 in PEM and in BMDM, macrophages were cultured with 20 ng/ml IFN-γ and 10 ng/ml LPS for 18 h before preparation of cell lysate for immunoblotting using an Ab specific for NOS2 (Santa Cruz Biotechnology, Santa Cruz, CA).

Flow cytometry for surface markers

Flow cytometry was used to examine surface markers expression on macrophages as described (17). Briefly, adherent macrophages were removed using cell dissociation buffer and gentle scraping, and placed in PBS containing 0.1% BSA. For analysis of IFN-γ induction of MHC class II, BMDM were cultured with 20 ng/ml IFN-γ for 48 h before harvest (29). Resuspended cells were incubated with 2 μg/ml PE-conjugated anti-mouse CD11b, FcγRII/III (CD32/CD16), MHC class II (I-A/I-E), Gr-1, or PE-conjugated anti-mouse IgG2b isotype (BD Pharmingen) for 30 min on ice. After washing cells twice with PBS containing 0.1% BSA, flow cytometric analysis was performed using a FACSscan (BD Biosciences, San Jose, CA). A minimum of 10,000 events were recorded and analyzed using CellQuest (BD Biosciences).

Nitrite assay

BMDM seeded at 1 × 106 cells per well in 96-well plates were cultured with IFN-γ (10–20 ng/ml) and LPS (0.1–10 ng/ml) for 18 h (30). NO2 in the supernatant was measured with the Griess reagent (Promega, Madison, WI).

Oxpsionization of particles

Ab-coated SRBC (IgG-SRBC) were freshly prepared in PBS by incubating SRBC (Cappel/ICN Biomedicals, Costa Mesa, CA) with a subagglutinating concentration of IgG rabbit anti-SRBC Ab (Cappel/ICN Biomedicals) as previously described (17). For opsonization of glass beads (2-μm diameter Glass Microsphere beads; Duke Scientific, Palo Alto, CA) with IgG, the protocol of Lennartz et al. (31) was used. Serum-opsonized zymosan was prepared as described (17).

Measurement of phagocytosis

Synchronized phagocytosis assays were performed as described, with slight modifications (32, 33). Briefly, PEM or BMDM in eight-well chamber slides at 1 × 105 cells/500 μl/well were placed on ice. The medium in
each well was replaced with 500 μl of 2 × 10^6/ml IgG-SRBC, unopsonized SRBC, or serum-opsonized zymosan, and the chambers spun at 100 × g for 5 min at 4°C. To initiate phagocytosis, supernatant was replaced with prewarmed DMEM (37°C), followed by incubation at 37°C, 5% CO₂ atmosphere. To stop phagocytosis, chamber slides were transferred onto ice. Unengulfed SRBC were lysed with cold water for 5 s, then washed after replacing water with cold PBS. Unengulfed serum-opsonized zymosan was removed by rinsing with chilled PBS. Ingested SRBC or serum-opsonized zymosan was counted under microscope at ×400 magnification field. Slides were stained with Diff-Quik and the percentage of cells undergoing phagocytosis was enumerated, and the phagocytic index was scored as the total number of ingested SRBC in 100 macrophages. Assessment of IgG-SRBC binding to macrophages was performed as described (34). IgG-SRBC were allowed to bind for 10 min on ice. Unbound IgG-SRBC were removed by three washes with chilled PBS, and slides were examined without hypotonic lysis to quantitate the percentage of macrophages with attached IgG-SRBC.

Analysis of macrophage morphology

PEM cultured overnight in RPMI 1640 without serum were allowed to adhere to microscope cover glasses coated with fibronectin fragment CH296 (25 μg/ml); Takara Bio, Shiga, Japan) in six-well culture plates for 15 min at 37°C. PEM were stimulated by replacing the medium with RPMI 1640 with 10 ng/ml M-CSF (or without M-CSF for unstimulated samples) for 60 s, then fixed with 3.9% paraformaldehyde in PBS for 10 min at room temperature. For some experiments PEM were plated as above, and cultured overnight in IMDM with 20% FCS overnight, and the culture medium was replaced with IMDM without serum, or IMDM with M-CSF (10 ng/ml) 15 min before fixation. Samples were permeabilized with 5% Triton X-100 in PBS for 5 min at room temperature, blocked with 5% BSA, 5% goat serum in PBS for 30 min, and incubated with 0.66 μM rhodamine phalloidin (Molecular Probes, Eugene, OR) for 1 h. Samples were imaged using a Zeiss LSM 510 laser scanning confocal microscope with a ×63 1.4 aperture oil-immersion objective. Overall morphology was evaluated by analysis of the images using Metamorph software (version 6.1; Universal Imaging, Brandywine, PA). Cell outlines were defined by automated segmentation, and the area and elliptical form factor (EFF, ratio of the longest chord through the cell to the caliper width perpendicular to that chord) determined using the Integrated Morphometry Analysis suit of tools. Similar results were obtained using PEM adhered to gelatin.

Measurement of F-actin

To monitor FcγR-stimulated F-actin formation, a previously published protocol for measuring FITC-phalloidin staining using a microplate reader (35) was adapted to flow cytometric analysis. Adherent PEM in 12-well plates were cultured on ice and spun with chilled PBS (1:100 cell ratio) at 50 × g, for 5 min at 4°C. Phagocytosis of IgG-SRBC was initiated by replacing the medium with prewarmed medium. At 5 min, the medium was replaced with 1 ml of chilled 4% paraformaldehyde in PBS for 15 min. Cells were then scraped off, transferred to 1.5-ml tubes, and incubated another 15 min at room temperature before washing with PBS containing 0.1% BSA and staining with 160 nM FITC-labeled phalloidin and PE-conjugated anti-CD11b Ab. Cells were washed and resuspended in PBS containing 0.1% BSA and the fluorescence was measured using FACS Calibur (BD Biosciences). Data were analyzed by CellQuest software (BD Biosciences) and mean cellular fluorescence (MCF) of FITC-phalloidin staining of CD11b-positive cells was compared using Microsoft Excel software. For measurement of M-CSF-stimulated F-actin formation, serum-starved PEM in 12-well plates were stimulated by the addition of prewarmed (37°C) 10 ng/ml M-CSF in PBS-G. After 1 min, the medium was replaced with 4% paraformaldehyde, and cells stained and analyzed as above.

Measurement of NADPH oxidase activity

A chemiluminescent method for detecting PMA-stimulated superoxide production using isoluminol was performed as previously described (18, 36). For reactive oxygen species (ROS) production by BMDM, the medium on a 35-mm dish was replaced with prewarmed buffer (0.1 mM l-ascorbate and 0.03% BSA in PBS-G) with or without 200 ng/ml PMA, and incubated at 37°C. Chemiluminescence was measured over a 5-s interval every 5 min using TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). For adherent PEM, the medium on 96-well plate was replaced with PBS-G containing 100 μM isoluminol and 4 U/well HRP and warmed at 37°C for 10 min before adding PMA (0.2 μg/ml final concentration) or PBS-G. Chemiluminescence was monitored over a 30-min period using an Lmax plate reader (Molecular Devices, Sunnyvale, CA) and SOFTMax Pro software (Molecular Devices). Unstimulated cells plated in parallel were treated with 1 N NaOH overnight and protein concentration was determined based on BSA standards by BCA protein assay kit (Pierce, Rockford, IL). Signals reflecting superoxide production were normalized as relative fluorescent unit per milligram total cellular protein. Isoluminol chemiluminescence was not detected in unstimulated macrophages or in PMA-stimulated macrophages from X-linked chronic granulomatous disease mice (our unpublished observations).

IgG-BSA immune complex-elicted ROS production in response to FcOxyBurst reagent (Molecular Probes) was measured by flow cytometry following the manufacturer’s recommended protocol. Adherent PEM on 10-cm diameter plate were detached using cell dissociation buffer, washed with PBS, resuspended, and OxyBurst reagent added, followed by incubation for 2 h at 37°C or on ice. Fluorescence related to oxidation of FcOxyBurst was assayed by flow cytometry.

Homovanillic acid (HVA) fluorescence was used to detect phagocytosis-associated hydrogen peroxide production as described (37, 38). Adherent PEM in 12-well plates were incubated with HVA assay solution (100 μM HVA, 4 U/ml HRP in PBS-G) with or without stimulant (1 × 10⁵ particles/ml IgG-coated glass beads, or 0.125 mg/ml serum-opsonized zymosan). Wells containing varying hydrogen peroxide concentrations were run in parallel to construct a standard curve. After 30 min incubation at 37°C with 5% CO₂, 500 μl of supernatant was transferred to an Eppendorf tube containing 75 μl of stop solution (glycine-EDTA buffer, pH 12), followed by a brief spin to pellet any debris. Then 200 μl was transferred to black wall and bottom 96-well plate and fluorescence was measured by SpectraMax Gemini EX fluorometer (Molecular Devices). Unstimulated cells plated in parallel were treated with 1 N NaOH overnight and protein concentration was determined as above. Hydrogen peroxide production was calculated as nanomoles H₂O₂ per milligram total cellular protein. No hydrogen peroxide production was detected by this assay in IgG-SRBC or serum-opsonized zymosan stimulated X-linked chronic granulomatous disease macrophages.

Measurement of Rac levels

Adherent PEM were stimulated with 1 ng/ml recombinant Rac1 and Rac2 used as standard (0, 20, and 40 ng/lane) from at least five experiments. Blots were probed with monoclonal anti-Rac1 or polyclonal anti-Rac2 Ab. Rac2^−/− (-/-) and rac2^+/+ (+/+) are shown. Note that the Ab for Rac2 has some cross-reactivity with Rac1 (16), which accounts for the faint bands seen in rac2^−/− macrophage extracts probed for Rac2. B, Rac1 and Rac2 levels were estimated by densitometry of immunoblots of macrophage extracts and several dilutions of isoprenylated Rac standards. Mean ± SEM is shown. **p < 0.0001, *p < 0.005 (Rac1 vs Rac2) for n ≥ 5 by unpaired t test.

**FIGURE 1.** Expression Rac1 and Rac2 in neutrophils and in exudate and BMDM. A, Representative immunoblot of human and murine polymorphonuclear neutrophils, murine BMDM and PEM, and isoprenylated recombinant Rac1 and Rac2 used as standard (0, 20, and 40 ng/lane) from at least five experiments. Blots were probed with monoclonal anti-Rac1 or polyclonal anti-Rac2 Ab. rac2^−/− (-/-) and rac2^+/+ (+/+) are shown. Note that the Ab for Rac2 has some cross-reactivity with Rac1 (16), which accounts for the faint bands seen in rac2^−/− macrophage extracts probed for Rac2. B, Rac1 and Rac2 levels were estimated by densitometry of immunoblots of macrophage extracts and several dilutions of isoprenylated Rac standards. Mean ± SEM is shown. **p < 0.0001, *p < 0.005 (Rac1 vs Rac2) for n ≥ 5 by unpaired t test.
FIGURE 2. Exudate formation and MHC class II expression in WT and Rac2-null murine macrophages. A, Sodium periodate-elicited peritoneal exudate formation in WT and rac2<sup>−/−</sup> mice. The numbers of mononuclear phagocytes in peritoneal exudate 96 h after challenge with sodium periodate were determined by counting and by examining differential in cytopsin preparations. Mean ± SEM is shown. *p < 0.05 (rac2<sup>−/−</sup> vs rac2<sup>+/+</sup>) by unpaired t test (n = 11). B, MHC class II (I-A/I-E) expression on BMDM. Representative histograms of MHC class II staining of WT and rac2<sup>−/−</sup> BMDM from three or more experiments, as determined by flow cytometry are shown. The filled histogram, the open histogram with solid line, and the open histogram with dotted line show isotype control, without IFN-γ treatment, and following IFN-γ treatment (20 ng/ml) for 48 h, respectively.

Statistical analysis
The two-tailed Student t test (either paired or unpaired, as indicated) was performed using Microsoft Excel software (Redmond, WA).

Results

Rac1 is the predominant isoform in BMDM and exudate murine macrophage

We first compared the expression of Rac isoforms in WT Rac2-null macrophages in cell lysates with serial dilutions of purified recombinant isoprenylated human Rac1 and Rac2 (Fig. 1A). In human neutrophils, Rac2 is the predominant isoform, whereas, in murine neutrophils, Rac1 and Rac2 are expressed in similar amounts (10, 18) (see also Fig. 1A). Rac3 has not been detected by RT-PCR in murine macrophages (39) or murine and human neutrophils (see Ref. 18). Rac2 expression in both WT PEM and BMDM was ∼4-fold lower than Rac1, and expression of the Rac1 isoform in Rac2-null PEM and BMDM was not significantly different from WT counterparts (Fig. 1B). These results indicate that Rac1 is the predominant isoform in murine macrophages and that genetic deletion of Rac2 is not associated with up-regulation of Rac1 expression in PEM and BMDM.

PEM are decreased in number in Rac2-null mice

Because Rac2 is required for normal accumulation of neutrophils in the early phases of exudate formation in vivo (16), we compared exudate formation in WT with Rac2-null mice at 96 h following i.p. injection of sodium periodate, a time when macrophages are the predominant inflammatory cell. In both WT and rac2<sup>−/−</sup> mice, almost all mononuclear exudate cells were macrophages as assessed by morphology. The percentage of mononuclear cells was not significantly different in Rac2-null mice compared with WT (91.1 ± 0.1% vs 87.4 ± 0.1%; mean ± SD, n = 11). However, the total number of exudate cells in Rac2-null mice was decreased compared with WT mice (4.4 ± 2.2 × 10<sup>6</sup> per mouse vs 6.6 ± 2.9 × 10<sup>6</sup>, respectively; mean ± SD, n = 11–15, p < 0.05). Thus, the number of exudate macrophages recovered from Rac2-null mice was decreased by approximately one-third compared with WT mice (Fig. 2A), which suggests that Rac2 plays a nonredundant role in macrophage accumulation in vivo during peritoneal exudate formation.

Culture of BMDM, cell surface Ag expression and morphology of exudate and BMDM are similar in WT and rac2<sup>−/−</sup> mice

There was no difference in the number of macrophages generated from WT and rac2<sup>−/−</sup> bone marrow in the presence of M-CSF (2.5 ± 0.57 × 10<sup>6</sup> per mouse vs 2.6 ± 0.11 × 10<sup>6</sup>, respectively; mean ± SD, n = 11). Expression of cell surface markers (CD11b, FcyRII/RIII, and MHC class II) was analyzed by flow cytometry in exudate and BMDM from WT and rac2<sup>−/−</sup> mice. Both the fraction of cells expressing these markers (Table I) and the staining intensity (data not shown) were similar in both genotypes. Treatment with IFN-γ for 48 h enhanced MHC class II expression in both WT and Rac2-null BMDM to a similar degree (Table I and Fig. 2B). The induction of NOS2, as assessed by immunoblotting for NOS2 expression in PEM and BMDM and by production of nitrite in BMDM was also similar in WT and Rac2-null cells (data not shown).

No differences were observed in the morphologic appearance of WT and Rac2-null BMDM (data not shown) and exudate macrophages (Fig. 3A). Spreading, ruffling, and lamellipodia formation...
were similar in macrophages of both genotypes cultured in the presence of serum (Fig. 3A, bottom panels) or upon stimulation with M-CSF following overnight serum starvation (Fig. 3A, top and middle panels). No difference between the genotypes in adhesive area or in the overall shape of the cells was observed. Because Rac1-deficient macrophages have been observed to have a more elongated morphology than WT (19), we made quantitative measurements of EFF, a measure of whether the cells are roughly round (low EFF) or elongated (high EFF), of WT and Rac2-null PEM. No significant difference was observed between M-CSF-stimulated serum-starved WT (EFF = 1.8 ± 0.8, n = 50) or Rac2-null (EFF = 2.1 ± 0.9, n = 150) cells, or between WT (EFF = 2.0 ± 0.8, n = 6) or Rac2 null (EFF = 1.6 ± 0.7, n = 6) cells cultured overnight in serum.

Rac2-null macrophages have decreased ingestion of IgG-opsonized but not serum-opsonized particles

Because macrophages are professional phagocytes and contribute to clearance of pathogens by their ingestion, we examined the phagocytic capability of Rac2-null PEM and BMDM using IgG-SRBC and serum-opsonized zymosan (Fig. 4). The fraction of cells undergoing phagocytosis of IgG-SRBC was similar in WT and Rac2-null macrophages, and phagocytosis by WT macrophages peaked by 10 min (data not shown). However, Rac2-null macrophages contained fewer IgG-SRBC at 10 min, resulting in a phagocytic index that was 50–65% of WT macrophages (Fig. 4A). A longer incubation of Rac2-null macrophages with IgG-SRBC (up to 30 min) did not further increase the phagocytic index (data not shown). Cell surface expression of FcγR, as measured by fluorescence staining intensity, was not significantly different between WT and rac2−/− PEM and BMDM (data not shown). The attachment of IgG-SRBC to WT and rac2−/− PEM was also similar (82 ± 3% vs 81 ± 3% of cells had bound IgG-SRBC, respectively; mean ± SD, n = 3). Thus, reduced receptor expression or binding of IgG-SRBC does not account for the decrease in IgG-SRBC phagocytosis by rac2−/− macrophages. As expected, binding and phagocytosis of unopsonized SRBC was much lower (50- and ~1000-fold, respectively) than IgG-coated SRBC in both WT and Rac2-null BMDM (data not shown). In contrast to results with IgG-coated particles, serum-opsonized zymosan phagocytosis was similar in Rac2-null and WT macrophages (Fig. 4B).

F-actin formation in response to IgG SRBC and to M-CSF is normal in Rac2-null macrophages

Rac plays an important role in regulating actin formation in many cells, and actin polymerization is important in the early phases of phagocytosis (40, 41). Defects in F-actin formation in response to chemotactants are also a prominent feature of the Rac2-null neutrophil phenotype (16, 18). We thus examined the increase in macrophage F-actin during actin cup formation after initiation of phagocytosis of IgG-SRBC (42). However, the F-actin content of Rac2-null PEM undergoing phagocytosis was similar to WT PEM (Fig. 3B). We were unable to detect an increase in F-actin over background in WT PEM in response to either PMA or RANTES (data not shown). M-CSF has been shown to stimulate F-actin formation and ruffling in macrophages in a Rac-dependent manner (12, 26, 39). Although both the basal and M-CSF-stimulated levels were modestly reduced in Rac2-null PEM compared with WT, Rac2-null macrophages exhibited a similar relative increase in F-actin in response to M-CSF (Fig. 3C), consistent with the similar degree of ruffling and lamellipodia noted morphologically in both genotypes upon M-CSF stimulation (Fig. 3A).

Rac2-null macrophages have defects in both phorbol ester- and FcγR-elicited oxidant production

Macrophages play an important role in innate immunity for pathogen killing by producing ROS via the NADH oxidase. We compared ROS production from Rac2-null PEM and BMDM to WT counterparts as stimulated by PMA or by phagocytic receptor agonists, PMA-elicited ROS production from both Rac2-null PEM and BMDM was substantially decreased (2- to 3-fold) compared with WT (Fig. 5). BSA-IgG immune complex elicited superoxide production in Rac-null PEM was almost undetectable compared with WT (Fig. 5A). ROS production elicited by ingestion of IgG-opsonized particles by Rac2-null PEM was also significantly reduced (Fig. 6B). However, serum-opsonized zymosan elicited ROS production was equivalent in WT and Rac2-null PEM (Fig. 6C).

Discussion

We report that although Rac1 is the predominant isoform in murine macrophages and is present in ~4-fold excess of Rac2, Rac2 is essential for normal regulation of superoxide production, phagocytosis, and macrophage accumulation at inflamed sites in vivo. This is the first study to identify a role for Rac2 in these macrophage functions, and supports the concept that each of the two closely related Rac isoforms contributes to regulating biologic responses of phagocytes.

The abundance of Rac1 in BMDM and exudate macrophage, on a per milligram protein basis, is similar to the amount of Rac1 in murine neutrophils (18), whereas Rac2 expression in macrophages is much lower. Wells et al. (39) also recently reported that murine BMDM had 3-fold more Rac1 protein compared with Rac2, and Rac1 is the predominant isoform in human monocytes (43). These results contrast with murine neutrophils, which have similar amounts of Rac1 and Rac2 (18), and human neutrophils, which have predominantly Rac2 (10). Thus, the expression ratio of Rac isoforms depends on species and cell type, with Rac1 being the predominant isoform at the protein level in both human and murine mononuclear phagocytes. We also found that the Rac1 expression in Rac2-null macrophages was not affected by the deletion of rac2 gene, which suggests that Rac1 and Rac2 expression is regulated independently in murine macrophages, in contrast to findings in murine mast cells (44).

Table I. Cell surface Ag expression in wild-type and Rac2-null BMDM and PEM

<table>
<thead>
<tr>
<th></th>
<th>rac2+/+ (%)</th>
<th>No. of Mice</th>
<th>rac2−/− (%)</th>
<th>No. of Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMDM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>78.0 ± 16.6</td>
<td>5</td>
<td>79.4 ± 11.2</td>
<td>3</td>
</tr>
<tr>
<td>FcγRII/III</td>
<td>74.1 ± 13.2</td>
<td>4</td>
<td>71.8 ± 14.3</td>
<td>3</td>
</tr>
<tr>
<td>MHC class II</td>
<td>12.7 ± 10.2</td>
<td>4</td>
<td>23.8 ± 13.2</td>
<td>3</td>
</tr>
<tr>
<td>MHC class II (IFN-γ treatment)</td>
<td>58.6 ± 6.5*</td>
<td>4</td>
<td>64.0 ± 2*</td>
<td>3</td>
</tr>
<tr>
<td>Gr-1</td>
<td>0.02 ± 0.05</td>
<td>4</td>
<td>0.1 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>PEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>94.1 ± 1.7</td>
<td>4</td>
<td>92.8 ± 3</td>
<td>4</td>
</tr>
<tr>
<td>FcγRII/III</td>
<td>93.3 ± 1.9</td>
<td>4</td>
<td>93.0 ± 1.7</td>
<td>4</td>
</tr>
<tr>
<td>MHC class II</td>
<td>63.0 ± 7.7</td>
<td>4</td>
<td>76.5 ± 10.8</td>
<td>4</td>
</tr>
<tr>
<td>Gr-1</td>
<td>0.5 ± 0.9</td>
<td>4</td>
<td>1.1 ± 0.9</td>
<td>4</td>
</tr>
</tbody>
</table>

* Surface Ag expression on BMDM cultured for 14 days was evaluated by flow cytometry using specific Abs. Surface Ag expression on adherent PEM was also evaluated by flow cytometry of PEM 48 h after harvesting from peritoneal exudate. For MHC class II molecule expression, cells were treated with or without 20 ng/ml IFN-γ for 48 h, followed by flow cytometric analysis. Data for the percentage of cells expressing the indicated surface Ag are shown as mean ± SD. The unpaired t test was used to compare data. There was no significant difference between the rac2+/+ and rac2−/− macrophages in any of the parameters listed above.

* P ≤ 0.005 (no IFN-γ treatment vs treatment).
The accumulation of macrophages at an inflamed site in vivo was diminished in Rac2-null mice, similar to findings for Rac2-null neutrophils, which are present in reduced numbers in the acute phase of the inflammatory response (16). Pradip and coworkers recently reported that Rac2-null BMDM plated on matrix proteins in vitro have a marked decrease in haptotaxis directed by binding to either $\alpha_5\beta_1$ or $\alpha_4\beta_1$ integrin receptors (45), which may contribute to decreased migration in vivo.

FcγR-stimulated phagocytosis and NADPH oxidase activation were also defective in Rac2-null macrophages, in contrast to phagocytosis and production of reactive oxidants elicited by serum-opsonized zymosan particles, which can bind to both the $\beta_2$ integrin receptor Mac-1 (CD11b/CD18) and FcγR (46, 47). These data suggest that Rac2 is selectively required for signaling events downstream of FcγR ligation, but not for responses to Mac-1, and that Rac1 is unable to serve as a replacement. These findings are similar to observations in Rac2-null neutrophils (17). The agonist selectivity of phagocytic defects in Rac2-null macrophages is also consistent with studies showing that Rac and Cdc42 GTPases participate in FcR-mediated phagocytosis, but not ingestion via the Mac1 receptor (12, 14, 15), where instead Rho appears to play an important role (14, 48).

Previous studies using dominant-negative derivatives of Rac and Cdc42 indicate that each contributes to actin remodeling and coordination of membrane extension leading to particle engulfment upon Fc receptor ligation (12, 14, 15, 24). We found that Rac2 was not absolutely required for phagocytosis of IgG-opsonized particles, but the number of particles ingested was reduced overall by ~2-fold in Rac2-null macrophages. However, the increase in F-actin content during particle ingestion was similar to WT macrophages, suggesting that the Rac1 isoform is sufficient for mediating Rac-dependent actin assembly during FcγR-induced phagocytosis. Rac2 must play a role distinct from Rac1 in regulating additional events that enhance the ingestion of IgG-opsonized particles. These may include events associated with phagosome closure and/or the membrane remodeling and vesicle trafficking, which accompany particle ingestion (49).
proteins (50). The agonist-selective dependence of NADPH oxidase activity on Rac2 in whole cells could reflect a nonredundant usage of this isoform for incorporation into the oxidase complex or for regulating events upstream of oxidase assembly in the context of specific signaling pathways. In the absence of Rac2, at least some Rac1 is presumably incorporated into the NADPH oxidase complex to support residual enzymatic activity with these agonists, and murine neutrophils with a combined deficiency of both Rac1 and Rac2 have extremely low superoxide production (51).

Rac1 was recently suggested to function as the main isoform for superoxide production in human monocytes, because it translocates to the membrane following activation with either serum-opsonized zymosan or phorbol ester and interacts with the p47phox and p67phox NADPH oxidase subunits (43). This result is consistent with the normal oxidant production we observed for serum-opsonized zymosan-stimulated Rac2-null macrophages. Although our studies suggest that Rac2 plays a role in phorbol ester-stimulated superoxide production by murine phagocytes, Rac2 was not detected in human monocyte membranes following phorbol ester stimulation (43). This may reflect the small amounts of Rac2 present in human monocytes; alternatively, these cells may differ from murine macrophages in the Rac isoforms used in the phorbol ester-stimulated respiratory burst.

The molecular mechanisms that account for the inability Rac1 to compensate in certain responses of Rac2-null macrophages are likely to reflect isoform-specific differences in subcellular localization, the relative affinities with interacting proteins, or both. The largest region of sequence divergence between Rac1 and Rac2 in involves sequences in the C terminus adjacent to the prenylation site. This domain has been shown to confer differential localization of Rac1 and Rac2 to membrane domains in fibroblasts and epithelial cells (52), as well as to modulate interactions with at least one downstream target of Rac GTPases, the serine-threonine kinase PAK1 (53). Rac1 and Rac2 have an identical switch 1 domain, which is a critical site of interaction with both guanine nucleotide exchange factors and downstream effector proteins (54). Comparison

**FIGURE 4.** Phagocytosis of PEM and BMDM using IgG-opsonized SRBC and serum-opsonized zymosan. The phagocytic index was scored as the number of ingested particles per 100 macrophages after 10 min of synchronized phagocytosis. Phagocytic index for BMDM (left panels) and phagocytic index for PEM (right panels) are shown. Mean ± SEM is shown. A, Phagocytosis of IgG-opsonized SRBC. ***, p < 0.005 (rac2 (-/-) vs rac2 (+/+)), unpaired t test (n = 8 for BMDM and n = 4 for PEM). B, Phagocytosis of serum-opsonized zymosan (n = 4 for both BMDM and PEM) is shown.

**FIGURE 5.** Phorbol ester-elicited NADPH oxidase activity in PEM and BMDM. NADPH oxidase activity was measured using an isoluminol chemiluminescence assay. Mean ± SEM is shown. A, NADPH oxidase activity in PEM stimulated for 30 min with PMA. Values represent integrated relative luminescent units (RLU) for 30 min per one milligram of cellular protein. ***, p < 0.05 (rac2 (-/-) vs rac2 (+/+)), unpaired t test (n = 5). B, NADPH oxidase activity in BMDM stimulated for 20 min with PMA. Values shown are the relative luminescent units (RLU) for 5 s per one milligram of cellular protein at 20 min, which were similarly reduced in Rac2-null macrophages throughout the entire incubation period compared with WT (data not shown). ***, p < 0.02 (rac2 (-/-) vs rac2 (+/+)) for unpaired t test (n = 4).
distinct role of Rac2 isoform in murine macrophages

FIGURE 6. Oxidant production by exudate macrophages stimulated via the FcγR or complement receptor 3. A. IgG-BSA immune complex—elicited superoxide from PEM was measured by fluorescent Fc OxyBurst reagent using flow cytometry. The values represent the MCF 30 min after stimulation. Error bars represent SEM. *, p < 0.05 (rac2+/− vs rac2+/+), B and C, IgG-opsonized glass beads (B) or serum-opsonized zymosan (C) elicited ROS from PEM was measured by fluorescent HVA method. The numbers indicate the amount of 
H2O2 (nanomoles) from PEM samples. Unstimulated macrophages (given PBS only) assayed in parallel, released 2.5 ± 3.1 and 2.2 ± 3.2 nM H2O2/mg of protein (rac2+/− and rac2+/+, respectively). Error bars represent SEM. *, p < 0.02 (rac2+/− vs rac2+/+) for unpaired t test (n = 7) (B), or (n = 3) (C).

of Rac1 and Rac2 activation in chemoattractant-stimulated murine neutrophils supports the concept that these two isoforms have both differential activation and signaling profiles (18). We are currently investigating whether Rac isoforms are also differentially activated in macrophages, but demonstrating Rac2 activation has been technically challenging due to the small amount of Rac2 present in these cells (our unpublished observations).

In contrast to the phenotypic defects described above, other macrophage responses regulated by signaling cascades in which Rac has been implicated were unaffected by deletion of Rac2. We did not observe morphologic differences between cultured WT and Rac2-null macrophages. In contrast, Rac1-null BMDM, obtained from mice with a conditional Rac1 allele deleted by an IFN-inducible Cre transgene, exhibited a distinctive elongated morphology when cultured on tissue culture plastic (39). Rac1-null macrophages also had decreased spreading and actin ruffling in response to M-CSF, but random migration and M-CSF-induced chemotaxis in vitro were unaffected (39). The altered morphology upon deletion of Rac1, but not Rac2, could indicate a distinct role for Rac1 in regulation of macrophage cell shape, or simply reflect the effects of a marked reduction in overall cellular Rac content in Rac1-null macrophages. Expression of the D57N dominant-negative derivative of Rac2 in WT murine BMDM also produced an elongated morphology (25). Because macrophages lacking Rac2 did not exhibit these changes in this current study, it likely is that the Rac2D57N effect on morphology is mediated primarily by sequestering guanine nucleotide exchange factors that activate Rac1. This is also consistent with the lack of evidence that dominant negative forms of Rac are specific for a given isoform, and illustrates the importance of using genetic deletion to examine specific contributions of Rac isoforms.

M-CSF (CSF-1) is a primary regulator of proliferation and differentiation of mononuclear phagocytes and its receptor signals to many downstream intracellular signaling molecules including Vav, a guanine nucleotide exchange factor for Rac, and the p85 subunit of PI3K (55). Although these molecules are potential upstream regulators of Rac GTPases, our data showed that there was no significant difference between WT and Rac2-null macrophages in proliferation and differentiation of BMDM by M-CSF, as was also reported for Rac1-null BMDM (39). Thus, neither of these two Rac isoforms appears to play a nonredundant role in these responses to M-CSF. In addition, although IFN-γ has been reported to activate Vav1 in murine macrophages (56), IFN-γ-induced NOS2 and MHC II class expression were normal in Rac-2 null macrophages.

In conclusion, using mice with a genetic deletion of the hematopoietic-specific Rac2 isoform, we show that Rac2 plays a nonoverlapping and agonist-specific role to regulate murine macrophage functions, although it is a minor isoform compared with Rac1. Although Rac2 is dispensable for ingestion and oxidant production elicited by serum-opsonized particles, actin responses, and maintaining normal macrophage morphology, this isoform is essential for normal accumulation of macrophages during peritoneal inflammation, FcγR-mediated phagocytosis, and oxidant production elicited by phorbol ester or FcγR stimulation.

Acknowledgments

We greatly thank Natalie Stull for taking care of mice used and Shari Upchurch for managing preparation of this manuscript.

References