



# Rac1 Deletion in Mouse Neutrophils Has Selective Effects on Neutrophil Functions<sup>1</sup>

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Defects in myeloid cell function in Rac2 knockout mice underline the importance of this isoform in activation of NADPH oxidase and cell motility. However, the specific role of Rac1 in neutrophil function has been difficult to assess since deletion of Rac1 results in embryonic lethality in mice. To elucidate the specific role of Rac1 in neutrophils, we generated mice with a conditional Rac1 deficiency restricted to cells of the granulocyte/monocyte lineage. As observed in Rac2-deficient neutrophils, Rac1-deficient neutrophils demonstrated profound defects in inflammatory recruitment in vivo, migration to chemotactic stimuli, and chemoattractant-mediated actin assembly. In contrast, superoxide production is normal in Rac1-deficient neutrophils but markedly diminished in Rac2 null cells. These data demonstrate that although Rac1 and Rac2 are both required for actin-mediated functions, Rac2 is specifically required for activation of the neutrophil NADPH oxidase. *The Journal of Immunology*, 2003, 170: 5652–5657.

The Rho family of small GTPases consists of three major members: Cdc42, Rac, and Rho (1). Within the Rac subclass there are three members; Rac1, Rac2, and Rac3 (2). Rac1 is ubiquitously expressed while Rac2 expression is restricted to cells of the hemopoietic lineage. Rac3, the most recently described Rac isoform, is expressed in brain, lung, liver, and pancreas (2) but not in neutrophils (U. Knauss, unpublished observations). Although Rac2 is the predominant isoform in human neutrophils (3), murine neutrophils express similar amounts of Rac1 and Rac2 (4). Rac1 and Rac2 share 92% amino acid identity with the major divergence occurring in the C terminus. Importantly, this region determines the subcellular localization and interactions of Rac with some downstream effector proteins (5, 6).

Racs are known to be key regulators of the actin cytoskeleton and of the NADPH oxidase system in neutrophils (7–10). Recent studies using Rac2-deficient mice and neutrophils from a patient with a naturally occurring mutation in Rac2 have demonstrated that this isoform is a key regulator of several antimicrobial functions including dynamic alterations of the actin cytoskeleton required for cell migration, cell shape, and generation of reactive oxygen species by the NADPH oxidase complex (11–14). However, the specific role of Rac1 in neutrophils remains obscure. The

high degree of homology in the effector regions of Rac1 and 2 has led to the hypothesis that these two proteins function interchangeably. Furthermore, in in vitro cell-free assays of the NADPH oxidase, prenylated Rac1 and Rac2 are indistinguishable. Using purified neutrophil membranes and recombinant Rac1 and Rac2, Heyworth et al. (10) demonstrated that both isoforms have equal activity in reconstitution of superoxide production, although Rac2 was more efficient in the presence of neutrophil cytosol. In permeabilized human neutrophils, Rac1 and Rac2 activated actin assembly similarly and catalytically inactive forms of Rac1 and Rac2 were equally effective at inhibiting fMLP-mediated actin assembly (15). However, the conditions used for all these in vitro systems may not replicate conditions present in intact cells.

Recent studies using Rac2-deficient neutrophils suggest that Rac1 and Rac2 have discrete functions inasmuch as activation and signaling profiles for each isoform in intact neutrophils are unique (4). In fMLP-activated murine neutrophils, 4-fold more Rac2 is activated compared with Rac1. In addition, using Rac2 null and Rac2 heterozygous mice, Li et al. (4) demonstrated that the level of activated Rac2 is rate limiting for fMLP-induced F-actin polymerization, chemotaxis, and superoxide generation (4). However the role of Rac1 in regulating neutrophil functions has not been evaluated. Investigation of the specific roles of these two Rac isoforms and specifically Rac1 in primary neutrophils requires an alternative model to the in vitro models used previously.

Targeted gene disruption in the mouse is an alternate approach to elucidate the distinct functions of Rac1 and 2, because in theory each gene can be disrupted individually. However, Rac1 deficiency results in embryonic lethality (16). To examine the specific role of Rac1 in neutrophil function, we generated mice in which the *rac1* gene is selectively disrupted in cells of granulocyte/monocyte lineage. As described herein, this was accomplished using a conditional (“floxed”) allele of *rac1* and a mouse expressing the Cre recombinase under the control of a neutrophil/monocyte-specific promoter (Lysozyme M). We demonstrate here for the first time that Rac1 null neutrophils have significant defects in inflammatory recruitment in vivo, migration to chemotactic stimuli, and chemoattractant-mediated actin assembly. However, superoxide production is normal in Rac1-deficient neutrophils in contrast to

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Received for publication December 12, 2002. Accepted for publication March 20, 2003.

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<sup>1</sup> This work was supported by Canadian Institutes of Health Research grants (to M.G. and G.P.D.) and National Institutes of Health Grants HL54188 (to D.J.K.), ROI HL45635 (to M.D.), and POI HL069974 (to M.D.). M.G. is a Canadian Institutes of Health Research Clinician Scientist.

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Rac2 null cells. These new data, combined with comparisons to Rac2 null mice, are the first demonstration in functional primary neutrophils that Rac2 is specifically required for superoxide generation in neutrophils, whereas Rac1 and Rac2 are both important regulators of actin-mediated neutrophil functions.

## Materials and Methods

### Gene targeting in embryonic stem (ES)<sup>3</sup> cells

A 10-kb fragment of the murine *Rac1* gene containing the first exon was isolated from a  $\lambda$  phage library made from J1 ES cell DNA (Fig. 1). This fragment was cloned and engineered as shown to contain a loxP sequence inserted at a *SpeI* site and a loxP-flanked neomycin resistance-thymidine kinase cassette inserted at a *StuI* site. Following electroporation of the targeting construct into the J1 ES cell line, ~10% of clonal lines selected with neomycin demonstrated evidence of homologous recombination using a flanking *Rac1* fragment in Southern blot experiments. One of these clones was expanded and transfected with a cre recombinase expression cassette, and subclones were isolated and analyzed to identify clones with either a conditional, loxP-flanked allele or a deleted allele (Fig. 1). The integrity of the *Rac1* genomic region was assessed by multiple Southern blot analyses (data not shown) and targeted ES cell clones were injected into blastocysts for generation of chimeras and germline transmission. Genotyping of the initial offspring was performed by Southern blot (data not shown), and later by PCR genotyping, using the indicated primers (Fig. 1, B and C). Chimeras were bred to C57BL/6 mice and maintained as a mixed strain population.

### PCR genotyping analyses of ES cells and mice

Tail snips were used to prepare DNA for PCR analysis (17). PCR genotyping was performed by simultaneous amplification of the wild-type (*Rac1*<sup>+</sup>), conditional (*Rac1*<sup>c</sup>), and null (*Rac1*<sup>-</sup>) alleles using three primers: PO33, TCCAATCTGTGCTGCCCATC; PO45, CAGAGCTCGAATCCA GAAACTAGTA; and PO91, GATGCTTCTAGGGGTGAGCC (Fig. 1B). All three primers were included in a single PCR and yielded products of sizes 115 bp *Rac1*<sup>+</sup>, 140 bp *Rac1*<sup>c</sup>, and 242 bp *Rac1*<sup>-</sup>, which were analyzed on 3% agarose gels. The presence of the *LysM*<sup>cre</sup> allele was assessed by a PCR using primers: cre-8, CCCAGAAATGCCAGATTACG and MLys1, CTTGGGCTGCCAGAATTTCTC, which yielded a product of size 620 bp (described previously in Ref. 18).

### Animal care procedures

All procedures were conducted in accordance with the *Guide for the Humane Use and Care of Laboratory Animals*. These studies were approved by the Harvard Medical Area Standing Committee on Animals, the University of Toronto Animal Care Committee, and the Indiana University Animal Care and Use Committee.

### Mouse breeding

Following establishment of Rac1 chimeras, germline transmission of both *Rac1*<sup>c</sup> and *Rac1*<sup>-</sup> alleles was readily achieved. Breeding studies indicated that no viable embryos at E8.5 (embryonic age in days) or later and no live offspring with a *Rac1*<sup>-/-</sup> genotype were obtained, similar to a previous study (16) indicating that the null allele was likely completely nonfunctional. In contrast *Rac1*<sup>c/c</sup> mice demonstrated no apparent pathology with normal fertility and survival past 2 years of age. Breeding the *Rac1*<sup>c/-</sup> with a mouse expressing the *LysM*<sup>cre</sup> allele (described previously in Ref. 18) resulted in generation of mice with genotypes of *Rac1*<sup>c/+LysM</sup><sup>cre/+</sup> and *Rac1*<sup>+/-LysM</sup><sup>cre/+</sup>. These mice were interbred to yield mice with the genotype *Rac1*<sup>c/-LysM</sup><sup>cre/+</sup> as well as littermate controls of several genotypes. *Rac1*<sup>c/+LysM</sup><sup>+/+</sup> and *Rac1*<sup>+/+LysM</sup><sup>+/+</sup> were used exclusively as controls. *Rac2*<sup>-/-</sup> mice previously described (14) were backcrossed (>12 generations) into C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Me). All experiments were performed with mice 6–13 wk old.

### Neutrophil preparations

Heterozygous and wild-type littermates were euthanized by CO<sub>2</sub> inhalation. Femurs and tibias were removed and bone marrow was isolated. Erythrocytes were lysed using E-Lyse (Cardinal Associates, Phoenix, AZ) and the remaining bone marrow cells were layered onto discontinuous Percoll (Sigma-Aldrich, Ontario, Canada) gradient of 82%/65%/55% (19). Mature neutrophils were recovered at the 82%/65% interface and were

demonstrated to be positive for Gr-1 and Mac-1 by flow cytometry. More than 85% of cells isolated were neutrophils as assessed by Wright-Giemsa staining. Viability as determined by trypan blue exclusion was >90%.

### Measurement of neutrophil NADPH oxidase activity

Neutrophil NADPH oxidase activity was measured as superoxide dismutase-inhibitable superoxide production using an isoluminol chemiluminescence assay for cells activated by 10  $\mu$ M fMLP or the cytochrome *c* reduction assay for cells activated with 200 ng/ml PMA (Sigma-Aldrich) as described previously (4). NADPH oxidase activity was compared in neutrophils isolated from *Rac1*<sup>c/-LysM</sup><sup>cre/+</sup> mice and control littermates. In addition, neutrophils from Rac2 null and wild-type C57BL/6J mice were assayed in parallel. There was no significant difference in neutrophil NADPH oxidase activity between Rac1 colony control littermates and C57BL/6J wild-type mice.

### Chemotaxis assays

Bone marrow neutrophils were placed into the upper well of a Transwell chamber which was separated from the bottom chamber, containing the indicated fMLP (Sigma-Aldrich) concentration by a membrane with 3- $\mu$ m pores (Corning, Corning, NY) (14). The Transwell plates were incubated at 37°C for 1 h and cells migrating through the membrane and onto the bottom coverslip were fixed with 3.7% paraformaldehyde. The number of cells per field was counted for each condition. A checkerboard approach was used which included having chemoattractant present only in the bottom well (chemotaxis) or in the top and bottom wells (chemokinesis). This approach verified that the fMLP-induced cell recruitment into the bottom chamber was a result of chemotaxis and not chemokinesis.

### F-actin assembly assay

Bone marrow neutrophils were incubated at 37°C for 30 min following isolation in HBSS. One micromolar fMLP was added for the indicated time (0–30 min). Cells were immediately fixed with 3.7% paraformaldehyde, permeabilized (0.1% Triton X-100), and stained with 1 U/500  $\mu$ l of Alexa-phalloidin (Molecular Probes, Eugene, OR) to detect F-actin content. Cellular fluorescence intensity was measured with a BD Biosciences FACScan flow cytometer (14) (BD Biosciences, Franklin Lakes, NJ). fMLP-mediated F-actin assembly was completely inhibited by cytochalasin B in all samples, verifying that we were observing actin assembly.

### Sodium periodate peritonitis

To induce an experimental peritonitis, 1 ml of 5 mM sodium periodate (Sigma-Aldrich) in PBS was injected i.p.. The mice were killed 3 h later and the peritoneal exudate was collected by lavage with chilled PBS (20 ml/mouse). Neutrophils were counted by hemocytometer and electronic cell counter (BD Biosciences).

### Quantification of Rac1 and Rac2 by immunoblot assay

Cell lysates of murine bone marrow neutrophils were prepared and subjected to 12% SDS-PAGE and immunoblotting as previously described (4). For quantification of Rac1 levels in cells, serial dilutions of rRac1 were loaded in adjacent lanes. Blots were probed with a mouse mAb for Rac1 and an anti-mouse secondary Ab conjugated with HRP and developed using ECL (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry was used to determine the intensity of signals for scanned films using NIH Image software (Research Services Branch, National Institute of Mental Health, Bethesda, MD). Multiple exposures were analyzed to ensure that relative signal intensities measured were in the linear range.

### Statistics

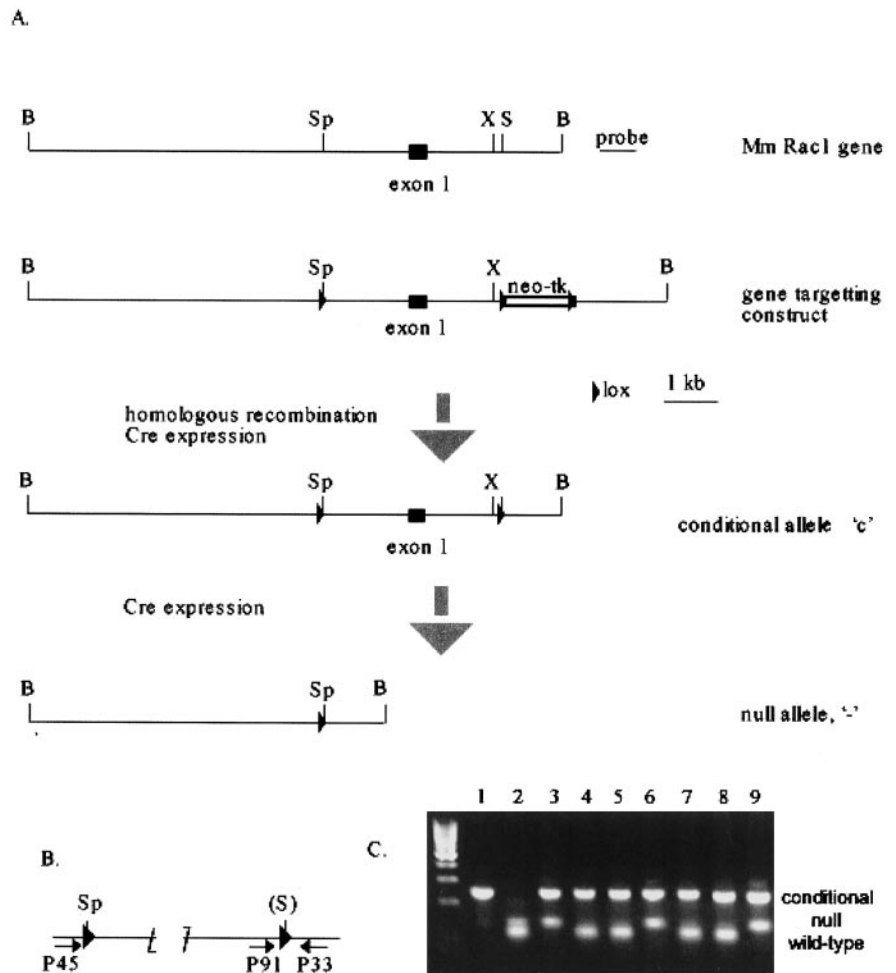
ANOVA and Student's *t* tests were performed. The Bonferroni test was used for post hoc testing. All experiments were repeated on at least three separate occasions and error bars in figures represent SEM. There were no observed significant differences between the two wild-type control mouse genotypes (*Rac1*<sup>c/+LysM</sup><sup>cre/+</sup> or *Rac1*<sup>c/+LysM</sup><sup>+/+</sup>) in any of the experiments performed.

## Results

### Generation of mice with neutrophils lacking Rac1 expression

To investigate the function of *Rac1* in neutrophils, we bred *Rac1*<sup>c/c</sup> mice (Fig. 1) with mice bearing a targeted insertion of the cre recombinase cDNA into the *LysM* gene (*LysM*<sup>cre</sup>) (18). The *LysM* gene encodes lysozyme, an enzyme that is expressed at high levels

<sup>3</sup> Abbreviation used in this paper: ES, embryonic cell.



**FIGURE 1.** Construction of a conditional allele of Rac1. *A*, The genomic map near exon 1 of Rac1 is shown at the top, then the gene targeting construct, then the conditional allele, and last the map of the null allele generated by recombination in the conditional allele. *B*, *Bam*HI; *Sp*, *Spe*I; *X*, *Xho*I; *S*, *Sac*I sites are indicated. *B*, Diagram of PCR genotyping strategy used, including the positions of three primers. *C*, PCR genotyping of *Rac1<sup>cc</sup>* and *Rac1<sup>+/-</sup>* parents (lanes 1 and 2, respectively) and *Rac1<sup>cc/+</sup>* (lanes 4, 5, 7, and 8) and *Rac1<sup>cc/-</sup>* (lanes 3, 6, and 9) offspring. *Rac<sup>c</sup>* designates the conditional allele, *Rac<sup>+</sup>* designates the wild-type allele, and *Rac<sup>-</sup>* designates the null allele.

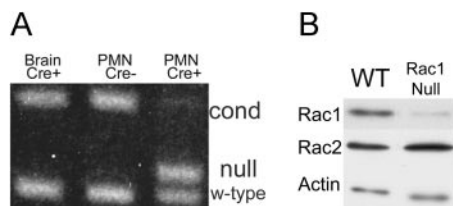
in myeloid cells. Previous studies have shown that the *LysM<sup>cre</sup>* allele leads to deletion efficiencies of 83–98% in mature macrophages and near 100% in granulocytes of floxed alleles (18). Mice were obtained with genotype *Rac1<sup>cc/-</sup>LysM<sup>cre/+</sup>* (*Rac1* null neutrophils) and were compared with littermate control mice of genotypes *Rac1<sup>cc/+</sup>LysM<sup>cre/+</sup>* or *Rac1<sup>cc/+</sup>LysM<sup>+/+</sup>*.

PCR analysis of genomic DNA from peritoneal neutrophils from *Rac1<sup>cc/+</sup>LysM<sup>cre/+</sup>* mice demonstrated near complete conversion of the conditional allele into the null allele (Fig. 2A). Rac1 protein expression was assessed by Western blots of bone marrow

(data not shown) and peritoneal neutrophils (Fig. 2B). Western blot analysis using rRac1 as a standard demonstrated that wild-type neutrophils contain  $1.15 \times 10^{-4} \pm 1.1 \times 10^{-5}$  ng of Rac1, similar to previous reports (4). A very faint residual Rac1 band was detected in lysates from *Rac1* null (*Rac1<sup>cc/-</sup>LysM<sup>cre/+</sup>*) neutrophils corresponding to  $1.11 \times 10^{-5} \pm 1.2 \times 10^{-6}$  ng of Rac1. Since recombination mediated by Cre recombinase either occurs or does not occur, either neutrophils have no Rac1 or have normal Rac1 expression. As described previously and shown here (Fig. 2A), the *LysM* Cre mouse eliminates the flanked loxP DNA in >95% of neutrophils (18). The residual Rac1 protein detected in the Western blots is likely to result from a combination of non-neutrophil cell contamination (as our isolation procedure has ~10% contamination of other cell types) or Rac1 Ab cross-reactivity with Rac2 (4, 14) and small numbers (<5%) of neutrophils that retain Rac1 expression.

#### Deficient cellular inflammatory exudate and neutrophil chemotactic responses

The number of circulating leukocytes and neutrophils were equivalent in unstimulated wild-type and *Rac1<sup>cc/-</sup>LysM<sup>cre/+</sup>* mice (Table I). However, 3 h following induction of peritonitis, circulating neutrophil and corresponding leukocyte counts increased significantly in wild-type controls but not in *Rac1<sup>cc/-</sup>LysM<sup>cre/+</sup>* mice (Table I;  $p < 0.04$ ). Whereas the peripheral blood neutrophil counts increased more than 3-fold from  $1.67 \pm 1.8 \times 10^9/L$  to  $6.2 \pm 1.2 \times 10^9/L$  in wild-type mice, only a small change in the neutrophil count was observed in the *Rac1<sup>cc/-</sup>LysM<sup>cre/+</sup>* mice from  $1.57 \pm$



**FIGURE 2.** Recombination of the *Rac1c* allele to a null allele: genotyping and immunoblot analysis. *A*, PCR genotyping of DNA from peritoneal neutrophils and brain from *Rac1<sup>cc/+</sup>LysM<sup>cre/+</sup>* mice and control (*Rac1<sup>cc/+</sup>LysM<sup>+/+</sup>*) neutrophils using primers indicated in Fig. 1. In *Rac1<sup>cc/+</sup>LysM<sup>cre/+</sup>* neutrophils, there is conversion of the *Rac1<sup>c</sup>* (conditional) allele to the *Rac1<sup>-</sup>* (null) allele. *Rac1<sup>+</sup>* denotes the wild-type (w-type) allele. *B*, Representative immunoblot of Rac1 and 2 expression by peritoneal neutrophils. Note: There is no compensatory increase in Rac2 expression in the *Rac1* null neutrophils. *Rac<sup>c</sup>* designates the conditional allele, *Rac<sup>+</sup>* designates the wild-type (WT) allele, and *Rac<sup>-</sup>* designates the null allele.



Table I. Circulating leukocytes and neutrophils before and 3 h after sodium periodate injection (SP)

	Leukocytes (10 <sup>9</sup> /L)	Neutrophils (10 <sup>9</sup> /L)	Leukocytes (SP, 10 <sup>9</sup> /L)	Neutrophils (SP, 10 <sup>9</sup> /L)
Controls	7.3 ± 1.5	1.67 ± 1.8	11.5 ± 0.6*	6.2 ± 1.2*
Rac1 null	7.4 ± 4.5	1.57 ± 1.0	8.0 ± 1.6	2.8 ± 0.6

\* ,  $p < 0.04$ : Rac1 null vs control leukocytes and neutrophils after SP injection ( $n = 6$  for all groups shown).

$1.0 \times 10^9/L$  to  $2.8 \pm 0.6 \times 10^9/L$  (Table I). The acute increase in peripheral blood neutrophil counts in response to an inflammatory stimulus largely reflects mobilization from the bone marrow storage pool (20). The impaired response of *Rac1*<sup>−/−</sup>*LysM*<sup>cre/+</sup> mice suggests that neutrophils lacking Rac1 are not able to leave the bone marrow at the same rate as wild-type control neutrophils.

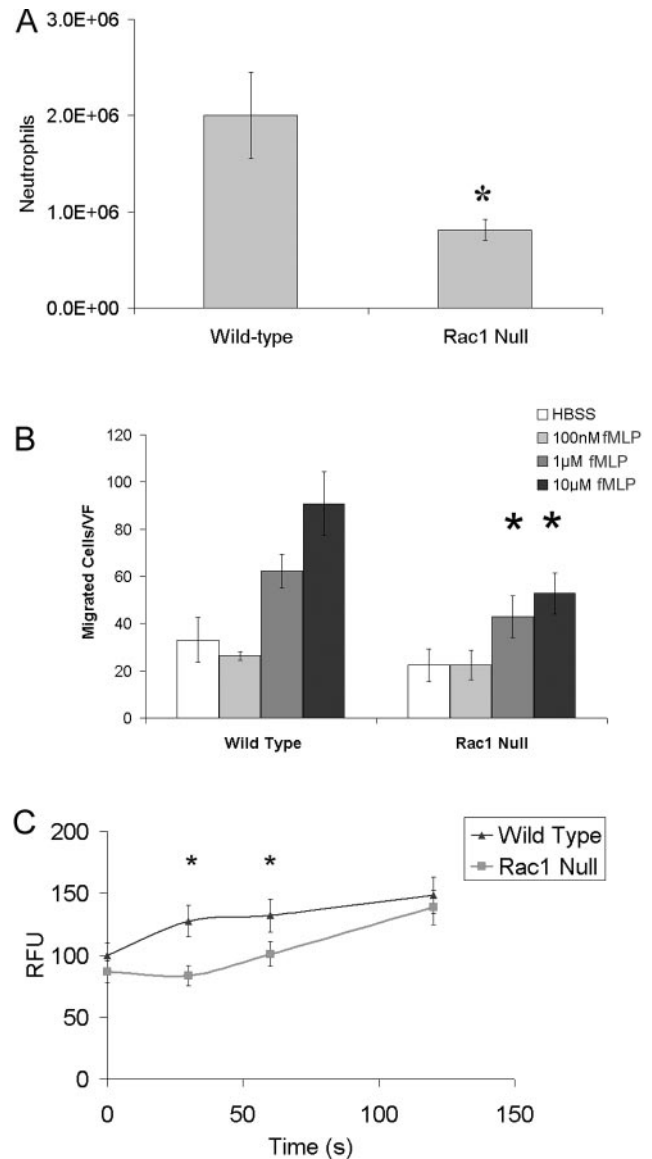
Since Rac2-deficient neutrophils have defects in their migratory capacity (14), we assessed the ability of Rac1 null neutrophils to migrate to a site of inflammation in vivo (14). In a peritoneal inflammation model (14), we observed a >50% reduction in neutrophil accumulation 3 h after sodium periodate injection into the peritoneum of *Rac1*<sup>−/−</sup>*LysM*<sup>cre/+</sup> mice (Fig. 3A). This strongly suggests that Rac1 plays an important role in the steps required for neutrophil accumulation at sites of inflammation. Reduced numbers of peritoneal exudate neutrophils in Rac1 null mice could in part reflect the smaller numbers of neutrophils seen in peripheral blood following induction of peritonitis. To determine whether impaired neutrophil migration might also contribute to residual exudate formations, we assessed neutrophil chemotaxis in vitro. The fMLP-directed movement of Rac1-deficient bone marrow neutrophils was reduced by almost 50% as compared with control littermates both at 1 and 10  $\mu$ M fMLP (Fig. 3B). This result implicates Rac1 as a key mediator of neutrophil chemotaxis.

#### Altered F-actin generation

Since the Rac small GTPases have been implicated as key regulators of the actin cytoskeleton and to determine the underlying role by which Rac1 regulates the chemotactic process of neutrophils, we assessed fMLP-induced actin polymerization in Rac1 null neutrophils using flow cytometry. Rac1 null neutrophils demonstrated a significant reduction in fMLP-induced F-actin formation and the kinetics of assembly were delayed compared with those of wild-type controls (Fig. 3C). This reduced early actin polymerization, which is crucial to the chemotactic process (21), may in part explain the observed defects in neutrophil chemotaxis and in vivo recruitment to sites of acute inflammation.

#### Superoxide production is normal in Rac1-deficient neutrophils

Since Rac1 and Rac2 have each been implicated as key regulators of superoxide production in studies using cell-free assays and since Rac2 null neutrophils have deficiencies in superoxide generation, we assessed whether Rac1 is also required for superoxide generation in intact primary neutrophils. Neutrophils from Rac2 null mice previously described (14) along with C57BL/6J controls were compared with Rac1 null neutrophils and matching littermate controls. NADPH oxidase activity was measured in bone marrow neutrophils stimulated with either PMA or fMLP. Rac1 null neutrophils demonstrated no significant impairment in superoxide production in response to PMA when compared with wild-type littermates in contrast to the marked defect observed in Rac2 null neutrophils (Fig. 4A). fMLP-induced superoxide production was also similar in wild-type and Rac1 null mice (Fig. 4). This again contrasts with the substantially diminished NADPH oxidase activ-

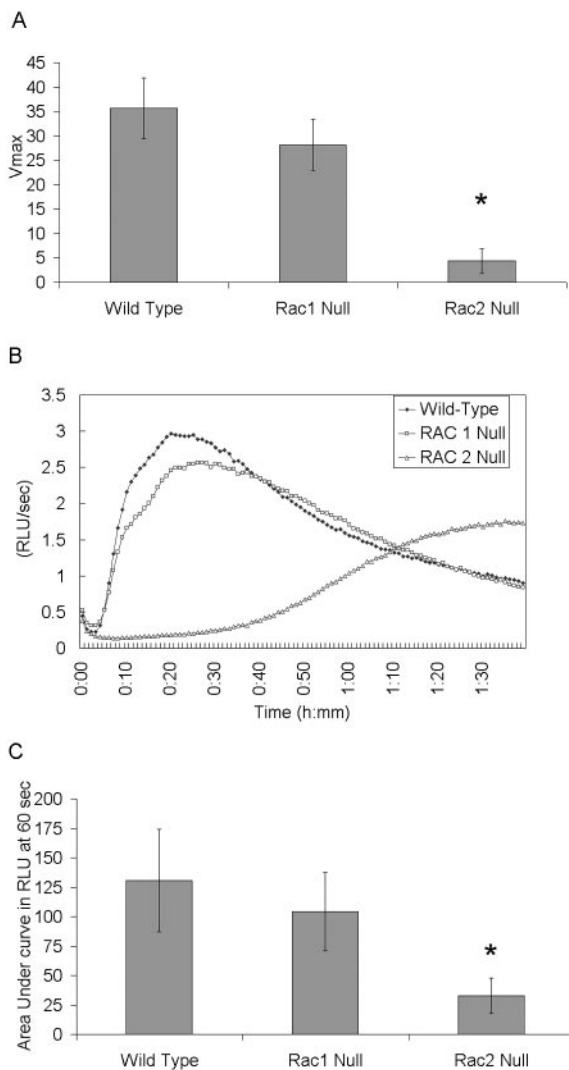


**FIGURE 3.** Analysis of Rac1 null neutrophil function. *A*, In vivo response to sodium periodate-induced inflammation. Peritoneal neutrophils were counted 3 h after i.p. injection of 1 ml of sodium periodate. Mean  $\pm$  SD ( $n = 6$ ; \*,  $p < 0.01$ ). *B*, Bone marrow neutrophil chemotaxis was assessed in a Transwell migration assay using gradients of fMLP as described (mean  $\pm$  SEM of five mice per group; \*,  $p < 0.05$ ). *C*, Reduced fMLP-induced actin polymerization kinetics in mouse neutrophils lacking Rac1. Note the slower rate of actin polymerization in Rac1 null neutrophils compared with control neutrophils (representative of three experiments; \*,  $p < 0.01$ ).

ity in fMLP-stimulated Rac2 null neutrophils, which have both a delay in reaching maximal enzyme activity (Fig. 4B) as well as an overall decrease in oxidant production (Fig. 4C). There was no significant difference between wild-type littermate controls and wild-type C57BL/6J controls, indicating that strain differences were not important in these measurements (data not shown).

## Discussion

Neutrophils are critical cells of the innate immune system carrying out a coordinated set of functions following their recruitment and migration to sites of infection which result in the elimination of invading pathogens. Neutrophil migration and oxygen radical generation through the NADPH oxidase are regulated by two members of the small GTPase Rac family, Rac1 and Rac2. The critical



**FIGURE 4.** Rac1 is not required for superoxide production. *A*, The rate of superoxide production by bone marrow neutrophils following stimulation with 0.2  $\mu\text{g/ml}$  PMA was monitored by the superoxide dismutase-inhibitable reduction of cytochrome *c*. The units for  $V_{\text{max}}$  are equal to nanomoles of superoxide per minute per  $10^7$  cells (mean  $\pm$  SEM of five mice per group (\*,  $p < 0.01$ , Bonferroni's test). Although the overall magnitude of PMA-elicited NADPH oxidase activity was decreased in Rac2 null mice, the overall kinetics were not affected (data not shown). *B*, fMLP-induced superoxide production in bone marrow neutrophils is only perturbed in neutrophils lacking Rac2 but not Rac1. The time course of superoxide production for neutrophils lacking Rac1 was similar to that of wild-type cells in contrast to Rac2 null neutrophils. A representative kinetic plot for the fMLP-induced superoxide production is presented. RLU, Relative luminescence units. *C*, Data shows the relative luminescence units integrated over 60 s as measured in fMLP-stimulated neutrophils using superoxide dismutase-inhibitable isoluminol chemiluminescence. The three neutrophil genotypes are as indicated (mean  $\pm$  SEM of six mice per group; \*,  $p < 0.01$ ).

importance of Rac2 in neutrophil chemotaxis and NADPH oxidase function has been established *in vivo* using Rac2 null mice (4, 14). Because Rac1 and Rac2 have 92% homology (5) as well as similar biochemical functions in reconstituted systems *in vitro* (22), it has been hypothesized that there is significant functional overlap between these two proteins. However, the level of *in vivo* redundancy between these two proteins has not been studied previously. In this report, we investigate the specific roles of Rac1 in neutro-

phil function using a conditional gene targeting approach to generate mice with Rac1 null neutrophils.

#### *Rac1 is a key regulator of neutrophil actin assembly and migration*

Our studies demonstrate that Rac1 null neutrophils have defects in regulation of the actin cytoskeleton and in neutrophil migration similar to defects seen in Rac2 null neutrophils (14). However, our data also confirm that Rac2 cannot compensate completely for Rac1 deletion and vice versa. Although it is possible that the similar phenotypes may be due to a reduction in total Rac (Rac1 and Rac2), recent evidence (4) demonstrates that Rac1 and Rac2 are differentially activated downstream of chemoattractant receptors, suggesting that Rac1 and Rac2 may have nonoverlapping roles in signaling pathways between membrane receptors and downstream targets. The notion of distinct functions for Rac1 and 2 is further supported by our observation that NADPH oxidase activity is normal in the absence of Rac1, whereas Rac2 deletion results in severely diminished oxidase activity (4).

It is important to note that although Rac2 is the predominant Rac isoform in human neutrophils (10), it has recently been demonstrated that between 20 and 25% of total Rac in human neutrophils is Rac1 (4). This suggests that although Rac2 is the predominant isoform, Rac1 may also participate in regulating neutrophil function.

#### *Role of Rac in superoxide generation*

Cell-free NADPH oxidase studies have used Rac1 and 2 interchangeably to reconstitute a functional oxidase complex (9, 10, 23). Dorseuil et al. (24), using a yeast two-hybrid system, demonstrated that nonprenylated Rac2 has a 6-fold higher affinity for p67<sup>phox</sup>, a key oxidase complex protein, than nonprenylated Rac1. These data suggest that Rac2 may be the preferential Rac for regulating the NADPH oxidase complex. Heyworth et al. (10) also demonstrated that recombinant prenylated Rac2 is more efficient at regulating the NADPH oxidase complex than Rac1 in the presence of neutrophil cytosol. This suggests that there could be a preferential interaction of Rac2 with cytosolic components that promote assembly of the active NADPH oxidase complex. Our results demonstrate for the first time, in primary intact neutrophils, that Rac1 is not required for NADPH oxidase function *in vivo*.

#### *Mechanisms for nonoverlapping functions*

Two possible mechanisms may explain why Rac1 and Rac2 are not able to completely compensate for the loss of the alternate isoform in chemoattractant-activated F-actin assembly and chemotaxis. First, the majority of sequence heterogeneity between these two proteins occurs in the C-terminal hypervariable region that has been implicated in the targeting of the small GTPases to subcellular membrane domains (5). Michaelson et al. (5) have demonstrated that activated Rac1 and 2 differentially localize to unique domains in fibroblast and epithelial cells; Rac2 accumulates predominantly on endomembranes whereas Rac1 is located predominantly at the plasma membrane. Localized targeting of each Rac isoform to unique membrane domains may be essential for normal regulation of cell migration and chemotaxis since actin-mediated cell locomotion is composed of a complex series of events involving cycles of actin polymerization and depolymerization localized to discrete cellular domains. Although actin polymerization at the leading edge is believed to drive membrane protrusion (21), actin assembly in other cytoplasmic regions including at cell-extracellular tissue matrix receptor sites (integrins) and rearward recycling membrane domains are also critical to normal cell locomotion (25). This may explain why these proteins have similar functional

biochemical properties *in vitro* but are unable to compensate for the others loss *in vivo*.

Another possibility is that Rac1 and Rac2 may have different downstream targets. The C-terminal domain of Rac is also involved in binding to downstream effector targets. Rac1 has a 2-fold greater ability to bind and a 5-fold greater ability to stimulate the kinase activity of PAK1 compared with Rac2 (26). PAK1 translocates to areas of active cytoskeletal rearrangement (27) and microinjection of active PAK1 resulted in the induction of lamellipodia and membrane ruffling in fibroblasts (28). Furthermore, recent studies have demonstrated that the Rac2 C-terminal polybasic TRQKRP motif is required for normal Rac2 localization and function (6). These data suggest that differential targeting and unique downstream protein targets may explain the nonoverlapping roles of these isoforms.

In conclusion, this study demonstrates that in intact neutrophils, Rac1 and 2 play significant roles in regulation of the actin cytoskeleton and neutrophil migration, but that Rac2 is the isoform responsible for regulating the NADPH oxidase complex. The lack of balancing compensation by the remaining Rac isoform in the knockout mice, the preferential role of Rac2 as the isoform involved in NADPH oxidase, and recent reports of the differential activation of Rac1 and Rac2 (4) strongly suggest that Rac1 and Rac2 each have unique roles in neutrophil functions.

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