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Rac2 is required for Formation of extracellular traps by neutrophils

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Summary Sentence: Ablation of Rac2 has negative impact on generation of ROS and NO, leading to impaired extracellular traps formation in neutrophils

Running Title: Absence of Rac2 impairs NET Formation *in vitro*

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Abbreviations: NET, neutrophil extracellular traps; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; LPS, Lipopolysaccharide; TEM, transmission electron microscope; DAF-2DA, 4,5-diaminofluorescein diacetate;

ABSTRACT

Neutrophils play a critical role as a first line of defence against invading pathogens. Recently, a new defence strategy of neutrophils was described in which pathogens are trapped and killed by neutrophil extracellular traps (NETs). However, the exact underlying mechanisms leading to the formation of NETs remain elusive. Here, we explored the role of Rac small GTPases in the formation of NETs using neutrophils that lack Rac1, Rac2 or both isoforms. Efficient NET formation was observed in both wild-type and Rac1null neutrophils. In contrast, NET formation was markedly impaired in cells lacking either Rac2 or both Rac2 and Rac1. The defect in NET formations in Rac2null cells could be rescued by exogenous ROS sources suggesting that Rac2 mediated ROS generation is required for NET formation. In addition, we assessed the role of nitric oxide in NET formation in murine neutrophils. Blocking nitric oxide (NO) production with the nitric oxide synthase inhibitor, L-NAME significantly reduced NET formation. Moreover, we show that Rac2null cells produce significantly less NO than Rac1null cells or their wildtype counterparts. Our data suggest that Rac2 is essential for NET formation via pathways involving both ROS and NO.

INTRODUCTION

Neutrophils play a critical role as a first line of defence against invading pathogens in the innate immune system of the host. During inflammation or infection, neutrophils are recruited to the site of injury and provide protection by eliminating potential threats via phagocytosis or release of bactericidal

substances [1]. Recently, a novel defence mechanism of neutrophils was described in which web-like structures (neutrophil extracellular traps; NETs) consisting of chromatin and proteins are produced to trap and kill pathogens [2, 3]. However, our knowledge of the underlying mechanisms regulating NET formation is still limited. Studies using neutrophils from patients with chronic granulomatous disease (CGD) have provided evidence that ROS are essential for the formation of NETs [3, 4]. Neutrophils from CGD patients lack significant ROS formation due to genetic defect in components of the NADPH oxidase resulting in an inability to produce NETs which may partly account for their increased susceptibility to microbial infections. This finding led us to investigate the specific roles of Rac1 and Rac2, members of the Rho family of small GTPases in the formation of NETs as Rac1 and Rac2 are a key regulator of ROS generation via NADPH oxidase system. Despite the fact that Rac1 and Rac2 share 92% homology in amino acid sequence, recent studies using Rac2-deficient neutrophils suggest that each isoform has unique roles in neutrophil functions [5-7]. To determine the involvement of Rac1 and Rac2 in the formation of NETs, we used mice in which Rac1 or Rac2 or both genes are selectively disrupted in cells of the granulocyte/monocyte lineage. We demonstrate here for the first time that Rac2null neutrophils have significant defects in formation of NETs *in vitro*. Moreover we demonstrate that NET formation in Rac2null cells can be partially rescued by stimulation with exogenous ROS, suggesting that activation of NOX2 by Rac2 plays a pivotal role. In addition, we present data on the role of nitric oxide (NO) in the formation of NETs. Several studies including Lee et al.[8] and Pieper et al. [9] have identified NO as a crucial ROS modulator in neutrophils. Both studies demonstrated that

different levels of NO have different effects, either stimulating or inhibiting ROS generation by neutrophils. Importantly, NO has been shown to play an important role in NET formation in human neutrophils [10]. Here, we confirm that NO production is also required for formation of NETs in murine neutrophils and that Rac2 is required for efficient NO production. Our data suggest that Rac2 is an essential regulator of NET formation through dual regulation of ROS and NO generation in Rac isoform-specific manner.

MATERIALS AND METHODS

Mice and neutrophil isolation

Conditional Rac1 mice were generated according to protocols described in Glogauer et al [5]. In brief, selective disruption of Rac1 granulocytes/neutrophils was achieved by using a conditional Rac1^{cre}-LysM^{cre} in which the Cre recombinase is expressed under the control of the murine lysozyme M gene regulatory region. Rac2null mice were previously generated according to protocols described in Roberts et al [11]. Mice were euthanized and their bone marrow was flushed out of femurs and tibias. They were layered onto discontinuous Percoll (Sigma-Aldrich, Ontario, Canada) gradients of 80%/65%/55% [12] and centrifuged for 30 min at 1300g at 4°C. Mature neutrophils found at 80% and 65% interface were collected. Routinely, more than 85% of isolated cells were neutrophils, confirmed by Wright-Giemsa staining. All experiments were performed with mice older than 4 weeks. All mice were used in

accordance with the Guide for the Human Use and Care of Laboratory Animals and the approval of the University of Toronto animal care committee.

Neutrophil Extracellular Traps (NETs) assay

To quantify NETs, neutrophils in 1x HBSS (Invitrogen, Ontario, Canada) containing $\text{CaCl}_2/\text{MgCl}_2$ were seeded in a black 96-well plate (Cayman Chemical, Michigan, USA). The cells were treated with 100nM PMA (Sigma-Aldrich, Ontario, Canada) or 100ng/ml LPS (Sigma-Aldrich, Missouri, USA) or left untreated. Cells were also lysed with 0.1% triton X-100 (Union Carbide Chemicals, Ohio, USA) as a control for the total DNA in different wells for each time points. They were then incubated at 37°C for 3 hours. The cell-impermeable DNA-binding dye, Sytox green (Invitrogen, Ontario, Canada) was added (5 μM) at each time points to bind extracellular DNA, and fluorescence was quantified using a fluorescence spectrophotometer (Fisher Scientific, Ontario, Canada). To ensure that the NET assay only detected extracellular NET-DNA, neutrophils were incubated with 5 units of DNase I, RNase-free (Fermentas, Ontario, Canada) for 1 hour at 37 °C prior to the extracellular DNA measurement.

Nitric Oxide (NO) quantification

To measure the amount of nitric oxide in live cells, neutrophils were incubated with 1 μM DAF-2DA (Cayman Chemical, Michigan, USA) for 30 min at 37 °C. Culture media were then replaced to remove excess dye and the neutrophils were either incubated in the presence or absence of 100nM PMA. NO was measured every 15 min for a period of 3 hours using a fluorescence spectrophotometer (Fisher Scientific, Ontario, Canada).

Confocal Microscopy

To visualize NETs using immunofluorescence, neutrophils were seeded on 13-mm cover slips (Fisher Scientific, Ontario, Canada) pre-coated with 0.001% poly-l-lysine (Sigma-Aldrich, Ontario, Canada) in 1x HBSS with $\text{CaCl}_2/\text{MgCl}_2$. Cells were washed, fixed with 4% paraformaldehyde and then blocked with 100nM glycine and 1% BSA for 1 hour each at room temperature. The cells were incubated with Histone H3 antibody (1:150) (Cell Signaling, Massachusetts, Canada) and Alexa Fluor 568 goat anti-rabbit IgG at (1:200) (Invitrogen, Ontario, Canada) for 1 hour each to stain for histones. 5 μM Sytox green was used to stain for DNA. Prepared samples were analyzed using Confocal microscopy.

Transmission Electron Microscopy

Samples were fixed with Karnovsky's fixative, dehydrated using a graded series of ethanol and infiltrated with mixture of Epon-Araldite and ethanol. After the subsequent polymerization step, samples were sectioned and stained using saturated uranyl acetate followed by Reynold's lead citrate. The sections were then examined and photographed in a Hitachi H7000 transmission electron microscope.

Statistical analysis

For experiments in which there were multiple observations per sample, numerical results were expressed as mean $\pm$ SEM. Each experiment had a sample size of $n \geq 3$. Statistical analysis was performed using Student's t- test unless otherwise specified. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Mouse neutrophils form NETs in vitro

To measure NET formation, we adapted assay developed by Brinkmann et al. [2] based on fluorescence quantification of extracellular DNA, the major structural component of NETs. To validate the NET assay, DNase was added after the induction of NETs. DNase mediated degradation of extracellular DNA was able to abolish the fluorescent signal from Sytox Green, demonstrating the specificity of the assay for extracellular DNA (data not shown). NET formation in murine neutrophils cells was induced with PMA (100 nM) or LPS (100 ng/ml) and extracellular DNA was quantified after 90 and 180 min (see methods). Unstimulated neutrophils were used as base line for normalization and NET formation was expressed as the ratio of extracellular DNA divided by total DNA. To measure total DNA, replicates were pre-treated with Triton X-100 prior to staining with Sytox green. NET formation was undetectable 15 min after stimulation (data not shown). However, we found that approximately 4% and 15% of the total DNA is released as NETs after 90 and 180 min of PMA stimulation, respectively (**Fig. 1**). In contrast, LPS stimulated neutrophils released only about 1% and 5% of total DNA after 90 and 180 min stimulation, respectively (**Fig. 1**). To confirm the results obtained with the NET assay, we applied confocal laser microscopy to visualize NET formation at various time points. Although microscopic visualization of NETs is useful for qualitative assessment of NETs, it may not be very suitable for quantitative purposes since NETs are very fragile [13]. To detect the appearance of NETs and investigate the morphological changes leading to the NET formation by confocal microscopy, we

used Sytox green to stain DNA and an antibody against histone H3, one of major structural component of NETs [14]. In unstimulated cells NET formation was absent and nuclei maintained their normal donut-like shape. In addition, histone H3 was seen to co-localize with nuclei at all time points (**Fig. 2 A**). Fifteen minute after induction of NET formation, nuclei of neutrophils still exhibited their normal donut-like shape comparable to unstimulated neutrophils. In contrast, we observed apparent changes in nuclei and overall shape of neutrophils at later time points after stimulation with PMA or LPS (**Fig. 2A**). Ninety minute after stimulation, extracellular DNA release by a small number of neutrophils was observed. The nuclei of some neutrophils no longer maintained their initial shape, rather started to expand and fill the cytoplasmic space. The overall cell shape also changed from circular to elongated shape (**Fig. 2A**). Three hours after stimulation, the nuclei of activated neutrophils expanded and filled most of the cytoplasmic space. At this time point, we also found that histone H3 co-localizes with DNA in NETs (**Fig. 2A**).

Transmission electron microscopic (TEM) images confirmed the change in shape of nucleus in the process of NET formation (**Fig. 2B**). After 15 min of stimulation, no apparent morphological changes were detected. However, after 90 min the plasma membrane of some neutrophils started to rupture, possibly marking the initial process of NET release. Extracellular structures suggestive of NETs were also observed at this time point (**Fig. 2B**). At 180 min after stimulation, more abundant NET release was observed than at the 90 min time point and many of the neutrophils seemed to have lost their

initial smooth plasma membrane structure. At this time point, we also observed that the nuclei of many of neutrophils filled most of the cytoplasmic space (**Fig. 2B**).

To confirm the requirement for ROS [3] during NET formation in our assay, we stimulated murine neutrophils with PMA (**Fig. 3A**) or LPS (**Fig. 3B**) in the presence of the ROS scavenger, Trolox. Trolox significantly reduced the formation of NETs both at 90 and 180 min after stimulation, demonstrating ROS as an important requirement for efficient NETs formation (**Fig. 3A,B**). *N*-Acetyl cysteine (NAC) was used as an alternative ROS scavenger and also resulted in significant reduction of NET formation (data not shown).

Since the Rac small GTPases are key regulators of ROS generation through activation of NADPH oxidase in neutrophils, we hypothesized that Rac is also important for NET formation. Murine neutrophils express two Rac isoforms, Rac1 and Rac2 [5]. In order to identify the isoform-specific contribution of Rac, NET formation was induced in neutrophils deficient in Rac1, Rac2 or both isoforms. Approximately, 2% and 16% of total DNA was released as NETs in Rac1null neutrophils in response to PMA after 90 and 180 min stimulation, respectively (**Fig. 4A**). In Rac1null neutrophils, the amount of NETs released was slightly lower at 90 min and higher at 180 min compared to wild-type neutrophils but the difference was statistically insignificant. Similar amounts of NET formation were also observed in WT and Rac1 deficient cells after stimulation with LPS (**Fig. 4B**). Rac1null neutrophils released about 2% and 4% of total DNA as extracellular traps, similar to amounts observed in wild-type neutrophils at 90 and 180 min after stimulation (**Fig. 4B**). In contrast, NET formation in Rac2

neutrophils was dramatically reduced in both PMA and LPS stimulated cells. In response to both stimuli, less than 1% and 2% of the DNA was released as NETs at 90 and 180 min after stimulation, respectively. NET formation was virtually undetectable in cells that lack both Rac isoforms (Rac1/2null) (**Fig. 4A, B**).

LPS or PMA are known activators of NADPH oxidase and induce the production of ROS [15]. Since Rac2 is the major isoform involved in NADPH oxidase complex activation [16] and ROS production is severely impaired in Rac2null neutrophils [5], we tested whether reduced NET formation in Rac2null cells is caused by defective ROS generation. Since it was reported that ROS alone can trigger NET formation [3] we induced NET formation in wild-type neutrophils using hydrogen peroxide and glucose oxidase (GO), a hydrogen peroxide producing enzyme. Both hydrogen peroxide and GO induced NETs in wild-type neutrophils (**Fig. 5A**). Stimulation with hydrogen peroxide (25 μ M - 150 μ M) triggered 6% of total DNA to be released as NETs after 180 min stimulation (**Fig. 5A**). GO (10 mU/ml) induced NET formation slightly more effective (10% DNA release) (**Fig. 5A**). NET formation induced by hydrogen peroxide could be confirmed by immunofluorescence microscopy (data not shown). To address whether the defect in NET formation in Rac2null cells is caused by their inability to generate adequate levels of ROS, we carried out rescue experiments by adding H₂O₂ or GO. H₂O₂ and GO were able to partially rescue NET formation (6% of total DNA), significantly more than PMA stimulated Rac2null neutrophils (**Fig. 5B**). Analysis of NET release by confocal microscopy also confirmed these results, demonstrating that impaired NET formation in Rac2null cells is – at least partially – caused by reduced ROS generation (**Fig. 5C**).

Recently, nitric oxide (NO) was shown to be important for NET formation in human neutrophils [10]. To validate if NO has a comparable role in NET formation in murine neutrophils, we induced NETs in wild-type neutrophils while blocking the activity of nitric oxide synthase (NOS). Using the pharmacological inhibitor L-NAME to block NO generation, we observed a significant reduction in NET formation at both 90 min and 180 min after stimulation with PMA (**Fig. 6A**), demonstrating a similar requirement for NO in NET generation in mouse neutrophils. Interestingly, Rac is able to interact and regulate the activity of constitutive NO synthases [17]. Therefore, we investigated the possible impact of Rac deficiency on NO production in Rac1null and Rac2null neutrophils. After stimulation with PMA, the amount of NO was monitored every 15 min over a period of 3 hours by measuring the fluorescent emission of the NO-probe, DAF2-DA. NO production in PMA stimulated WT and Rac1null neutrophils constantly increased over a period of 3 hours (**Fig. 6B**). NO production in Rac2null neutrophils was markedly lower and after 180 min the amount of NO generated in Rac2null neutrophils was about half of compared to wildtype neutrophils (**Fig. 6B**).

We show here that Rac2 is required for the formation of NETs in an isoform-specific manner. Indeed, NET formation in Rac1null cells was not affected. Because NET formation is tightly linked to generation of ROS, it is likely that the defect in NET formation is caused by lack of ROS formation in Rac2null cells [6]. This is supported by the finding that exogenous addition of ROS partially rescued NET formation in Rac2null cells. In addition, we identified an isoform-specific function for Rac2 in NO generation in neutrophils. Rac2null cells produce roughly half the amount of NO than WT cells. Since

NO plays an important role in stimulating ROS production required for NET formation [10], we propose a dual role for Rac2 in NET formation. Besides being the preferential Rac isoform for NADPH oxidase, Rac2 also augments ROS production through NO-mediated stimulation of NADPH-oxidase.

AUTHORSHIP

M.B.H.L designed and performed the experiments, analyzed data, prepared the figures and wrote the manuscript. J.W.P.K conceived the project, designed experiments and wrote the manuscript; A.L.K and H.G helped in performing experiments; M.G coordinated the project and edited the manuscript.

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Figure Legends

Figure 1. *Neutrophil Extracellular Trap (NET) release is induced by phorbol 12-myristate 13-acetate (PMA) and Lipopolysaccharide (LPS).* Neutrophils were isolated from wild-type and were treated with 100 nM PMA, 100 ng/ml LPS or mock-treated. Staining of extracellular DNA with Sytox Green was used to quantify NET formation (see methods). The percentage of extracellular DNA was calculated by subtracting the fluorescence of unstimulated neutrophils from the stimulated neutrophils at each time point and divided by the total DNA (n=3, stars indicate p<0.05).

Figure 2. *Microscopic analysis of neutrophil extracellular trap formation in murine neutrophils.*

Isolated neutrophils were seeded on poly-L-lysine coated glass slides, stimulated with 100 nM PMA for the indicated time and fixed before processing for confocal laser microscopy or transmission electron microscopy. (A) DNA was stained with Sytox Green and histone 3 was detected by a specific antibody against histone 3, followed by an Alexa-568 conjugated antibody (in red). Confocal laser scanning

microscopy demonstrated initial NET formation after 90 min with co-localization of extracellular DNA and histone 3 in NETs. **(B)** Transmission electron microscope (TEM) images show the changes in nucleus and the formation of NETs at 90 min and 180 min after stimulation. Scale bars = 10 μ m.

Figure. 3 *Reactive Oxygen Species (ROS) are required for NET formation.* Neutrophils from the wild-type mice were stimulated with PMA (100 nM) or LPS (100 ng/ml) in the presence of 100 μ M Trolox or vehicle. NET formation was measured at 90 minutes and 180 minutes post stimulation. (n=3, p<0.05)

Figure 4. *Rac2 is required for neutrophil extracellular trap formation.* Neutrophils were isolated from WT, Rac1null, Rac2null and Rac1/2null mice, and NET formation was induced with **(A)** 100 nM PMA or **(B)** 100 ng/ml LPS. Formation of NETs was quantified at 90 minute and 180 minute post stimulation. (n=3, * indicates p<0.05)

Figure 5. *Hydrogen Peroxide induces NET formation in wild-type and Rac2null neutrophils.* **(A)** Wild-type neutrophils and **(B)** Rac2null neutrophils were stimulated with 100 nM PMA, 25, 50, 150 μ M hydrogen peroxide and 10 mU/ml glucose oxidase (GO) to induce NET formation and extracellular NETs were quantified at the indicated time points (n=3, stars indicate p<0.05). **(C)** Partial rescue of NET formation in Rac2null neutrophil by hydrogen peroxide was confirmed by confocal microscopic images (DNA stained by Sytox Green, Histone 3 by Alexa 568 in red)

Figure 6. *Nitric oxide generation is involved in NET formation and is decreased in Rac2null cells. (A)*

PMA-induced NET formation was significantly reduced by inhibiting nitric oxide generation with the nitric synthase inhibitor, L-NAME (5mM). **(B)** Nitric oxide formation was measured every 15 minutes in wild-type, Rac1null and Rac2null neutrophils after stimulation with PMA (n=3, stars indicate $p<0.05$).

A **B**