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Research article

Mouse macrophage polarity and ROCK1 activity depend on RhoA and non-apoptotic Caspase 3

Yianzhu Liu [a,b,c], Laurie J. Minze [a,b], Lindsay Mumma [a,b], Xian C. Li [a,b], Rafik M Ghobrial [a,b,d], Malgorzata Kloc [a,b,*]

[a] The Houston Methodist Research Institute, Houston, TX, USA
[b] Department of Surgery, The Houston Methodist Hospital, Houston, TX, USA
[c] Department of Neurology, Xiangya Hospital, Central South University, Changsha, 410008 Hunan, China
[d] Sherrie and Alan Conover Center for Liver Disease and Transplantation, Houston, TX, USA

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ABSTRACT

The macrophages have different subtypes with different functions in immune response and disease. It has been generally accepted that M1 macrophages are responsible for stimulation of immune system and inflammation while M2 macrophages play a role in tissue repair. Irrespective of the type, macrophage functions depend on actin cytoskeleton, which is under the control of small GTPase RhoA pathway and its downstream effector ROCK1. We generated RhoA-deleted macrophages and compared the effect of RhoA deletion on M0, M1 and M2 macrophage phenotype. Our studies showed that, unexpectedly, the RhoA deletion did not eliminate macrophage ROCK1 expression and increased ROCK1 activity. The RhoA deletion effect on macrophage phenotype, structure and polarity was different for each subtype. Moreover, our study indicates that the up-regulation of ROCK1 activity in RhoA-deleted macrophages and macrophage phenotype/polarity are dependent on non-apoptotic Caspase-3 and are sensitive to Caspase-3 inhibition. These novel findings will revise/complement our understanding of RhoA pathway regulation of cell structure and polarity.

1. Introduction

The vertebrate macrophages and their invertebrate equivalent – the immunocytes [1] are the motile and professional phagocytes, which play a critical role in animal immune response [2]. Out of a spectrum of macrophage subsets identified recently in vertebrates [2–4] the M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages are the classical and most studied. While, *in vivo,* the acquisition of M1 and M2 properties by circulating monocytes (M0) depend on IFNs/Toll-like receptor or IL-4/IL-13 signaling [3,4] in *in vitro* system, the M1 and M2 macrophages can be easily induced from the bone marrow or peritoneal M0 macrophages by incubation with respective recombinant signaling molecules. Numerous studies showed that various macrophage functions depend on the RhoA signaling pathway and actin cytoskeleton [5–8]. The small GTPase RhoA and its downstream effector the Rho-associated protein kinase ROCK regulate actin filaments polymerization status, which in turn, controls cell polarity, movement, phagocytosis, endocytosis, antigen presentation and matrix degradation [5,9–12]. Our studies and studies from other laboratories showed that inhibition of RhoA/ROCK pathway using ROCK inhibitor Y-27632 influences macrophage polarity, matrix degradation and motility and inhibits chronic rejection of cardiac allografts in rodent model system [5,11]. The RhoA signaling pathway is extremely elaborate and it is influenced by or influences other actin-related pathways, such as mTOR and Rac1 [6–10,12]. In addition, besides the RhoA, which under normal circumstances increases ROCK activation, there are several potential alternative activators of ROCK [13–17] one of them being Caspase-3, which constitutively activates ROCK by proteolytic cleavage of its C terminus [18,19]. Although the most recognized role of caspases (including Caspase-3) is their involvement in apoptosis, it is also known that these enzymes play role in a caspase-mediated incomplete apoptosis events regulating cell cycle and cell differentiation, including differentiation and maturation of macrophages [20,21]. Here we studied how macrophage specific deletion of RhoA affects phenotype and structure of mouse M0, M1 and M2 macrophage subtypes and how Caspase-3 inhibition influences macrophage phenotype.

* Corresponding author at: Houston Methodist Hospital, Department of Surgery, 6550 Fannin St., Houston, TX 77030, USA.
E-mail address: mkloc@houstonmethodist.org (M. Kloc).

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2. Material and methods

2.1. Generation of mice with RhoA-deficient macrophages

RhoA$^{flox/flox}$ mice were gift from dr Richard A. Lang from UC Department of Pediatrics and UC Department of Ophthalmology, Cincinnati Children's Hospital, Cincinnati, Ohio. They were crossbred with transgenic B6.129P2-Lyz2tm1(cre)Ifo/J mice purchased from JAX® Mice (Bar Harbor, Maine, USA) to generate Lyz2$^{Cre+/-}$ RhoA$^{flox/flox}$ mice.

Breeding and all experiments were performed according to The Methodist Hospital Research Institute's animal care and use NIH standards as set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication no. (NIH) 85-23 Revised 1985). The Institute also mandates concordance with the PHS "Policy on Humane Care and Use of Laboratory Animals" and the NIH "Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training."

2.2. Genotyping

Mice were genotyped for RhoA flox and Lyz2Cre using following primers and PCR conditions: RhoA flox primer set: 5′TCTCT GCACTGAGGGAGTTAGG and 5′GTACATACAGGGAATGGAAACAAGG and TaKaRa LA Taq polymerase (Clontech) using following PCR cycle: 94 °C – 4 min initialization step and 35 cycles: 94 °C 30 sec, 58 °C – 30 sec, 72 °C – 30 sec and 72 °C – 7 min final elongation step. Expected PCR products were: WT – 482 bp and RhoA flox=633 bp. Lyz2Cre primer set: mutant 5′CCC AGA AAT GCC AGA TTA CG, common 5′CTT GGG CTG CCA GAA TTT CTC and wild type 5′ TTA CAG TCG GCC AGG CTG AC using following PCR cycle: 94 °C – 3 min initialization step and 35 cycles: 94 °C 30 sec, 62 °C – 1 min, 72 °C – 1 min and 72 °C – 5 min final elongation step. Expected PCR products were: mutant=700 bp, heterozygote=700 bp and 350 bp, and wild type=350 bp.

2.3. Macrophages isolation and culture

All experiments were performed on macrophages derived from (RhoA$^{flox/flox}$ no Cre, further referred to in the text as the "wild type"), heterozygous Lyz2$^{Cre+/-}$ RhoA$^{+/flox}$ and Lyz2$^{Cre+/-}$ RhoA$^{flox/flox}$ transgenic mice and they were repeated at least 3 times. Macrophage purity was confirmed by immunostaining with antibody against macrophage marker mucin-like hormone receptor-like 1 (F4/80) which belongs to the adhesion GPCR family of proteins and flow cytometry.

2.4. Peritoneal macrophages

Isolation of peritoneal macrophages from mouse peritoneal cavity was performed as described in [5]. Purified macrophages were seeded in 12/24 well plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100 μg/ml streptomycin. All media were from Thermo Fisher Scientific, Waltham, MA, USA. After overnight incubation and subsequent removal of the non-adherent cells macrophages were either fixed for staining or incubated for 24 h in the presence of either 20 ng/ml of recombinant murine IFN-γ (Peprotech, Rocky Hill, NJ, USA) and 100 ng/ml of Lipopolysaccharide (LPS) (Sigma Aldrich, St Louis, MO, USA) to polarize into M1 macrophages or with 20 ng/ml of recombinant murine IL-4 (Peprotech) and 20 ng/ml of recombinant murine IL-13 (Peprotech) to polarize into M2 macrophages, as described in Liu et al. [5].

2.5. Bone marrow derived macrophages (BMDMs)

Bone marrow cells were flushed out from mice bone marrow cavity of femurs and tibias using DMEM. Cell suspension was centrifuged at 1700 rpm for 5 min and cell pellet was re-suspended in DMEM containing 10% FBS, antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and 10 ng/ml M-CSF, seeded in culture dishes and incubated at 37 °C and 5% CO_2. After three days medium was changed and cells were incubated for another 3 days, Subsequently cells were washed with DPBS twice to remove the non-adherent cells. Adherent cells were detached (using cell scraper) in 10 ml DPBS and cell suspension was collected and centrifuged at 1700 rpm for 5 min. Cell pellet was re-suspended in fresh DMEM containing FBS and antibiotics (see above) and macrophages were seeded in 12/24 well dishes at density of 1 mln cells/ml.

After overnight incubation at 37 °C and 5% CO_2 the macrophages were incubated 6–24 h in the presence of either 20 ng/ml IFN-γ and 100ng/ml LPS to polarize into M1 or 20 ng/ml IL-4 and 20 ng/ml IL-13 to polarize into M2.

2.6. Actin staining

For actin staining macrophages grown on chamber slides were fixed in 4% formaldehyde (EM grade, Ted Pella, Inc. Redding, CA, USA) in PBS with 0.1% Triton X-100 for 30 min at room temperature stained with rhodamine-phalloidn and mounted as described in Liu et al. [5].

2.7. Immunostaining

For immunostaining macrophages were seeded on chamber slides and fixed in 1% formalin in PBS – 0.1% Triton X100 for 30 min at room temperature as described in Liu et al. [5]. After washing and blocking macrophages were incubated overnight with a 1:50 of FITC conjugated anti-vinculin, F7053 antibody (Sigma-Alrdich, USA). After washing macrophages were mounted in antifade with 10 μg/ml of Hoechst (both from Molecular Probes) and observed with Nikon fluorescence microscope as described in Liu et al. [5].

2.8. Western blotting and flow cytometry

Macrophages were lysed with lysis buffer (Proprietary pink tracking dye in 0.3 M Tris–HCl, 5% SDS, 50% glycerol, 100 mM dithiothreitol (DTT)). Cell lysates were separated by standard SDS-PAGE and analyzed by western blot. Antibodies to the following proteins were used: Rho A (Santa Cruz Biotechnology, Inc. Dallas, TX, USA), ROCK1 (Sigma, USA), CD206 (Abcam), Arg1 (R&D systems), iNos (eBioscience) and GAPDH (Cell Signaling Technology Inc., Danvers, MA, USA) as a loading control [5]. Flow cytometry was performed using F4/80, CD206 and Annexin antibodies (all from Biolegend, USA) as described below for annexin assay.

2.9. ROCK activity assay

Macrophages were lysed using RIPA buffer containing Halt Protease and Phosphatase inhibitor cocktail and the ROCK activity assay which assessed the amount of phosphorylated MYPT1 and ROCK1 was examined using western blot as described in Liu et al. [5].

2.10. ROCK double inhibition assay

M0, M1 and M2 macrophages were treated for 24 h with 30 μM of ROCK inhibitor Y-27632 (Selleckchem, USA) in combination with 10 μM Caspase-3 inhibitor (see below), fixed, stained with