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Research paper

The phenotype of peritoneal mouse macrophages depends on the mitochondria and ATP/ADP homeostasis

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ABSTRACT

Different macrophage subtypes have different morphologies/shapes and functions. Naïve M0 macrophages are elongated. Pro-inflammatory M1 that produce the bactericidal molecule iNos are round. Anti-inflammatory M2 macrophages that produce the pro-healing enzyme Arg-1 are highly elongated. We showed previously that the morphologies of M0 and M2 but not M1 macrophages are RhoA-dependent. Macrophage-specific deletion of RhoA causes the extreme elongation (hummingbird phenotype) of M0 and M2 but not M1 macrophages. The M1 and M2 macrophages also differ in their metabolic status. Here, we studied the effect of the oxidative phosphorylation inhibitors, antimycin A and oligomycin A, at a suboptimal dose, which depolarizes mitochondria but does not eliminate mitochondrial functions, on the mitochondria/energy production and phenotype of wild-type and RhoA-deleted M0, M1 and M2 peritoneal mouse macrophages. We found that, while untreated M1 macrophages had the lowest and the M2 had the highest level of ATP the ATP/ADP ratio was nearly identical between M0, M1 and M2 macrophages. Inhibitor treatment resulted in approximately 60% increase in ATP level and ATP/ADP ratio in M0 and M2 macrophages, and decrease in the level of filamentous (F) actin, and these changes correlated with a drastic shortening/tail retraction of M0 and M2 macrophages, and decreased expression of Arg-1 in M2 macrophages. The treatment of M1 macrophages caused only a 30% increase in the ATP level and ATP/ADP ratio, and while it did not affect the shape of M1 macrophages, it increased the production of iNos. This indicates that the maintenance of mouse macrophage phenotypes depends on mitochondrial function and ATP/ADP homeostasis.

1. Introduction

Macrophages are very heterogeneous phenotypically, functionally and metabolically. Until recently it had been believed that energy production in inflammatory/anti-bacterial M1 macrophages relied mainly on aerobic glycolysis, while anti-inflammatory/healing M2 macrophages used oxidative phosphorylation (OXPHOS) [1]. Although recent studies have shown that such a belief is a tremendous oversimplification and that both glycolysis, and OXPHOS play a role in shaping M1 and M2 phenotypes [2,3], the metabolic profile of M1 macrophages is related to their bactericidal activity and their production of the nitric oxide synthase iNos that kills intracellular pathogens, while the metabolic profile of M2 macrophages relates to the production of Arginase 1 (Arg-1) and its products, such as polyamines, ornithine and urea, which are involved in tissue repair and healing [1]. It has also been shown that the inhibition of oxidative metabolism blocks the M2 phenotype and reverses the macrophage to the M1 state [4,5]. We showed recently that the phenotype/morphology of M0 (slightly elongated), M1 (round) and M2 (highly elongated) macrophages depend on the actin cytoskeleton, which is regulated by the small GTPase RhoA pathway. We also showed that M0, M1 and M2 macrophages respond differently to RhoA pathway interference [6–9]. The macrophage-specific deletion of RhoA or inhibition of RhoA/ROCK kinase disrupts the actin cytoskeleton and causes the extreme elongation

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(hummingbird) phenotype of M0 and M2 mouse macrophages. In contrast, RhoA-deleted M1 macrophages remain round or roundish/multipolar [6–9]. It has also been shown that the mechanically enforced elongation of M0 macrophages induces the M2 phenotype [10]. Several studies have shown that cell elongation requires the relocation of the mitochondria to the most energy-demanding regions of the cell where the most intense reorganization of the actin cytoskeleton occurs [11–13]. Thus, we were interested in if or how the disruption of mitochondrial function would affect the phenotype of M0, M1 and M2 macrophages. We studied the effect of suboptimal doses of the oxidative phosphorylation inhibitors antimycin A, which inhibits cytochrome *c* reductase [14–16], in conjunction with oligomycin A, which inhibits ATP synthase [14,16,17], on the phenotype of wild-type and RhoA-deleted M0, M1 and M2 macrophages. At suboptimal doses, these drugs depolarize the mitochondria but do not completely eliminate mitochondrial functions [18]. We found that inhibitor treatment caused a statistically significant increase in the ATP level and ATP/ADP ratio in M0 and M2 macrophages and a much smaller increase in M1 macrophages. Inhibitor treatment caused a dramatic shortening (tail retraction) of wild-type and RhoA-deleted (hummingbird phenotype) M0 and M2 macrophages and inhibited the expression of Arg-1 in M2 macrophages. In contrast, while inhibitor treatment did not have a noticeable effect on the morphology of M1 macrophages, it increased the expression of the M1 marker iNos. In addition, inhibitor treatment lowered the level of filamentous (F) actin in favor of the globular (G) actin. This suggests that macrophage shortening is a direct effect of deficiency of filamentous actin, which is necessary for cell elongation.

This indicates that the phenotypes of M0/M2 macrophages and M1 mouse macrophages have different requirements not only for RhoA pathway activity but also for mitochondrial function and ATP/ADP homeostasis. Further studies are needed to confirm if the human macrophages have the same requirements like mouse macrophages.

2. Material and methods

2.1. Animals

Breeding and all experiments were performed according to Methodist Hospital Research Institute's animal care and use NIH standards in concordance with the "Guide for the Care and Use of Laboratory Animals" (DHHS publication No. (NIH) 85–23 Revised 1985), 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." All studies were performed according to the animal protocol AUP-0317*0006 (IS00003962) entitled "Tolerance Induction in a Rodent System" approved by Houston Methodist Institutional Animal Care and Use Committee. Mouse euthanasia was performed according to the HMRI Euthanasia of Rodents Procedure by isoflurane overdose via vaporizer inhalation, followed by cervical dislocation and thoracotomy to ensure death. For the genotyping, mice were anesthetized with 3–4% isoflurane. RhoA$^{flox/flox}$ mice (a gift from Dr. Richard A. Lang from the UC Department of Pediatrics and UC Department of Ophthalmology, Cincinnati Children's Hospital, Cincinnati, Ohio) were crossbred with B6.129P2-Lyz2tm1(cre)Ifo/J mice (JAX® Mice (Bar Harbor, Maine, USA) to generate Lyz2$^{Cre+/-}$ RhoA$^{flox/flox}$ mice and genotyped as described in Liu et al. [9].

2.2. Peritoneal macrophages

Mouse peritoneal macrophages from no Cre-negative mice (Lyz2$^{Cre-/-}$ RhoA$^{flox/flox}$), hereafter called the wild-type, or RhoA deleted Lyz2$^{Cre+/-}$ RhoA$^{flox/flox}$ mice, further called the RhoA-KO, were purified. Mice around 8 weeks old were used. 3 mice were used for single experiment Mouse peritoneal cavity was injected three consecutive times with 8 ml of cold DPBS and after 1 min the peritoneal

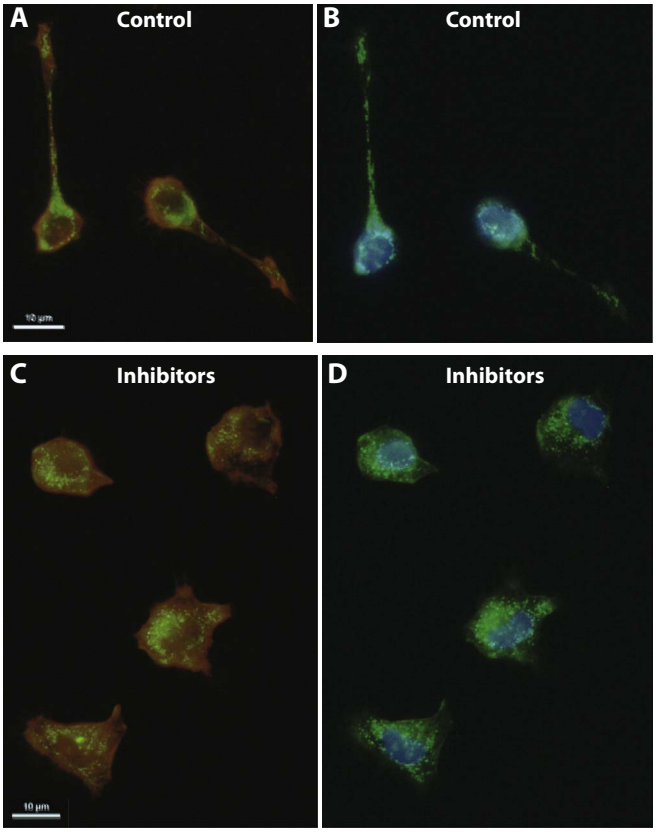

Fig. 1. The effect of antimycin A and oligomycin A on the morphology of M0 macrophages. Macrophages were immunostained with COX4 antibody and FITC (green)-conjugated secondary antibody to visualize the mitochondria and counterstained with rhodamine-phalloidin (red) to visualize actin and with DAPI (blue) to visualize nuclei. (A, B) Untreated M0 macrophages are elongated with interconnected mitochondria distributed around the nucleus and in the tail. (C, D) M0 macrophages treated with combination of antimycin A and oligomycin A show dramatic shortening and clearly separated, "granular" mitochondria. (E) Graph shows statistically significant difference between the average length of the untreated and treated M0 macrophages. We measured the lengths of several tens of macrophages from independent experiments and averaged the macrophage length. We used T test calculated by Graph pad prism 5 and Excel.Panel A and C are merged images of actin and mitochondrial staining. Panel B and D are merged imaged images of mitochondrial staining and DAPI staining. Bar is equal to 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

eluent was collected by centrifugation at 1700 rpm for 5 min. Cell pellet was re-suspended in 5–10 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100 µg/ml streptomycin and seeded in 12/24 well plates. Total number of cells from 3 mice was 3×10^6. All media were from Thermo Fisher Scientific, Waltham, MA, USA. After overnight incubation at 37 °C and 5% CO_2, plates were washed twice with DPBS to remove the non-adherent cells and incubated overnight. The total number