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Graphene oxide nanosheets increase *Candida albicans* killing by pro-inflammatory and reparative peritoneal macrophages

Rosalía Diez-Orejas[a,*], María José Feito[b], Mónica Cicuéndez[c,d], Laura Casarrubios[b], José María Rojo[e], María Teresa Portolés[b,*]

[a] Department of Microbiology and Parasitology, Faculty of Pharmacy, Universidad Complutense de Madrid, 28040, Madrid, Spain
[b] Department of Biochemistry and Molecular Biology, Faculty of Chemistry, Universidad Complutense de Madrid, Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC), 28040, Madrid, Spain
[c] CICECO-Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitario de Santiago, 3810-193, Aveiro, Portugal
[d] TEMA-Nanotechnology Research Group, Mechanical Engineering Department, University of Aveiro, Campus Universitario de Santiago, 3810-193, Aveiro, Portugal
[e] Department of Molecular Biomedicine, Centro de Investigaciones Biológicas, CSIC, 28040, Madrid, Spain

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ABSTRACT

Graphene oxide (GO) is a new nanomaterial with different potential biomedical applications due to its excellent physicochemical properties and ease of surface functionalization. Macrophages play key roles in the control of fungal infections preventing invasive candidiasis by both limiting the growth of the opportunistic fungal pathogen *Candida albicans* and activating other immune effector cells. In order to know if macrophages maintain their immunocompetence against this microorganism after GO uptake, we have evaluated the interactions at the interface of GO nanosheets, macrophages and *Candida albicans*. Poly (ethylene glycol-amine)-derivatized GO nanosheets labelled with fluorescein isothiocyanate (FITC-PEG-GO), were efficiently taken up by peritoneal macrophages inducing a significant increase of *C. albicans* phagocytosis by both pro-inflammatory macrophages (M1/stimulated with LPS/IFN-γ) and reparative macrophages (M2/stimulated with IL-4). On the other hand, after FITC-PEG-GO treatment and *C. albicans* infection, the percentages of GO^+ macrophages diminished when *Candida* uptake increased in every condition (macrophages with no stimuli, M1 and M2 macrophages), thus suggesting the exocytosis of this nanomaterial as a dynamic mechanism favoring fungal phagocytosis. For the first time, we have analyzed the effects of PEG-GO nanosheets on *Candida albicans* killing by unstimulated, M1 and M2 macrophages, evidencing that intracellular GO modulates the macrophage candidacidal activity in a multiplicity of infection (MOI) dependent manner. At MOI 1, the high intracellular GO levels increase the fungicidal activity of basal and stimulated macrophages. At MOI 5, as intracellular GO decreases, the previous pro-inflammatory or reparative stimulus predefines the killing ability of macrophages. In summary, GO treatment enhances classical M1 macrophage activation, important for pathogen eradication, and diminishes alternative activation of M2 macrophages, thus decreasing fungal persistence and avoiding chronic infectious diseases.

1. Introduction

Candida albicans is an opportunistic fungal pathogen that is a frequent cause of mucosal infections along with disseminated candidiasis with high mortality rates [1]. The importance of phagocytes in the immune response against the fungus and the resolution of the infection is well stablished [2], and their absence or defect clearly favors the infection [3]. Macrophages are particularly important because they are critical to prevent invasive candidiasis [4] by both limiting *C. albicans* burden and recruiting and activating other immune effector cells [5].

Macrophages are innate immune cells that possess unique abilities to polarize towards different phenotypes that allow them to respond to changes in their microenvironment. In the extremes of their activation spectrum, these phenotypes are classified as classical (M1) and alternative (M2) activated macrophages [6–8]. Whereas M1 macrophages play a major role in host defense against various microbial pathogens, including fungi, M2 macrophages are instrumental in immune-regulation and wound healing [6–8]. Thus, macrophages can modify their metabolic functions from killing/inhibitory (M1 cells) to healing/growth promoting (M2 cells) [9]. One characteristic difference between

* Corresponding authors.
E-mail addresses: rosaliad@farm.ucm.es (R. Diez-Orejas), portoles@quim.ucm.es (M.T. Portolés).

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these two phenotypes is that in M2 macrophages the arginine metabolism is shifted to the production of ornithine and polyamines, while in M1 cells it is shifted towards production of NO and citrulline [9].

Stimulation of macrophages *in vitro* with IFN-γ and lipopolysaccharide (LPS) results in macrophage polarization towards a pro-inflammatory M1 phenotype. M1 macrophages mediate host defense against foreign pathogens due to their microbicidal activity, related to the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) [10]. On the other hand, the increase of anti-inflammatory cytokines like IL-4 during the response to tissue damage caused by injury or infection induces the polarization of macrophages towards a reparative M2 phenotype [11,12]. This reparative phenotype can be also generated *in vitro* using IL-4 [13]. Interestingly, new biomaterials can be designed that selectively polarize macrophages to obtain the appropriate immune response [14,15].

Graphene oxide (GO) nanomaterials are actively investigated for different biomedical applications due to their small two-dimensional shape in addition to their electrical, mechanical and physicochemical properties, their large surface area and ease of surface functionalization [16]. The biomedical applications of GO include drug delivery [17,18], treatment of cancers and viral infections [19], tissue engineering [20], bioimaging [21] and biosensing [22].

Concerning the interaction of GO with macrophages, different studies have demonstrated the capability of phagocytes for internalizing this nanomaterial with significant effects on their metabolism and activity [23–26]. The interaction of GO with different cells of the immune system (macrophages, lymphocytes, neutrophils, and dendritic cells) has been evaluated [27–30], but many aspects concerning the impact of graphene-based materials like GO on immune responses have not been fully assessed.

Due to the essential role of macrophages during the immune response involved in the control of fungal infections, it is important to evaluate the effects of GO nanosheets on their anti-microbial functions in order to take into account these effects for future GO biomedical treatments. In this regard, we have recently shown that GO uptake by murine peritoneal macrophages modulates their phagocytic capability of the fungal pathogen *Candida albicans*, affecting differentially the subsequent ingestion of live or heat-killed *Candida* [31]. In the present study and for the first time, the effects of poly (ethylene glycol-amine)-derivatized GO nanosheets (PEG-GO) on the candidacidal activity of peritoneal macrophages in the absence or in the presence of pro-inflammatory (LPS/IFN-γ) and reparative (IL-4) stimuli have been analyzed.

2. Experimental

2.1. Preparation and characterization of pegylated GO nanosheets labelled with FITC

GO nanosheets were obtained from exfoliation of high-purity graphite in an acidic medium by a modified Hummers method [32]. Briefly, 2 g of graphite (99.99%; Sigma-Aldrich) was dispersed into a flask containing 50 ml of concentrated H_2SO_4 and 7 g of $KMnO_4$ and the solution was magnetically stirred for 2 h. After that, the solution was treated with H_2O_2 until the gas evolution ceased, then the resultant suspension was thoroughly washed, first with HCl solution 0.0:1 mol dm^{-3}/ and then with distilled water by filtration and centrifugation. The resulting GO suspension was then dialyzed at pH 7.0.

After 3 h of further ultrasonic treatment to reduce the lateral size, 50 ml of graphite oxide in water ≈1 mg ml^{-1}/ was activated with 2.5 g of chloroacetic acid ($Cl-CH_2-COOH$) under strongly basic conditions (2.5 g NaOH), in order to promote $-COOH$ groups at its surface, and bath sonicated for 3 h. The resulting GO solution was neutralized and purified by repeated rinsing and filtration. Activated GO was then functionalized by covalent bonding with non-toxic and non-immunogenic polymer poly (ethylene Glycol-amine) (PEG) to avoid the intercession with cellular functions or target immunogenicities and to decrease aggregation [24]. A diimide activation was performed by adding (EDAC:1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Sigma Inc. 0.106 g) to 45 ml of GO aqueous solution (1 mg ml^{-1}) and stirring vigorously for 3 h. Afterwards this solution was added to a 25 ml solution of polyethylene glycol-amine (11 mg ml^{-1}) and the reaction was allowed to take place for 48 h. Excess PEG was removed by centrifuge filtration. GO water suspensions were systematically washed by centrifugation through MWCO Amicon filters with a 100 kDa molecular weight cutoff.

All GOs were marked with fluorescein isocyanate (FITC) covalent bonded to the PEG in order to control and follow the GOs during the different steps in the *in vitro* cell internalization studies. Pegylated GOs were redispersed in a pH 7.5 phosphate buffer (0.02 M) (0:5 mg ml^{-1}) and reacted with the amine reactive dye FITC. The reaction was allowed to proceed overnight at room temperature in the dark. Excess dye molecules were removed by centrifugation filtration through 100 kDa MWCO Amicon filters and washed away with water several times, until no noticeable color remained in the filtrate solution.

X-Ray Photoelectron Spectroscopy (XPS) spectra were acquired in an Ultra High Vacuum (UHV) system with a base pressure of 2×10^{-10} mbar. NanoGO-PEG powder was dispersed in MQ H_2O and drop coated on a Si wafer. The system is equipped with a hemispherical electron energy analyzer (SPECS Phoibos 150), a delay-line detector and a monochromatic AlKα (1486.74 eV) X-ray source. High resolution spectra were recorded at normal emission take-off angle and with a pass-energy of 20 eV, which provides an overall instrumental peak broadening of 0.5 eV. The XPS spectra were calibrated in binding energy by referencing to the first component of the C 1s spectrum to 284.8 eV.

Atomic force microscopy (AFM) analysis of these nanoparticles was carried out in a Bruker AFM multimode Nanoscope III A. Zeta-potential (ζ), and dynamic light scattering (DLS) particle size analysis were performed in a Zetasizer Nano series instrument equipped with a 633 nm “red” laser from Malvern Instruments.

2.2. Isolation, culture and stimulation of murine peritoneal macrophages

Basal macrophages were obtained from the peritoneum of mice as described elsewhere [33]. Briefly, CL57/BL mice were killed, and the skin was removed from the abdominal area. Mice were then injected intraperitoneally with 4–5 ml of phosphate buffered saline (PBS) using 18 gauge needle. Without extracting the needle, the abdomen was gently massaged and then as much fluid from the peritoneum as possible was slowly withdrawn with the syringe. After removing, the peritoneal cells were gently washed with PBS before use. All procedures were approved by Institutional Animal Care and Use Committees. Murine peritoneal cells were seeded in 6 well culture plates (CULTEK S.L.U., Madrid, Spain) at 10^5 cells/ml in 2 ml of culture medium (Dulbecco's Modified Eagle Medium DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS, Gibco, BRL), and 1 mM L-glutamine, 200 µg/ml penicillin, and 200 µg/ml streptomycin, all from BioWhittaker Europe (Belgium), and incubated at 37 °C under a 5% CO_2 atmosphere.

Polarization of peritoneal macrophages was achieved by treatment for 24 h with different stimuli. “Classically activated” or M1 macrophages were obtained upon stimulation with *E. coli* lipopolysaccharide (LPS, 250 ng/ml, Sigma-Aldrich Corporation, St. Louis, MO, USA) and interferon gamma (IFN-γ, 100 ng/ml, Sigma-Aldrich Corporation, St. Louis, MO, USA), meanwhile “alternatively activated” M2 macrophages were generated by incubation of macrophages with interleukin-4 (IL-4, 20 ng/ml, Sigma-Aldrich Corporation, St. Louis, MO, USA). M1 and M2 macrophage phenotypes were characterized by flow cytometry by the expression of CD80 and CD206, respectively [34]. After detachment and centrifugation, cells were incubated in 45 µl of staining buffer (PBS, 2.5% FBS Gibco, BRL and 0.1% sodium azide, Sigma-Aldrich