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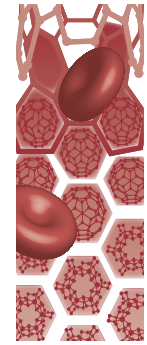

Copper oxide nanoparticles recruit macrophages and modulate nitric oxide, proinflammatory cytokines and PGE_2 production through arginase activation

Aim: In the present study, we examine the effects of copper oxide nanoparticles (CuNP) on macrophage immune response and the signaling pathways involved. **Materials & Methods:** A peritonitis model was used to determine *in vivo* immune cells recruitment, while primary macrophages were used as an *in vitro* model for the cellular and molecular analysis. **Results:** *In vivo*, CuNP induce significant macrophages recruitment to the site of injection. *In vitro,* in LPS-stimulated primary macrophages, the co-treatment with CuNP inhibited the production of NO in a dose-dependent manner. The mechanism underlying NO and proinflammatory cytokines inhibition was associated with an increased arginase activity. Macrophage stimulation with CuNP did not provoke any cytokine secretion; however, arginase inhibition promoted TNFα and MIP-1β production. In addition, CuNP induced the expression of COX-2 and the production of PGE_2 through arginase activation. **Conclusion:** Our results demonstrate that CuNP activate arginase and suppress macrophage innate immune response.

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Keywords: cyclooxygenase 2 • immunotoxicology • inducible nitric oxide synthase • nanomedicine

Sergio Arancibia*[1,2], Andrea Barrientos[2], Javiera Torrejón[2], Alejandro Escobar[3] & Caroll J Beltrán[4,5]

[1]Investigación y Desarrollo, Fundación Ciencia y Tecnología para el Desarrollo, Santiago, Chile

[2]Investigación y Desarrollo, Laboratorio Centrovet, Santiago, Chile

[3]Instituto de Investigación en Ciencias Odontológicas, Facultad de Odontología, Universidad de Chile, Santiago, Chile

[4]Facultad de Medicina, Universidad de Chile, Santiago, Chile

[5]Departamento de Gastroenterología, Hospital Clínico de la Universidad de Chile, Santiago, Chile

*Author for correspondence:
Tel.: +562 25837754
sergio.arancibia@centrovet.com
sergio.arancibia@fucited.cl

The rapid development of nanotechnology has led to the increased use of nanomaterials in several fields. The interaction of nanoparticles with components of the immune system can be considered appropriate when it may lead to advantageous applications. However, in certain cases, acute or prolonged stimulation with nanoparticles may produce severe complications [1]. Because of their antimicrobial properties, copper oxide nanoparticles (CuNP) are frequently included in many pharmaceutical products and medical devices, among other applications. Given their widespread use, there is an increasing demand to study the immunotoxicological influence of CuNP on human health. Previous reports have demonstrated the high toxicity of these particles, demonstrating their ability to induce cell death in several epithelial cell lines, mainly through the production of reactive oxygen species (ROS), a process that is dependent on both nanoparticle size and cell type [2–4]. *In vivo*, CuNP promote several toxicological events, including oxidative stress, changes in lipid profile and severe increases in liver, kidney and spleen toxicity levels [5,6]. In a model of pulmonary infection, CuNP impaired bacterial clearance by decreasing the antimicrobial capacity of macrophages, suggesting a negative regulation of macrophage effector functions [7]. Macrophages play an important role in pathogen clearance where the production of NO is crucial. High levels of NO produced by macrophages induce cytotoxicity in bacteria, viruses, parasites, protozoa and tumor cells [8,9]. In this regard, it has been reported that CuNP inhibit LPS-mediated NO production in microglia and bone marrow-derived macrophages (BMMs), respec-

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tively, suggesting that reduced production of NO may contribute to a deficiency in pathogen clearance [10,11]. Nonetheless, the mechanisms by which CuNP alter NO generation remains unclear.

Two enzymes, inducible nitric oxide synthase (iNOS) and arginase tightly regulate the metabolism of L-arginine in macrophages. iNOS catalyses the oxidation of L-arginine to NO and L-citrulline, while arginase, a manganese-containing enzyme, hydrolyses L-arginine to produce urea, L-ornithine and polyamines. Crucially, iNOS and arginase utilize the same substrate; therefore, the activity of both enzymes are intrinsically linked [12,13]. The activation of iNOS and arginase can be triggered by either pathogen-associated molecular patterns (PAMPs) or different cytokines, which influence the activation of the macrophage into a classical (M1) or alternative (M2) phenotype [14]. Moreover, the generation of NO has also been associated with the inhibition of COX-2-derived prostaglandin production, and recently it was shown that COX-2 induces the expression of arginase-1 in lung carcinoma cells, indicating the existence of molecular crosstalk between iNOS, arginase and COX-2 enzymes [15,16].

In the current study, we used primary macrophages to investigate the consequences of CuNP on triggering signaling proinflammatory molecules and modulating intracellular pathways through arginase activation. This process may lead to a less destructive form of immunity or it will produce a regulatory macrophage phenotype, attenuating host immune response.

Materials & methods

Reagents

CuNP, AlNP, ZnNP and TiNP nanoparticles (30–50 nm) were purchased from SkySpring nanomaterials Inc. (USA). The nanoparticles with a purity >99.5% were produced under sterile and endotoxin free conditions. Nanoparticles were dispersed on sterile ultrapure water (Hyclone, USA) and sonicated to diminish particle aggregation before each test. Lipopolysaccharide (LPS) from *Escherichia coli* serotype R515, NG-nitro-L-arginine methyl ester (L-NAME – iNOS inhibitor), N-Ω-Hydroxy-L-norarginine (nor-NOHA – arginase inhibitor), 2(S)-Amino-6-boronohexanoic acid (ABH – arginase inhibitor) and N-acetylcysteine (NAC – free radical scavenger) were all from Enzo Life Sciences (USA). Antibodies for COX-2, arginase-1 and β-actin were supplied by Cell Signaling Technology (USA) and the anti-arginase-2 by Abcam (UK). Phosphate buffered saline (PBS) was from Hyclone (USA).

Mice

C57BL/6 mice were obtained from Instituto de Salud Publica de Chile (ISP). Experimental mice (8–12 weeks old) were housed in a controlled access area at 22–24°C with 12/12h light/dark cycle and studied in compliance with institutional guidelines (CICUAL).

Cells

The isolation of thioglycollate-elicited peritoneal macrophages was prepared as previously described [17]. Briefly, C57BL/6 mice were injected intraperitoneally (ip.) with 1.5 ml of thioglycollate broth solution. Five days later macrophages were collected from the peritoneum cavity using cold sterile PBS and selected by adherence for 1 h at 37°C in complete medium. The purity of the culture was 95% (F4/80 and CD11b double positive cells). Peritoneal macrophages were cultured at 37°C in 5% CO_2 environment with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Hyclone). The murine macrophage/monocyte cell line J774.2 was kindly donated by Dra. Maria Inés Becker (Fundación Ciencia y Tecnología para el Desarrollo, Chile). The cells were maintained at 37°C in 5% CO_2 environment with RPMI-1640 complete medium.

Bone marrow-derived macrophages (BMMs) were produced according to Zhang *et al.* [18] and maintained at 37°C in 5% CO_2 environment with RPMI-1640 complete medium. Briefly, bone marrow were obtained from the femur and tibia from 3 months old mice. Bone marrow cells were differentiated in RPMI-1640 medium supplemented with 15% L929 supernatant containing 10% FSB, 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO_2 at 37°C. After 7 days, bone marrow-derived macrophages were harvested and analyzed by FACS (98%, F4/80-CD11b-positive cells).

Cell viability

Thioglycollate-elicited peritoneal macrophages, BMMs and J774.2 cells were used to determine the nanoparticles cytotoxicity. The cells were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with differing concentration of nanoparticles. After 24 h of treatment, each well was incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT solution – 2.5 mg/ml) for 4h at 37°C. The formed crystals were dissolved with acidified isopropanol (HCl 1N) and measured by colorimetric analysis at 570 nm with an Asys ELISA reader (USA).

PGE_2 & cytokines detection

Peritoneal macrophages were seeded at 7.5×10^5 cells/ml and stimulated with combinations of LPS (10 ng/ml) and CuNP (1 µg/ml). The pharmacological inhib-

itors or the free radical scavenger was used 1 h prior to LPS or CuNP challenge. After 24 h of treatment, the supernatants were collected and the level of PGE_2 was determined using a commercial ELISA kit according to the manufacturer's instructions (R&D systems). Cytokines and chemokines quantification were determined using a validated Multiplexing Laser Bead Assay (Eve Technologies, Canada).

Immune cells recruitment (peritonitis model)

C57BL/6 mice (n = 4) were injected ip. with PBS (100 µL) or CuNP (200 µg/mouse/100 µL). After 1 h, peritoneal lavage cells were collected and studied by flow cytometry. The cells were stained with several cell surface markers in order to determine the recruitment of different populations. The antibodies used in the analysis were CD11b-PE, Gr1-FITC, F4/80-PerCP Cy5.5, CD19-PE, CD3-FITC, CD4-APC, NK1.1-PerCP Cy5.5, CD117-APC (Biolegend). Samples were acquired with a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star Inc., USA). The results were expressed as a percentage of live cells (mean ± SE, $^*p < 0.05$ and $^{****}p < 0.0001$).

Nitric oxide measurement

The level of nitric oxide was detected using the Griess reaction. This method indirectly detects nitric oxide production by the accumulation of nitrite as a stable end product in cell culture supernatants. Macrophages were plated at 7.5×10^5 cells/ml in 96-well plates and stimulated for 24 h with combinations of LPS (10 ng/ml), nanoparticles (0.1, 1 and 10 µg/ml). Different pharmacological inhibitors or free radical scavengers were added 1 h prior to LPS or CuNP treatments. The supernatants were collected and mixed with the Griess reagent at room temperature for 10 min. The colorimetric product was measured at 570 nm with an Asys ELISA reader.

Arginase assay

Macrophages were seeded at 1×10^6 cells/ml in 6-well plates and stimulated for different time points with copper oxide nanoparticles (1 µg/ml). The cells were washed with PBS, lysate with a low detergent buffer (20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 150 mM NaCl and 0.5% Triton X-100, protease and phosphatase inhibitors), centrifuged at 10.000 rpm for 10 min at 4°C and the supernatants were collected for assay. Arginase activity was measured by the determination of urea formation as previously described [19]. Briefly, to activate arginase, cell lysates were incubated with $MnCl_2$ (1:1) at 56°C for 10 min. Two volumes of the enzyme substrate L-arginine were then added and incubated for 1 h at 37°C. The reaction was stopped with a 1:3:7 mixtures of H_2SO_4, H_3PO_4 and H_2O. Next, the samples were incubated with α-isonitrosopropiophenone (9% in ethanol) and heated in the dark for 45 min at 100°C. Finally, samples were analyzed at 570 nm with an Asys ELISA reader. Each result was normalized to protein content and expressed as a percentage of the control.

Western blot

Stimulated macrophages were lysed with RIPA buffer (Santa Cruz biotechnologies, USA) and mixed with a buffer-containing SDS and 4% β-mercaptoethanol, then heated for 5 min at 100°C, and analyzed by SDS-PAGE on 10% polyacrylamide separating gel, as described by Laemmli [20]. Protein samples were transferred onto a nitrocellulose membrane and developed with an anti-arginase-1, arginase-2 and COX-2 antibodies. An anti-β-actin antibody was used as a loading control.

Transmission electron microscopy (TEM)

To study nanoparticle internalization, 1×10^6 thyoglycollate-elicited peritoneal macrophages were challenged with several nanoparticles for 10 min, and then the cells were washed, collected and centrifuged at 1500 rpm for 5 min. Finally, the cells were washed with PBS, and fixed with PBS-2% glutaraldehyde solution (Merck, USA). Macrophages were postfixed with OsO_4, dehydrated and embedded in Epon (Polyscience, USA) according to Luft [21]. Then, the samples were stained with lead citrate according to the procedure by Reynolds [22]. The preparations were examined with a Philips Tecnai 12 electron microscope (Pontificia Universidad Catolica de Chile).

Statistical analysis

The results of the experiments were expressed as means ± SE. Comparisons between groups were made using unpaired *t*-test and one-way or two-way ANOVA and Tukey's or Sidak's post-test, respectively. Statistical significance was defined as a p value <0.05. Analyses were performed using GraphPad Prism software (USA).

Results

CuNP promote macrophages recruitment *in vivo*

To understand, *in vivo,* the modulatory properties of copper oxide nanoparticles on the immune responses, C57BL/6 mice were injected ip. with PBS or PBS containing CuNP suspension. The infiltrating cells in the peritoneal cavity were analyzed using flow cytometry. We observed that after 1 h of treatment with CuNP, there was a significant macrophage recruitment

(Figure 1A). In contrast, no significant changes were observed with T and B cells, mast cells or NK cells recruitment between CuNP and the control group. These results show that macrophages are among the first cells of the innate immune system recruited by CuNP at the site of injection, suggesting an important role in nanoparticle response and clearance (Figure 1B).

CuNP are internalized by peritoneal macrophages inducing cell death

Given that CuNP promote rapid macrophage recruitment and that this cell is the first line of defence against harmful invading substances, we studied the impact of CuNP in the function of macrophages. We employed transmission electron microscopy (TEM) to analyse and compare the uptake of silica, titanium, aluminium and copper oxide nanoparticles. The data showed that peritoneal macrophages readily internalized all the nanoparticles tested. Large aggregates were mainly internalized by macropinocytosis; however, it is possible that smaller aggregates use other processes simultaneously, such as clathrin-mediated endocytosis (Figure 2A) [23]. Once inside the cell, CuNP were found to localize within both endosome-like structures and the cytoplasm, whereas SiNP, TiNP and AlNP were mostly found only in endosome-like organelles (Figure 2A).

Nanoparticle toxicity was evaluated by measuring the viability of the monocyte/macrophage cell line J774.2 and comparing it with a primary culture of peritoneal macrophages. J774.2 cells exposed to high concentrations of CuNP (10 μg/ml) for 24 h, displayed a significantly reduced viability up to 50% (Figure 2B) compared with a 25% reduction in the viability of peritoneal macrophages (Figure 2C). The exposure of CuNP at concentrations lower than 10 μg/ml did not affect cell viability either in J774.2 or peritoneal macrophages, while challenging with TiNP, SiNP or AlNP produced an innocuous outcome in both cell lines, at all concentrations tested. Our data reveal that CuNP are rapidly internalized in peritoneal macrophages and macrophage-like cell lines and that the exposure of CuNP to these cells promote cytotoxic effects at high concentrations.

CuNP inhibit LPS-mediated NO production

The elimination of pathogens and extraneous molecules by macrophages requires the production of inflammatory mediators and free radicals such as nitric oxide. It has been reported that CuNP reduce the phagocytosis and LPS-mediated NO production from BMMs and microglial cells, respectively. In this context, we sought to evaluate whether the production of NO was altered by CuNP treatment in our cell model. Peritoneal macrophages were treated with several nanoparticles for 24 h to measure the nitrite content in the supernatant of stimulated cells, as an indirect method to determine NO production. The results showed that CuNP, TiNP, SiNP and AlNP did not induce the generation of NO after 24 h of treatment (Figure 3A–D). Given that nanoparticles may enter the body in conjunction with microorganisms or microbial products, we evaluated the response of macrophages to nanoparticles in the presence of LPS. Peritoneal macrophages were co-treated with LPS and nanoparticles to measure NO production following 24 h of stimulation. The results demonstrated that the LPS-mediated NO production was not affected by SiNP, AlNP or TiNP (Figure 3B–D). In contrast, CuNP significantly diminished the generation of LPS induced NO in a dose-dependent manner (Figure 3E).

CuNP inhibition of NO production is dependent on arginase activation

Given that iNOS and arginase use L-arginine as a common substrate, we hypothesized that CuNP may be acting by means of increasing arginase activity, thereby reducing L-arginine availability for NO production [12]. To address this hypothesis, peritoneal macrophages were preincubated with nor-NOHA and ABH, two specific arginase inhibitors, and then co-treated with CuNP and LPS for 24 h. The results demonstrated that the inhibition of arginase activity via nor-NOHA or ABH restored the production of NO induced by LPS, following stimulation with CuNP (Figure 4A & Supplementary Figure 1, respectively). The inhibitor L-NAME was used as a positive control in order to determine iNOS activity. The results showed that L-NAME completely abrogated NO production in LPS-stimulated macrophages (Figure 4B). These results indicated that CuNP diminished the production of NO by means of arginase activation, suggesting that copper may play a role in the intracellular modulation of arginase activity.

Given that CuNP induce high levels of intracellular free radicals [24]; we sought to evaluate their possible role in arginase activity. These molecules can influence the expression of several genes and signal transduction pathways. Indeed, it has been reported that hydrogen peroxide and the hydroxyl radical can activate or upregulate arginase, thereby affecting different extra- and intracellular processes [25,26]. In order to analyze the role of these molecules in arginase activity, N-acetylcysteine (NAC) was used as a free radical scavenger. Peritoneal macrophages were preincubated with NAC and then co-stimulated with CuNP and LPS for 24 h. The results demonstrated that free radicals were not involved in the inhibition of NO production by CuNP,

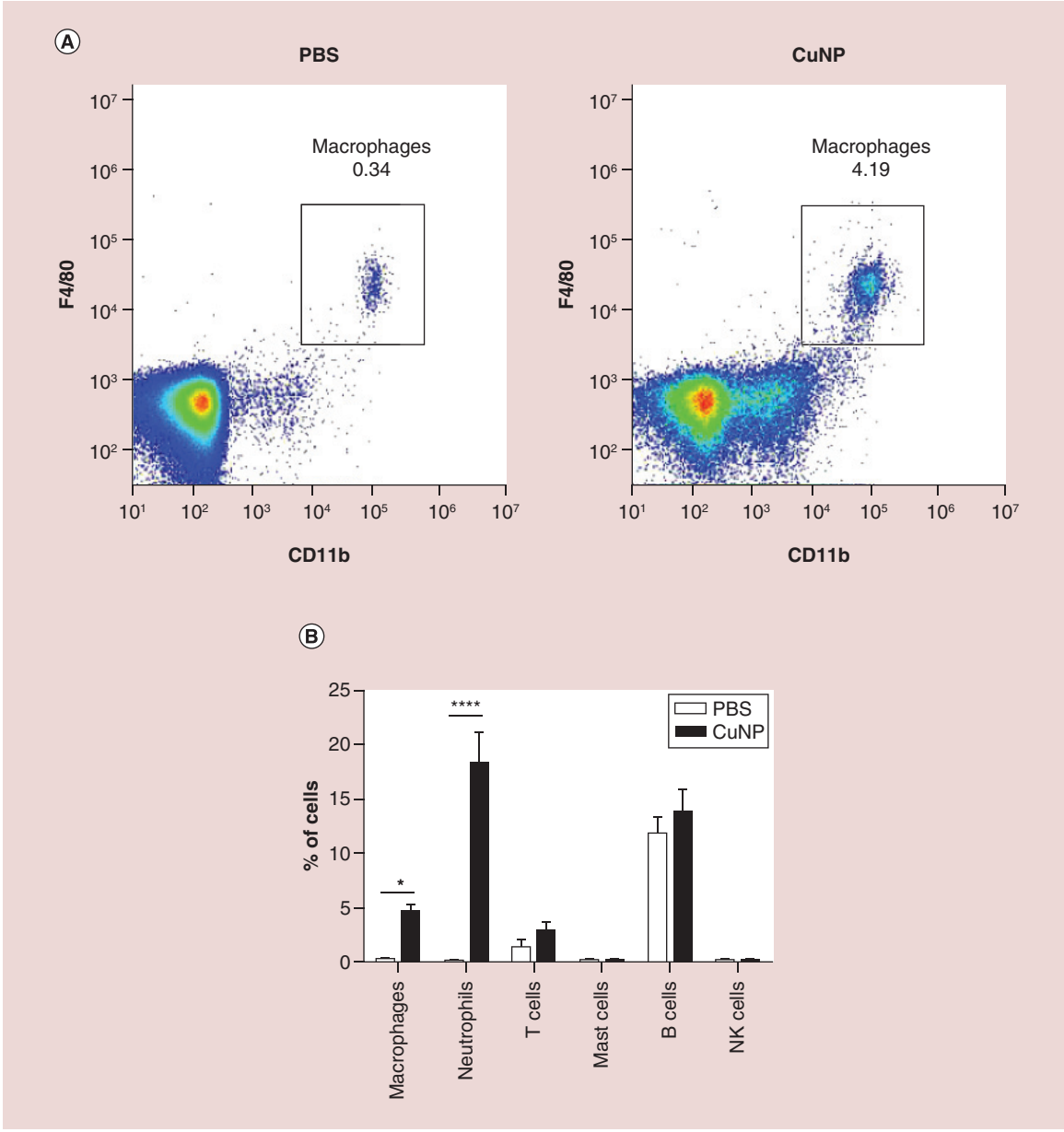

Figure 1. CuNP modulate immune cells infiltration into peritoneal cavity. (A & B) C57BL/6 mice were injected intraperitoneally with PBS (100 μL) or CuNP (100 μg/mouse/100 μL). After 1 h, infiltrating peritoneal cells were collected and analyzed by flow cytometry. Immune cells were characterized as follows: $CD11b^+/F4/80^+$ macrophages, GR-1+/CD11b+ neutrophils, $CD3^+/CD4^+$ T cells, $CD19^+$ B cells, NK1.1+ NK cells, $CD117^+$ mast cells. CuNP groups were compared with the PBS group and significance was assessed using two-way ANOVA Sidak's post-test, n = 4.
*$p < 0.05$; ****$p < 0.0001$.

implicating alternative mechanisms, which remain unidentified (Figure 4C).

CuNP augment arginase activity

We next studied whether CuNP diminished LPS-mediated NO production directly by modulating arginase activity or indirectly by increasing its protein expression. First, we determined the activity of arginase by measuring the formation of urea, as the end product of the enzymatic reaction. Peritoneal macrophages were incubated with CuNP for different time points. The results displayed a time-dependent increase in arginase activity, following exposure to CuNP. Peritoneal macrophages exposed for 6 and 24 h to the particles presented a significantly higher arginase activity than control cells (Figure 5A). Next, we sought to validate the

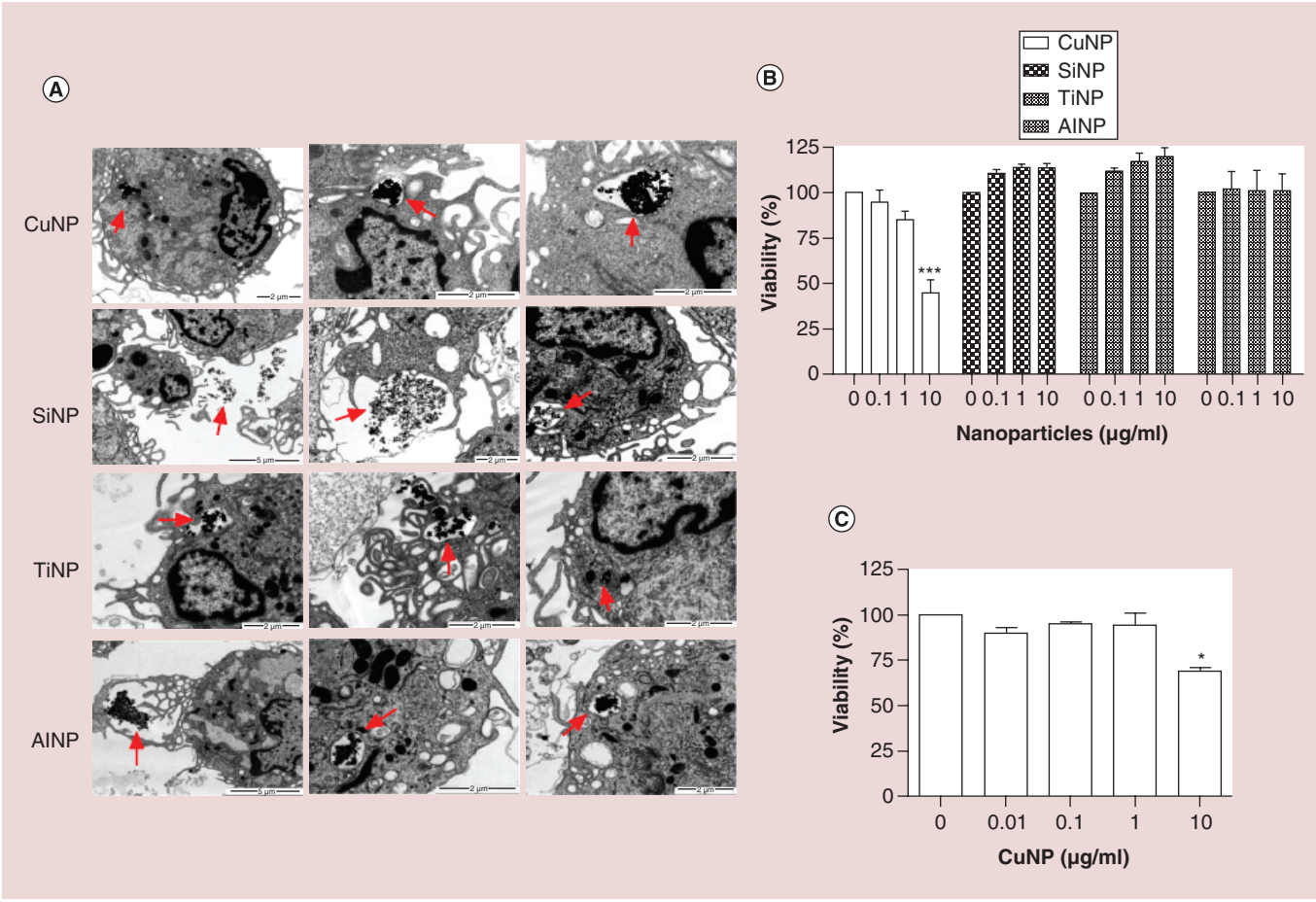

Figure 2. Copper nanoparticles are internalized by peritoneal macrophages. **(A)** TEM analysis of peritoneal macrophages exposed for 10 min to CuNP, SiNP, TiNP and AlNP nanoparticles (n = 2). The micrographs show nanoparticle aggregates being internalized by macropinocytosis into endosome-like structures. **(B)** J774.2 cells were incubated with the particles for 24 h to determine cell viability by the MTT assay (2.5 mg/ml). One-way ANOVA - Tukey's post-test, n = 3, ***p < 0.001 between CuNP and the control. **(C)** Peritoneal macrophages treated with CuNP for 24 h. Cell viability was expressed as a percentage of the control. One-way ANOVA – Tukey's post-test, n = 3, *p < 0.05 between CuNP and the control.

inhibitory properties of nor-NOHA, as arginase inhibitor. BMMs treated for 1 h with the inhibitor showed a significant reduction (50%) of arginase activity in comparison with the control group, demonstrating its properties (Figure 5B). In order to observe the effect of nor-NOHA in combination with CuNP on arginase activity, BMMs were stimulated at different time points. In BMMs, CuNP increased arginase activity more rapidly and to a greater extent than peritoneal macrophages, given that at 30 min of treatment the activity was more than 200%, reaching a maximum of 250% at 24 h. In addition, macrophages pretreated with nor-NOHA and then stimulated with CuNP significantly reduced the arginase activity after 0.5, 6 and 24 h of CuNP treatment, validating the pharmacologic inhibition of the enzyme by nor-NOHA (Figure 5C). Western blot analysis indicated that CuNP did not affect the expression of either arginase-1 or arginase-2 synthases, suggesting that the CuNP-mediated reduction of NO was driven by a direct increase in arginase activity (Figure 5D). Moreover, we evaluated the role of arginase activation on CuNP-induced cell death in primary macrophages. The results indicated that arginase was not implicated in macrophage cell death upon CuNP stimulation (Supplementary Figure 2).

CuNP modulate proinflammatory cytokine secretion through arginase

Given that CuNP influence nitric oxide production, we sought to evaluate whether CuNP can also modulate macrophage cytokines production. BMMs were stimulated with LPS, CuNP or PBS (control group) for 24 h and the supernatants were analyzed using a cytokine/chemokine array. Our study indicated that CuNP did not induce the production of cytokines or chemokines in comparison with the control group (Figure 6A). In contrast, LPS treatment promoted high levels of several proinflammatory mediators, such as

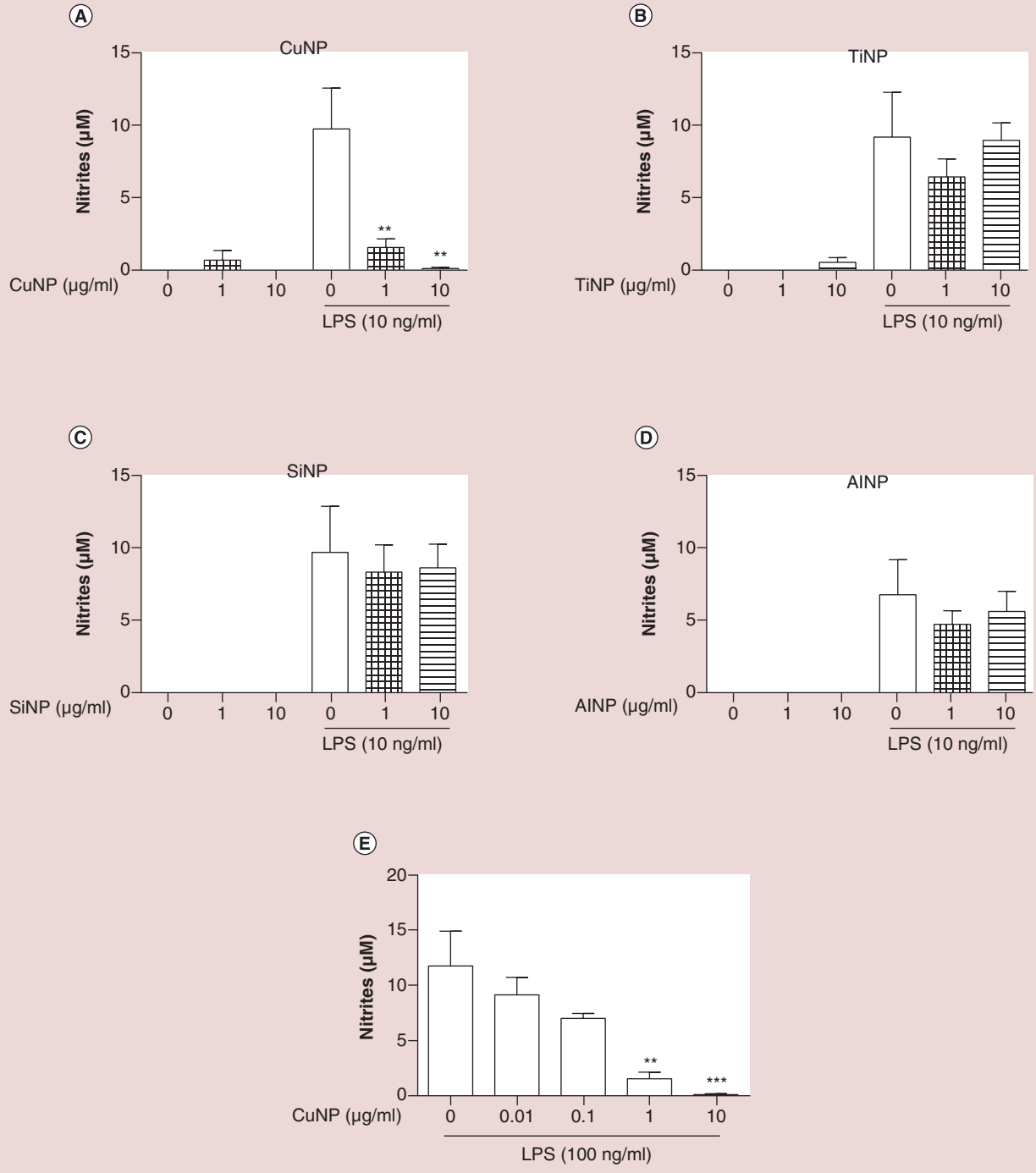

Figure 3. Copper nanoparticles inhibit LPS-mediated nitric oxide production. (A–D) Peritoneal macrophages challenged with different nanoparticles for 24 h. Only CuNP inhibited LPS-mediated nitric oxide production. Nitrite concentration was measured in triplicate by means of Griess reaction. One-way ANOVA – Tukey's post-test, n = 3, **p < 0.01 between control and CuNP (1 and 10 μg/ml). **(E)** Dose-dependent inhibition of NO generation by peritoneal macrophages stimulated with CuNP in combination with LPS (100 ng/ml) for 24 h. One-way ANOVA Tukey's post-test, n = 3, **p < 0.01 and ***p < 0.001 between CuNP treatment and unstimulated cells.

IL-6, TNFα, IL-12p40, RANTES, MIP-1α and MIP-1β. Interestingly, macrophages pretreated with arginase inhibitors (nor-NOHA and ABH) induced the secretion of high levels of TNFα and MIP-1β (Figure 6B & C, & Supplementary Figure 3, respectively). Taken together, these results demonstrated that arginase modulates CuNP-induced proinflammatory cytokine secretion in murine macrophages.

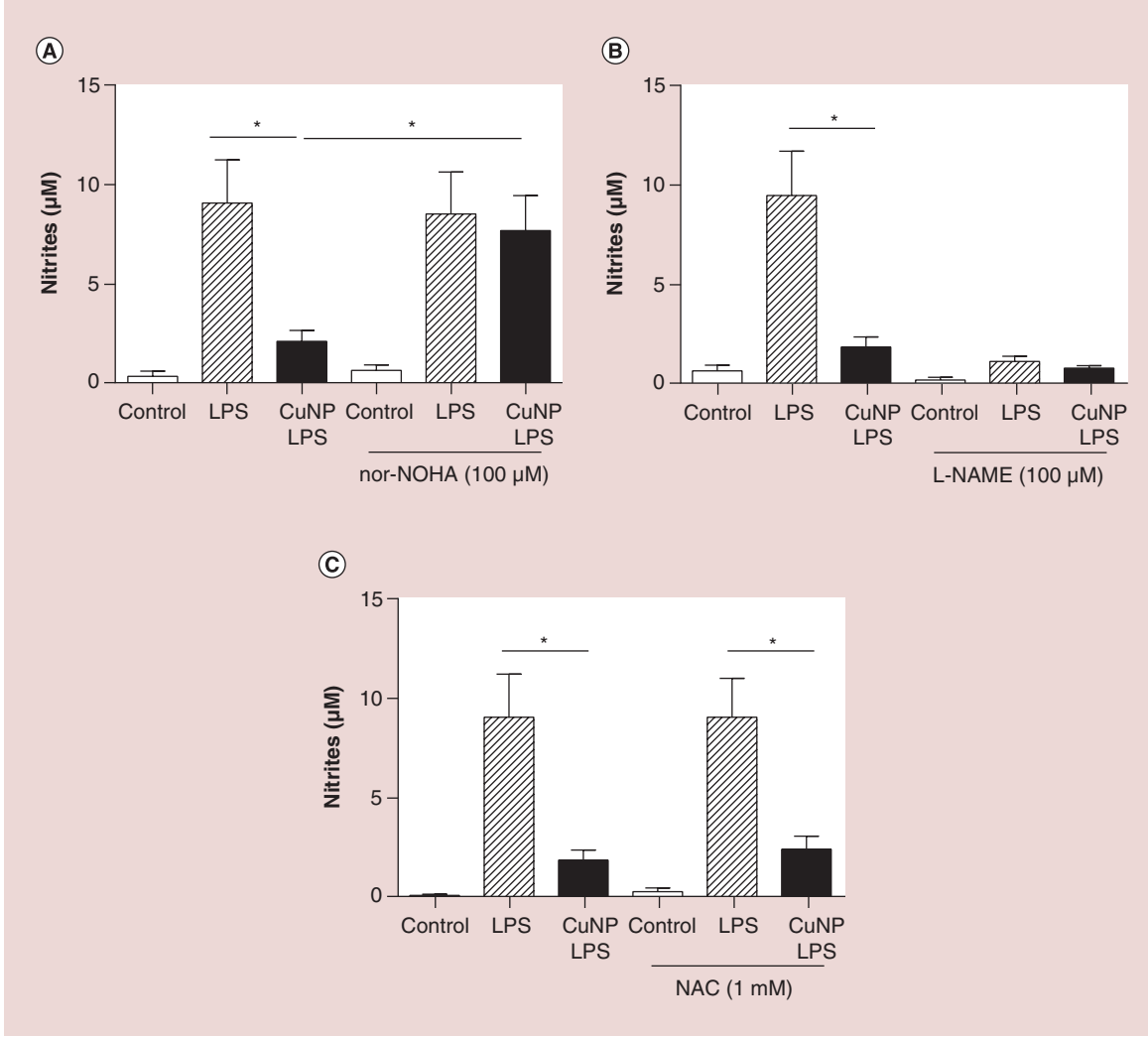

Figure 4. Copper nanoparticles suppress the production of NO. (A–C) Peritoneal macrophages were stimulated for 1 h with the pharmacological inhibitors of iNOS (L-NAME) and arginase (nor-NOHA) as well as NAC as an antioxidant prior to CuNP, LPS or combined treatments. After 24 h, nitrite concentration was measured in the supernatant of stimulated cells by the Griess reaction. One-way ANOVA – Tukey's post-test, n = 5, *p < 0.05.

CuNP induces PGE_2 via arginase activation

Prostaglandin E_2 (PGE_2) is most abundant in mice and humans and is crucial for a numerous biological activities. The synthesis of this versatile mediator is controlled by cyclooxygenases (COXs) and PGE_2 synthases [27]. A recognized molecular crosstalk exists between COXs and NOS signaling pathways in order to regulate tissue homeostasis and pathophysiological processes. A detailed study in macrophages demonstrated that NO activates COX-1 but inhibits COX-2-derived prostaglandin production [15]. Given that CuNP modulate NO production in peritoneal macrophages, we next analyzed whether CuNP treatment could modify COX-2 expression and thus PGE_2 generation. Peritoneal macrophages were treated with CuNP for 24 h and the supernatants were analyzed for PGE_2 production. CuNP significantly enhanced the prostaglandin production in comparison with the control (Figure 7A). Western blot analyses indicated that the generation of PGE_2 by CuNP was associated with an increased expression of COX-2 (Figure 7B). In these experiments, LPS treatment was used as a positive control of COX-2 activation. Given that arginase is a key factor involved in the inhibition of NO, we evaluated the role of this enzyme on PGE_2 production. The data indicated that arginase inhibition with nor-NOHA significantly abrogated CuNP-induced PGE_2 secretion in peritoneal macrophages, a process that was not observed with the LPS treatment (Figure 7C).

Discussion

To evaluate nanoparticle toxicity, the study of their interaction with the immune system becomes essential to understanding their final effects. Macrophages are

antigen-presenting cells that have a great capacity to recognize and internalize foreign molecules and pathogens in order to mount an appropriate immune response against them. Several nanoparticles have been reported to promote proinflammatory responses by altering different signaling pathways, such as silica, carbon, titanium and silver nanoparticles, among others [28]. However, few studies have focused on the effect of copper nanoparticles on primary macrophages.

In our study, we observed that *in vivo* CuNP recruit different innate immune cells such as macrophages and neutrophils. Macrophages, in particular, are crucial to initiate the immune response to different foreign molecules or harmful agents. Similar macrophage and neutrophils infiltration was observed in mice injected with different types of functionalized carbon nanoparticles [29], suggesting that macrophages play a crucial role in nanoparticles clearance. *In vitro*, all nanoparticles tested were rapidly internalized by peritoneal macrophages through macropinocytosis. Similar results have been observed with gold nanoparticles, indicating that particles of 50 nm were more rapidly and efficiently incorporated by the cell in comparison with particle of 14, 30, 74 and 100 nm [30]. The increased uptake of 50 nm nanoparticles was related to their capacity to bind specific cell surface receptors, which will finally produce the membrane-wrapping process [31]. Different effects have been observed between particles of differing sizes, while nanoparticles of 40–50 nm demonstrated strong effects in terms of alterations to intracellular processes,

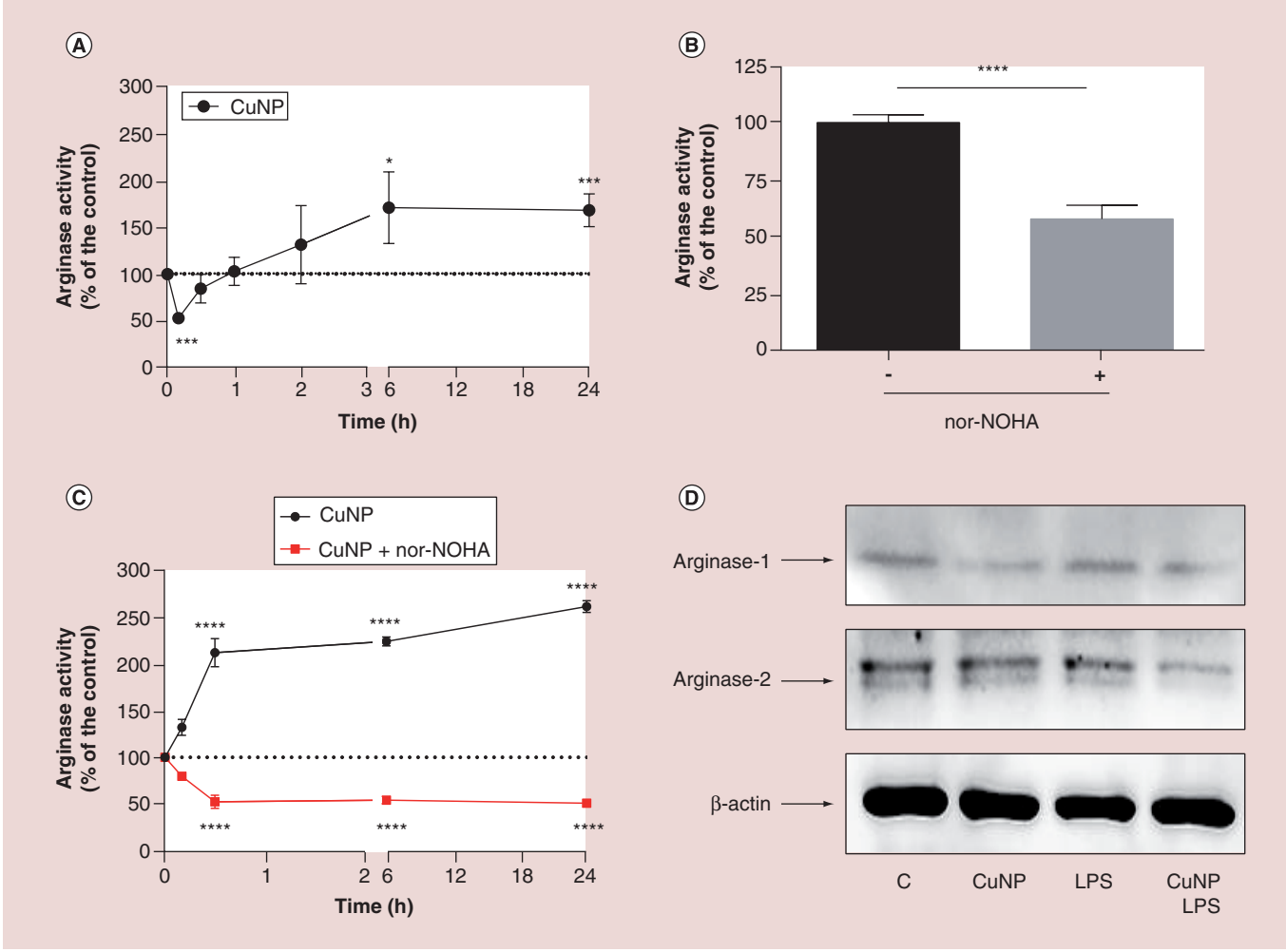

Figure 5. Copper nanoparticles activate arginase but do not induce its expression. (A) Peritoneal macrophages were stimulated at different time points with CuNP (1 μg/ml). Arginase activity was measured by determining urea formation. One-way ANOVA – Tukey's post-test, n = 3, *p < 0.05, **p < 0.01 and ***p < 0.001 between CuNP and unstimulated cells. **(B)** BMMs were exposed to nor-NOHA for 24 h to measure the arginase activity. Unpaired t-test, n = 2, ****p < 0.0001 between nor-NOHA and unstimulated cells. **(C)** BMMs were stimulated at different time points with CuNP (1 μg/ml) or a combined treatment with nor-NOHA 1 h prior to CuNP treatment. Arginase activity was measured by means of urea formation. One-way ANOVA – Tukey's post-test, n = 3, ****p < 0.001 between CuNP and CuNP+nor-NOHA. **(D)** Western blot analysis of peritoneal macrophages stimulated for 24 h with CuNP, LPS and combined treatments. The cell lysates were developed with anti-arginase-1 and arginase-2 antibodies. β-actin was used as a loading control (n = 4).

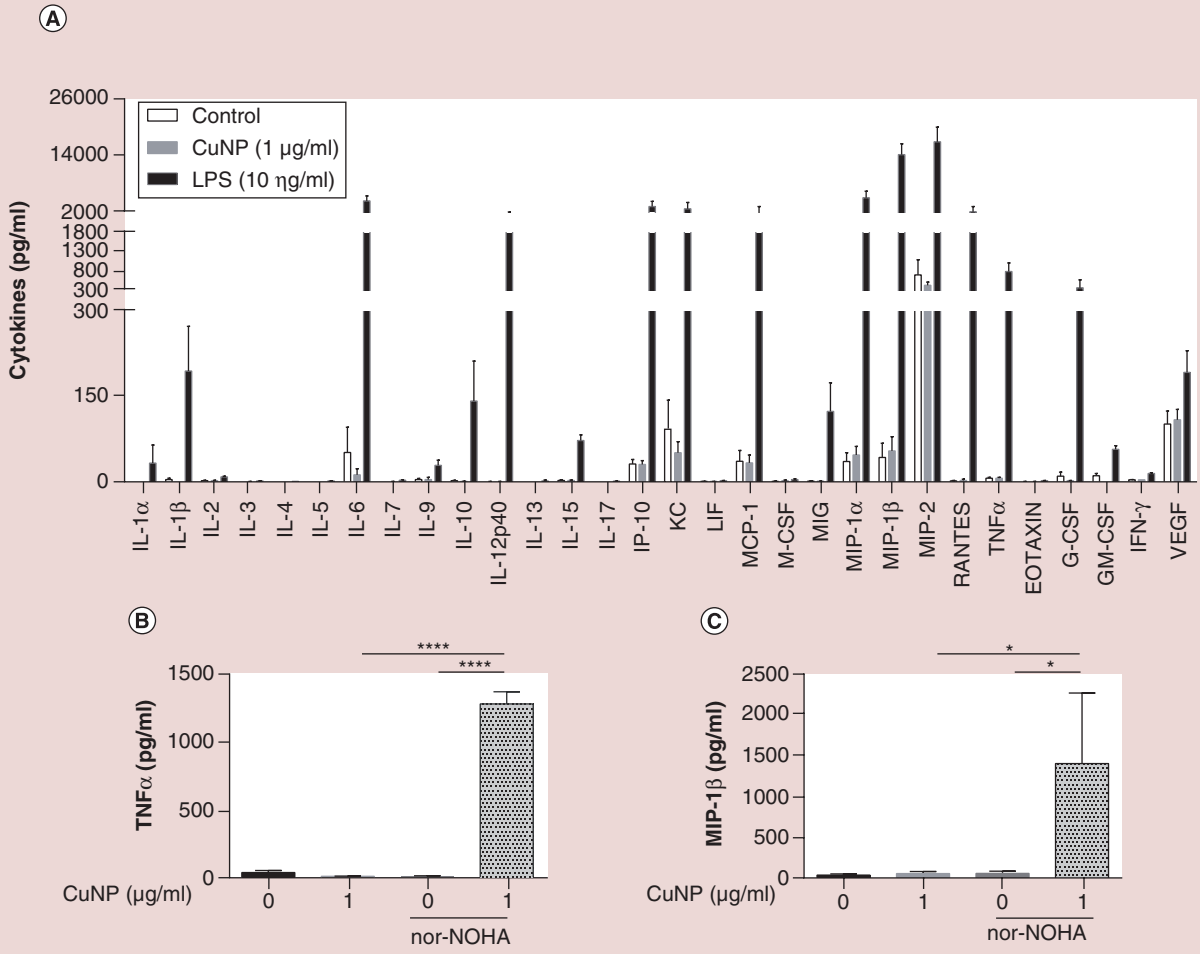

Figure 6. CuNP modulate proinflammatory cytokines secretion. (A) BMMs were stimulated with CuNP, LPS and PBS for 24 h to quantify cytokine and chemokine production in the supernatant. Samples were assessed with a validated cytokines and chemokines array (Eve technologies, Canada). **(B & C)** Arginase inhibition by nor-NOHA significantly increased the production of some proinflammatory cytokines such as TNFα and MIP-1β. One-way ANOVA – Tukey's post-test, n = 3, *p < 0.05 and ****p < 0.0001 between CuNP and CuNP+nor-NOHA and unstimulated cells+nor-NOHA and CuNP+nor-NOHA.

including cell death [30,32]. In our studies, although all nanoparticles tested were within the range of 30–50 nm, only CuNP promoted toxic effects to either primary macrophages or macrophage cell lines in concentrations equal or superior to 10 µg/ml. Nanoparticle toxicity does not only depend on their size and shape, but also on the intracellular solubility. Indeed, previous reports have indicated that CuNP are dissolved into the acidic pH of phagolysosomes promoting the release of toxic cupric ions [33]. The ions destabilize the organelle and permit the distribution of nanoparticle material into the cytoplasm, which, in part, would explain the cytotoxic effects of CuNP. Although the mechanism through which copper oxide nanoparticles promote cell death is not completely understood, it has been suggested that this process requires the activation of oxidative stress-dependent signaling pathways and the initiation of mitochondrial events, as well as autophagic processes [34].

The production of proinflammatory mediators such as cytokines and nitric oxide are crucial to eradicate invading pathogens and foreign molecules. Recently, it was demonstrated that CuNP exposure in a murine model diminished *K. pneumoniae* clearance in a dose-dependent manner, increasing the risk of pulmonary infection [7]. Similar effects were observed in mice pre-exposed to zinc oxide nanoparticles or single-walled carbon nanotubes (SWCNT) and challenged with nontypeable Haemophilus influenza and *L. monocytogenes*, respectively [35,36]. Although robust inflammation was observed in both reports, macrophages presented an impaired capacity to mount an appropriate antimicrobial response due to a diminished capacity for phagocytosis and nitric oxide production.

Triboulet *et al*. reported that CuNP and copper ions do not induce the production of NO by themselves, but both inhibit LPS-induced NO production in primary macrophages. They suggested that the reduction of NO production could be linked, at least indirectly, with the decrease in S-Adenosylhomocysteine hydrolase, which has been shown to reduce proinflammatory properties, and this can be linked, at least in part to a decrease in adenosine concentrations, as adenosine is a potent activator of nitric oxide production in macrophages [37].

Our *in vitro* data confirmed the results of the previous reports in relation to the inhibition of the secre-

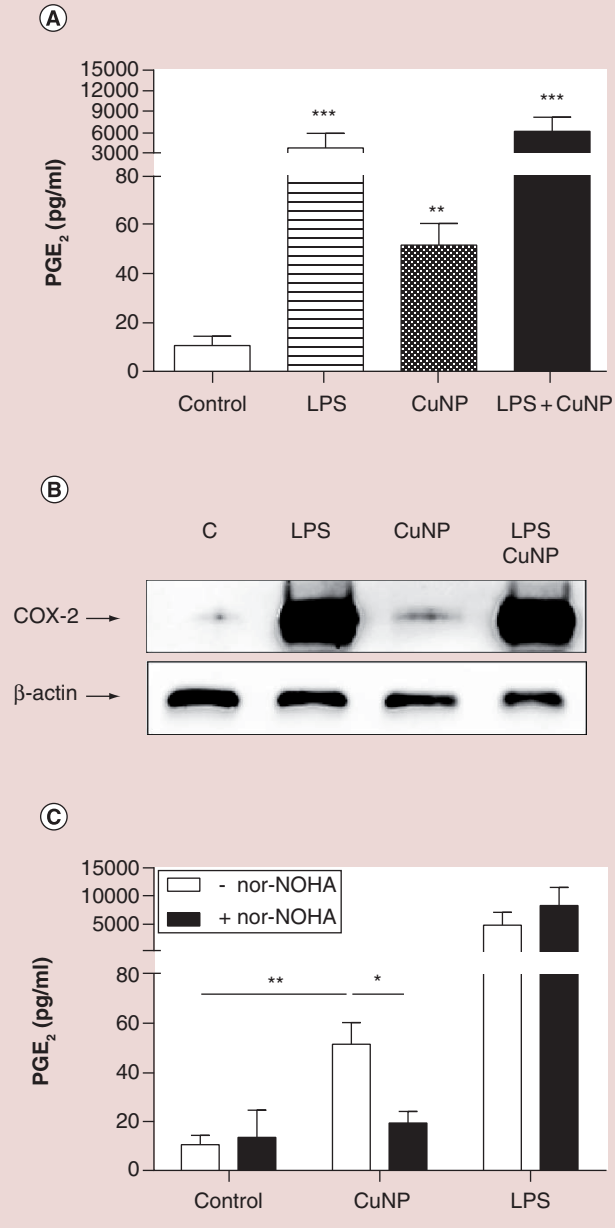

Figure 7. Copper nanoparticles promote PGE_2 production via arginase activation. **(A)** Peritoneal macrophages were exposed to CuNP, LPS and a combined treatment for 24 h to analyse PGE_2 production. One-way ANOVA – Tukey's post-test, n = 4, **p < 0.01 and ***p < 0.001 between CuNP and LPS, respectively, versus unstimulated cells. **(B)** Western blot analysis of peritoneal macrophages challenged with CuNP (1 μg/ml) and LPS (10 ng/ml) for 24 h. The cell lysates were analyzed for COX-2 expression (n = 3). **(C)** Arginase inhibition by nor-NOHA abolished CuNP-mediated PGE_2 generation. The cells were pretreated with nor-NOHA and then challenged with CuNP or LPS for 24 h. One-way ANOVA – Tukey's post-test, n = 3.
*p < 0.05 and **p < 0.01 between CuNP and CuNP+nor-NOHA and CuNP and unstimulated cells, respectively.

tion of proinflammatory cytokines and NO production by CuNP [10,37]. In addition, we observed that this process was dependent on an increase of arginase activation, confirming the antagonistic activity that exists between iNOS and arginase. Although the exact mechanism by which CuNP activate arginase remains unclear, it has been reported that metal ions can activate or inhibit enzymatic reactions due to a complex formation with the enzyme, where metal-substrate or metal-substrate-enzyme complexes may be formed if the substrate is the coordinating component. In the case of arginase, the presence of certain ions can alter urea production, indicating that these ions may modulate arginase activity. Indeed, it has been demonstrated that Co^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} ions enhance arginase activity in a pH-dependent manner. In the same conditions, Cu^{2+} was the only divalent cation that inhibits arginase activity [38]. Other reports indicate that copper ions can induce an allosteric inhibition of rat liver arginase-1 but not arginase-2 [39]. These studies, which present copper as an inhibitor of arginase, were carried out under *in vitro* conditions, in the presence of pure enzyme and substrate. However, it has been proven that copper ions can become activators of arginase in the presence of amine groups, a condition similar to that found inside the cell. We hypothesize that CuNP would eventually dissolve into the cytoplasm, allowing the cupric ions to interact with arginase and intracellular amines, thus activating the enzyme. However, the mechanisms through which amines facilitate copper to activate rather than inhibit arginase remain unclear. These results suggest that copper oxide nanoparticles activate arginase possibly by a direct interaction of the copper ions with the enzyme. This process would limit the bioavailability of L-arginine, suppressing the production of NO by macrophages.

Cytokine secretion is one of the key events in antigen-presenting cell activation in order to mount a robust immune response against different foreign agents. Some reports have stated that CuNP promote the production of proinflammatory cytokines such as IL-8 or TNFα in different cell lines [37,40]. These data may indicate a cell-type dependent effect on proinflammatory cytokine secretion by macrophages or may also evidence the presence of endotoxins in the nanoparticle preparation. Regarding the endotoxin content, it has been demonstrated that concentrations of LPS as low as 50 pg/ml can significantly stimulates proinflammatory cytokines secretion on dendritic cells, indicating that the use of endotoxin-free nanomaterials is crucial to avoid misleading conclusions [41]. Our results demonstrated that CuNP do not induce cytokines production in BMMs, in contrast to LPS, which promoted significant amounts of different cytokines. Interestingly, we observed that BMMs previously exposed to different arginase inhibitors produce high amounts of TNFα, indicating an anti-inflammatory effect of arginase [42]. Indeed, it has been shown that arginase is a negative regulator of LPS-dependent TNFα secretion in vascular smooth muscle cells. The mechanism associated to this regulatory pathway is the inhibition of iNOS activity, but not its expression, and NF-κB activation by preventing I-κB degradation [43].

Prostaglandin E_2 has been implicated in the modulation of key immune effector functions, the immunopathology of cancer and even chronic infections. In our study, we determined that CuNP augmented the expression of COX-2 and PGE_2 production, after 24 h of treatment, by means of arginase activation. Although the mechanism underlying arginase activation of COX-2 is unknown, it has been demonstrated that arginase-1 can be induced by COX-2, suggesting a molecular crosstalk between these two enzymes [16]. In this regard, it has been demonstrated that an increased PGE_2 production can decrease the phagocytic activity and pathogen-killing capacity of alveolar macrophages, and reduces TNFα and cysteinyl leukotriene production via scavenger receptor and TLR-dependent mechanisms [44]. Moreover, this is attributed to the ability of PGE_2 to selectively suppress macrophage effector function and Th1-dependent mechanisms, thus promoting Th2 and regulatory T cell responses [27]. These modulatory effects of PGE_2 are likely initiated either to facilitate a less destructive form of immunity or to promote the development of regulatory cells that could diminish tissue damage induced by CuNP.

Conclusion

Our results demonstrate that CuNP activate arginase, promoting the inhibition of NO and proinflammatory cytokines while enhancing PGE_2 production in murine macrophages. The alteration of these inflammatory mediators may explain previous reports, which describe the diminished capacity of macrophages exposed to CuNP to promote bacterial clearance upon infection. Thus, successive or chronic exposures to CuNP may increase susceptibility to certain infectious diseases by the inhibition of macrophage effector function and Th1-dependent mechanisms. These findings add further evidence in support of the concept that nanoparticles present a complex capacity to interact and modify different intracellular pathways and processes. Understanding how these nanoparticles interact with the immune system will be crucial to the development of novel therapeutics and indeed for the future of nanomedicine.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: http://www.futuremedicine.com/doi/full/10.2217/NNM.16.39x

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Inhalation of copper nanoparticles impaired bacterial clearance by decreasing the antimicrobial capacity of macrophages and host defences against bacterial infection.
- CuNP inhibit LPS-mediated NO production in macrophages, suggesting that reduced production of NO may contribute to a deficiency in pathogen clearance.
- How copper nanoparticles affect macrophage innate immune response was investigated.

Materials & methods

- A peritonitis mouse model was established to study innate immune cells recruitment.
- Primary macrophages were used to investigate the effect of CuNP on triggering signaling proinflammatory molecules through arginase activation.

Results

- CuNP were rapidly internalized by peritoneal macrophages through macropinocytosis.
- CuNP promoted toxic effects to primary macrophages in concentrations equal or superior to 10 μg/ml.
- CuNP inhibit the secretion of proinflammatory cytokines and NO production through arginase activation.
- CuNP augmented the expression of COX-2 and PGE_2 production by means of arginase activation.

Conclusion

- Our results indicate that exposure to CuNP diminish macrophage immune responses through arginase activation.

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