

Cerium dioxide nanoparticles selectively up-regulate C-C chemokine receptor 2 and CD16 expression on human monocytes

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ABSTRACT

Cerium dioxide nanoparticles (CeO₂ NPs) are known as scavengers of reactive oxygen species for the coexistence of Ce³⁺/ Ce⁴⁺ oxidation states. Cell treatments with CeO₂ NPs often lead to controversial pro-inflammatory and anti-inflammatory results. The aim of the study was to investigate the immune events following the administration of ceria nanoparticles to THP-1 monocytes. To address this issue, we performed flow cytometry, chemotaxis and ELISA experiments on THP-1 monocytes treated with different concentrations of CeO₂ NPs. CeO₂ nanoparticle treatments induced a significant pro-inflammatory C-C chemokine receptor 2 (CCR2) up-regulation within the first 6 hours lasting over-expressed for 24 hours. Differently, CCR5 showed no response at any concentration tested. Enhanced chemotaxis towards the CCR2 specific ligand MCP-1 reinforced the observation demonstrating a functional immune outcome. The pro-inflammatory profile of the treated monocytes was also supported by CD16 up-regulation but no differences in CX3CR1 or other monocyte receptors, like CD11b and CD14, were detectable. Moreover, CeO₂ NPs exposure did not promote any release of inflammatory cytokines suggesting a specific and direct effect of the nanoparticles on CCR2 and CD16.

Our *in vitro* results reveal a specific role of CeO₂ NPs in the up-regulation of CCR2, which might contribute to increase the pro-inflammatory monocyte/macrophage migration toward the sites of CCL2 expression.

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1. INTRODUCTION

Among the different nano-sized materials, cerium dioxide nanoparticles (nanoceria, CeO₂ NPs) have gained increased attention for their versatile properties. Nanoceria are widely used in electronics, electrochemistry, optics and industrial applications as catalysts, gas sensors, UV filters, fuel additives (Murray, 1999; Zheng, 2005). CeO₂ NPs can also behave as scavengers of reactive oxygen species (ROS) because of cerium auto-regenerative cycle of its two oxidation states, Ce³⁺ as Ce₂O₃ and Ce⁴⁺ as CeO₂. The presence of both Ce (III) / Ce (IV) valence forms and the oxygen vacancies on their surface (Esch, 2005; Fronzi, 2009) let these NPs store and release oxygen, being responsible for ceria catalytic and antioxidant properties. This specific property of the material stimulated research focused on CeO₂ NPs for the development of biomedical systems and pharmacological treatments such as antioxidant therapy and radio-protection (Celardo, 2011).

Many disorders are associated with impaired cellular redox balance and inflammation since excessive production of ROS within cells can lead to oxidative stress (Valko, 2007). Application of CeO₂ NPs in biomedicine are still a matter of debate, because of their controversial effects pro and anti-inflammatory results after *in vitro* and *in vivo* CeO₂ NPs treatment (Hirst, 2009; Ma, 2012; Niu, 2007; Poma, 2014).

Several studies describe cerium oxide NPs as extraordinary antioxidants. It has been also demonstrated that they are able to induce lung fibrosis and macrophage activation in rats and mice (Ma, 2012; Xue, 2013). Moreover, they can be internalised by human monocytes inducing apoptosis and autophagy in these cells (Hussain, 2012). Some studies have characterised the cytotoxicity and ROS scavenging properties of nanoceria in different cell types such as epithelial cells (Chigurupati, 2013), fibroblasts (Chigurupati, 2013), endothelial cells (Chen, 2014) and monocytes (Lord, 2012). However, little is known in literature regarding the effect of CeO₂ NPs on specific monocyte inflammatory receptor profile.

Monocytes are key cells of the innate immunity implicated in inflammatory processes in different tissues including rheumatoid arthritis, atherosclerosis and obesity (Ingersoll, 2011). Environmental signals at the site of inflammation mediate rapid monocyte mobilization and promote the differentiation of these cells into macrophages or dendritic cells that actively phagocytise non-self-molecules. Moreover, they regulate growth and

differentiation of the other immune cells through the release of cytokines, representing a crucial link between the innate and adaptive immune response against pathogens (Ingersoll, 2011). A critical role in the inflammatory process as modulators of the influx of monocytes to specific tissue and organ targets has been established for the (C-C motif) receptor 2 (CCR2) and its selective ligand CCL2 (formerly known as monocyte chemoattractant protein 1, MCP-1) (Ingersoll, 2011).

In the present study we studied the inflammatory effects of non-cytotoxic doses of nanoceria on a human monocyte cell line. To address this issue, we evaluated the expression of different inflammatory receptors, cell migration and cytokine release in THP-1 monocytes treated with different concentrations of CeO₂ NPs.

2. METHODS

CeO₂ nanoparticle solution

Cerium dioxide nanoparticles (CeO₂ NPs) were purchased from Sigma (cat. no #544841) and resuspended in PBS. Characterization has been already reported by Ciofani et al. (2013). Briefly, CeO₂ NPs have a crystalline nature showing a cubic morphology. Transmission electron microscopy measurements demonstrated a broad size dispersion of the particles, ranging from 5 to 80 nm. The amount of Ce³⁺ content on the NP surface was evaluated \sim 23%, by XPS spectra. Nanoparticles were dispersed through a mild sonication in ultrapure MilliQ water (Millipore) at a concentration of 10 mg/ml. Appropriate dilutions in the cell culture medium were performed just before the experiments.

Cell culture and CeO₂ NPs treatment

Human THP-1 monocytic cells were purchased from American Type Culture Collection (ATCC Manassas, VA, USA). Cells were maintained as a monocytic cell suspension in RPMI-1640 ATCC modified (Life Technologies, cat.no. #A10491-01) supplemented with 10% fetal bovine serum (FBS, Life Technologies cat. no #10108-165), 1% antibiotics and 0.05 mM 2-mercaptoethanol (Life Technologies cat. no. #21985) at 37°C in 5% CO₂, and cultures were split every 3 days. For each experiment cells were incubated with the indicated CeO₂ NPs concentrations (1, 10, 25, 50, 100 µg/ml) at different time points (2, 6 and 24 h). Cells were stimulated with 100 ng/ml of lipopolysaccharide (LPS) as positive control.

Cytokine release

MCP-1, TNF- α , IL-1 β and IL-6 levels released by THP-1 after CeO₂ NPs treatment were assessed by Bio-Plex Magpix System (Bio-Rad) following manufacturer's instructions.

Cell migration assay / Chemotaxis

Chemotaxis was performed in a 24-well plate after cell exposure to 10 and 25 µg/ml CeO₂ NPs. In the bottom wells 900 µl of chemotaxis medium (RPMI 1640 + 0.1% BSA) for controls and 900 µl of 10 nM human MCP-1 (Peprotech, cat. no 300-04) were added. Hanging cell culture inserts of polyethylene terephthalate (PET) with a pore size of 5 µm were placed over the wells (Millipore, cat. no. #PIMP12R48). Cells (1.2×10^5 /200 µl) were placed onto the filter wells and incubated for 2 h at 37°C.

After the incubation, filters were removed and 450 µl of resuspended cell suspension from the bottom wells were read by flow cytometry. The migrated cells were counted through MACS Quantify software provided by Miltenyi Biotec.

CCR2 internalization

Incubations of THP-1 cells treated with 10 µg/ml CeO₂ NPs with 10 and 50 nM MCP-1 have been performed for 30 min at 37°C, 5% CO₂ in RPMI medium supplemented with 1% BSA. Internalization of CCR2 was followed by flow cytometry using Alexa Fluor 647 conjugated mouse anti-human CD192/CCR2 (BD Pharmigen, cat no. #558406) resuspended in Running buffer (Miltenyi Biotec cat. no #130-092-747). Cell associated fluorescence was analyzed by flow cytometry (MACSQuant Analyzer, Miltenyi Biotec, Bergisch, Germany).

Antibody staining and flow cytometry

After CeO₂ NPs treatment, supernatants were collected and cells washed with RPMI at 8,000 rpm for 4'. Then, the pellets were resuspended in RPMI 1640/0.5% BSA and incubated for 30' on ice, in the dark, with the respective fluorescently labeled antibodies. After the incubation time, cells were washed with ice-cold RPMI at 8,000 rpm for 4' and resuspended in an appropriate volume with MACS Running buffer (Miltenyi, cat no. #130-092-747). Fluorescence activated flow cytometry (FACS) was performed with MACS Quant Analyzer (Miltenyi Biotec) flow cytometer and 20,000 events for each sample were analyzed. Live cells used for the analysis were gated based on Forward light scatter (FSC) and Side light scatter (SSC) and further analyzed using the MACS Quantify software (Miltenyi Biotec). The specific antibodies used in flow cytometric experiments were: FITC conjugated mouse anti-human CD195/CCR5 (BD Pharmigen, cat no. #555992), PE conjugated mouse anti-human CX3CR1 (Miltenyi Biotec, cat no. #130-096-432), AlexaFluor 647 conjugated mouse anti-human CD192/CCR2 (BD Pharmigen, cat no. #558406), APC conjugated mouse anti-human CD16 (Miltenyi Biotec, cat no #130-091-246), VioBlue conjugated mouse anti-human CD14 (Miltenyi Biotec, cat no #130-094-364), PE conjugated mouse anti-human CD11b (Miltenyi Biotec, cat no. #130-097-336). All the antibodies were used in the concentrations suggested by the suppliers.

Lactate dehydrogenase (LDH) and WST-8 assay

Cell membrane damage and metabolic activity of THP-1 were determined after 24 h of cell exposure to increasing concentration of CeO₂ NPs (1–100 µg/ml), as described recently (Bertero, 2014).

induce significant changes after 24 h treatments with 10 µg/ml, in agreement with the level of CCR2 expression at this time point using the same

Statistical analysis

Each experiment was performed in triplicate. Data are expressed as fold increase and mean ± SEM. Statistically significant difference was determined by performing 1 and 2-way ANOVA analysis followed by Bonferroni *post-hoc* test for more than two groups and Student t test for two groups. A P value of < 0.05 was considered significant.

3. RESULTS

CeO₂ NPs induce overexpression of CCR2 but not CCR5

THP-1 cells were treated with ceria nanoparticles from 1 to 100 µg/ml for 2, 6, and 24 h. This range of concentration was chosen based on nanoparticle dose dependent internalization and cytotoxicity results (Fig S1). CCR2 receptor expression was increased in a dose dependent manner starting from 10 µg/ml CeO₂ NPs, as measured by flow cytometry (Fig 1A). The highest peak of CCR2 surface expression was after the first 2 h at all the concentrations tested, to decrease within 24 h. On the other hand, the expression of the other inflammatory receptor expressed on the monocyte/macrophage lineage CCR5 was not affected by ceria nanoparticle treatments (Fig 1B). No variation of expression of a different chemokine receptor, CX3CR1, usually expressed on immature monocytes, was also found after CeO₂ NPs administration in the same range of concentration (Fig S2).

Ceria treatment increases CCL2 mediated migration

To see whether increased receptor expression might cause cell functional changes in CCR2 mediated signalling, we performed chemotaxis assay toward the receptor specific ligand CCL2 (Fig 2A and B). CCL2 mediated migration of THP-1 treated with 10 or 25 µg/ml of CeO₂ NPs for 2 h (Fig 2A) increased in a significant way with respect to the untreated cells. After 24 h from the beginning of the treatment, only 25 µg/ml of nanoceria, or higher concentration (data not shown), were still able to increase CCL2 chemotaxis (Fig 2B). It is worth to note that nanoceria did not

CeO₂ nanoparticle concentration (Fig 1A). Figure 2C shows CCL2 mediated down-regulation of CCR2 receptor in presence or absence of 10 µg/ml nanoceria. This result demonstrates that CCR2 specific ligand mediated internalization is not altered in presence of nanoceria, although the latter induce receptor over-expression on the cell surface.

CD16 up-regulation on THP-1 cells following CeO₂ NPs administration

CCR2 has a major role in mediating inflammatory responses and it is crucial for most of monocyte migration towards sites of inflammation. Once reached these sites, monocytes usually differentiate into macrophages with a defined receptor profile that depends on the specific organ or tissue environment. We investigated a possible role of CeO₂ NPs in monocyte differentiation by promoting inflammatory macrophage receptor profile. As shown in Fig 3A, 24 h treatment with nanoceria induces up-regulation of CD16, but no variations of other cell surface receptors involved in inflammatory responses, namely CD11b and CD14, have been observed in presence of CeO₂ NPs at the same concentration (50 µg/ml). Over-expression of CD16 starts at nanoceria doses comparable with the ones needed to up-regulate CCR2 and lasting for 24 h, as displayed in Fig 3B.

CeO₂ nanoparticle administration to THP-1 cells does not induce inflammatory cytokine release

Modulation of surface receptors on immune cells is often regulated by cytokines, which in turn activate signals leading to early gene expression, mRNA translation or protein localization on the cell membrane. To understand if inflammatory receptor up-regulation induced by the nanoceria treatment was dependent on soluble mediators, we analysed inflammatory cytokine release in THP-1 cells (Fig 4). No release of TNF-α, IL-1β, IL-6 or CCL2 was observed following CeO₂ NPs administration within 24 h at any concentration tested. This observation proved that no soluble mediators, possibly released by nanoceria treatment of THP-1 cells, were responsible for CCR2 expression, thus implying a direct effect of the nanomaterial. To exclude any other possible soluble molecule as potential mediator of chemokine receptor expression, we confirmed that cell culture medium from previously treated monocytes had no influence on CCR2 expression (Fig S3).

4. DISCUSSION

CeO₂ NPs have been studied by several laboratories with contentious results on their safety (Celardo, 2011; Hussain, 2012). Indeed, our results in the present work (Fig S1), as well as many of the previously cited reports, show no toxicity in different cell lines, even at high concentrations. Often, toxic or not toxic effects have been observed in different cell types (Chen, 2013; Hussain, 2012; Sabella 2014) and different time of nanoparticle exposure (De Marzi, 2013). Their involvement in anti-inflammatory (Hirst, 2009) or pro-inflammatory immune responses has been also shown, even if a pro-inflammatory role *in vivo* is becoming evident by several studies (Demokritou, 2013; Peng, 2013; Poma, 2014; Xue, 2013). In the present work, we focused our attention on the modulation of monocyte surface receptors involved in immune responses evoked by CeO₂ NPs. THP-1 monocyte cell model was chosen as innate immunity is the first biochemical barrier in case of accidental exposure with microorganisms, as well as nanomaterials. CCR2 is considered the "prototype" of pro-inflammatory chemokine receptors driving monocyte/macrophage cells to the sites of inflammation in several diseases (Ingersoll, 2011). We demonstrated ceria nanoparticles induce the up-regulation of CCR2 (Fig 1), increasing the possibility of THP-1 monocytes to migrate toward a gradient of its cognate ligand, CCL2 (Fig 2A and B). It is worth noticing that the functionality of CCR2 was not compromised by CeO₂ NPs, as shown by the normal internalization of the receptor in presence of increasing doses of chemokine (Fig 1C). In our *in vitro* cellular model, the increased expression of chemokine inflammatory receptors was restricted to CCR2 with no effects on CCR5 (Fig 1). However, the immunoglobulin receptor CD16, a receptor that belongs to the Fcγ receptors' family and mediates the phagocytosis of opsonized microbes defining activated sub-populations of monocyte/macrophage cells (Ingersoll, 2011), was also up-regulated in a dose dependent manner. Hussain et al describe how ceria dioxide nanoparticles are able to induce autophagy in human monocytes (Hussain, 2012), the main catabolic pathway in mammalian cells after internalization of exogenous material. Indeed, TEM revealed CeO₂ NPs internalized by monocytes and found either in vesicles or free in the cytoplasm, probably interacting directly with endosomal/lysosomal compartments (Hussain, 2013). The authors observed a significant increase in TNF-α, known to induce autophagy, after exposure to CeO₂ NPs (Hussain, 2013). In our study, we did not obtain any increase of this cytokine after nanoceria treatment, suggesting that a possible autophagic event is mediated by the direct effect of NPs on CD16 receptor. On the contrary, we did not observe any modulation of CD11b nor CD14 on the THP-1 cells treated with

nanoceria (Fig 3). Over expression of integrins and the chemokine receptor CXCR4 by metal oxide nanoparticles, including CeO₂ NPs, has also been recently observed on lymphocytes (Lozano-Fernández, 2014). These and our indications provide crucial information on the impact that nanoparticles may have on the regulation of the immune system at the cellular level. Here we show that nanoceria induce CCR2 and CD16 over-expression, and this event is not a secondary effect mediated by inflammatory cytokines released THP-1 monocytes (Fig 4). The latter result suggests a direct effect of the nanomaterial on the cell mechanisms that regulate CCR2 expression, although the molecular pathway remains unknown.

5. CONCLUSIONS

Our *in vitro* results reveal a specific role of CeO₂ NPs in the up-regulation of CCR2, which might contribute to increase the pro-inflammatory monocyte/macrophage migration toward the sites of CCL2 expression.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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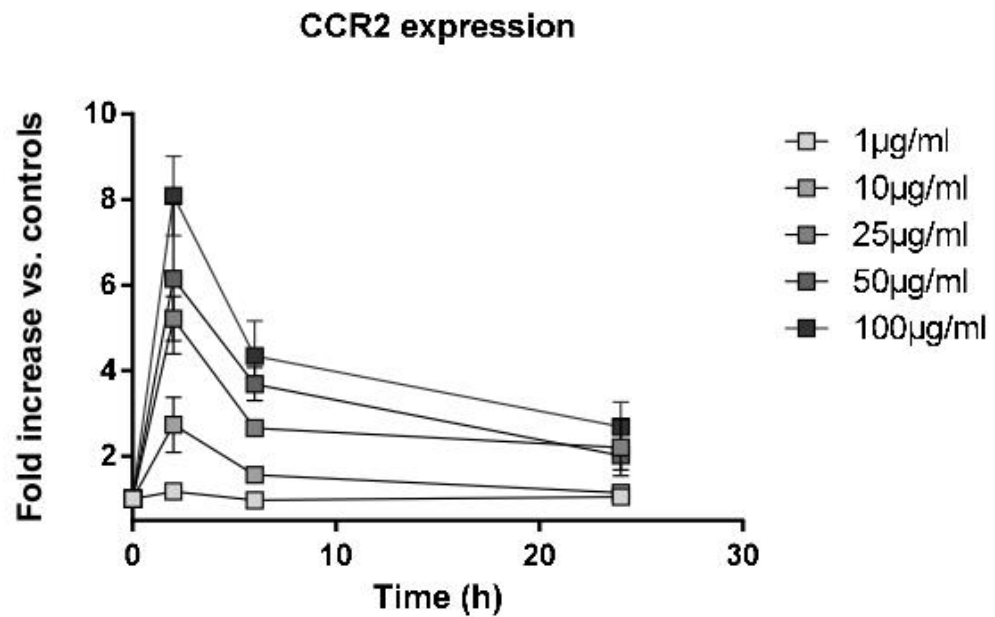
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Figures

Fig 1

A



B

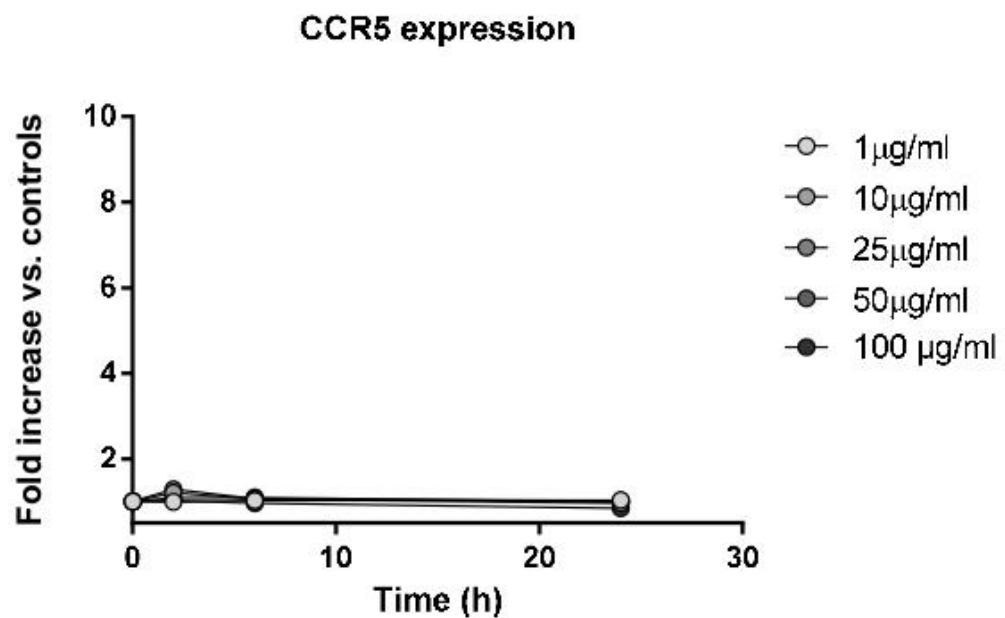


Fig. 1 CCR2 and CCR5 expression after CeO₂ NPs treatment.

Fold increase of CCR2 (A) and CCR5 (B) expression in THP-1 treated for 2, 6 and 24 h with increasing CeO₂ NPs concentrations and analyzed by flow cytometry. Each time and concentration point are expressed as average \pm SEM of at least 4 independent experiments.

Fig 2

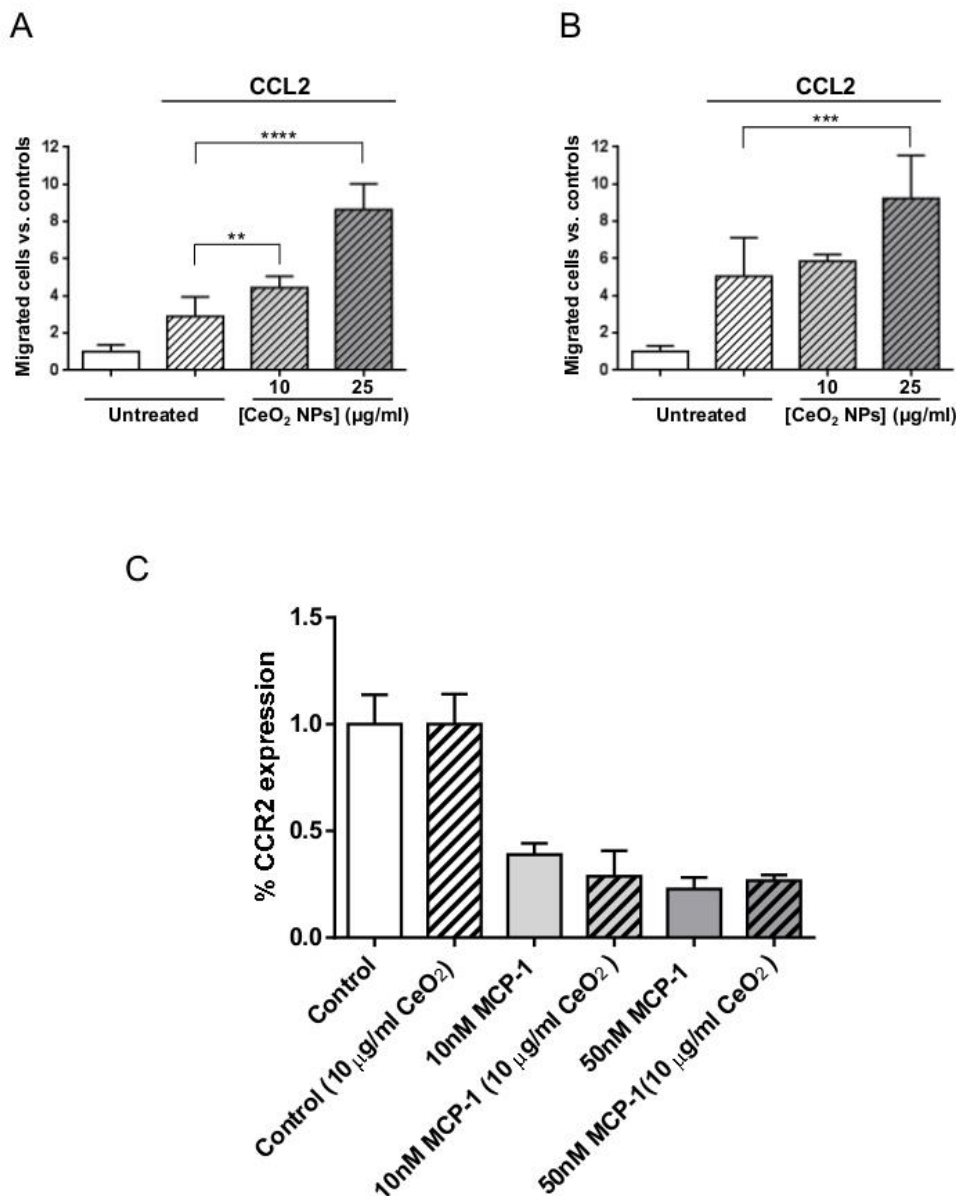


Fig. 2 CCL2 increased chemotaxis in CeO₂ NPs treated THP-1 cells does not compromise CCR2 internalization.

THP-1 were cultured for 2 h (A) and for 24 h (B) with the indicated concentrations of CeO₂ NPs. At the end of the treatment, cells were collected, washed and placed within a 5 μm-pore inserts in a 24-well plate loaded with 10 nM CCL2 chemokine. The experiment was performed for 30 min at 37°C, 5% CO₂ in chemotaxis medium (RPMI + 0.1% BSA). Data are expressed as fold increase of treatments vs. controls. One way ANOVA, Bonferroni post hoc test ** P < 0.01, *** P < 0.001, **** P < 0.0001. C) Bar graph showing the % of CCR2 surface expression in THP-1 untreated (not striped bars) and treated with 10 μg/ml CeO₂ NPs (striped bars) for 2 h and incubated with 10 (light grey bars) and 50 nM (dark grey bars) CCL2. No statistical difference between CeO₂ NPs concentration treatments and controls was observed in five independent experiments.

Fig 3

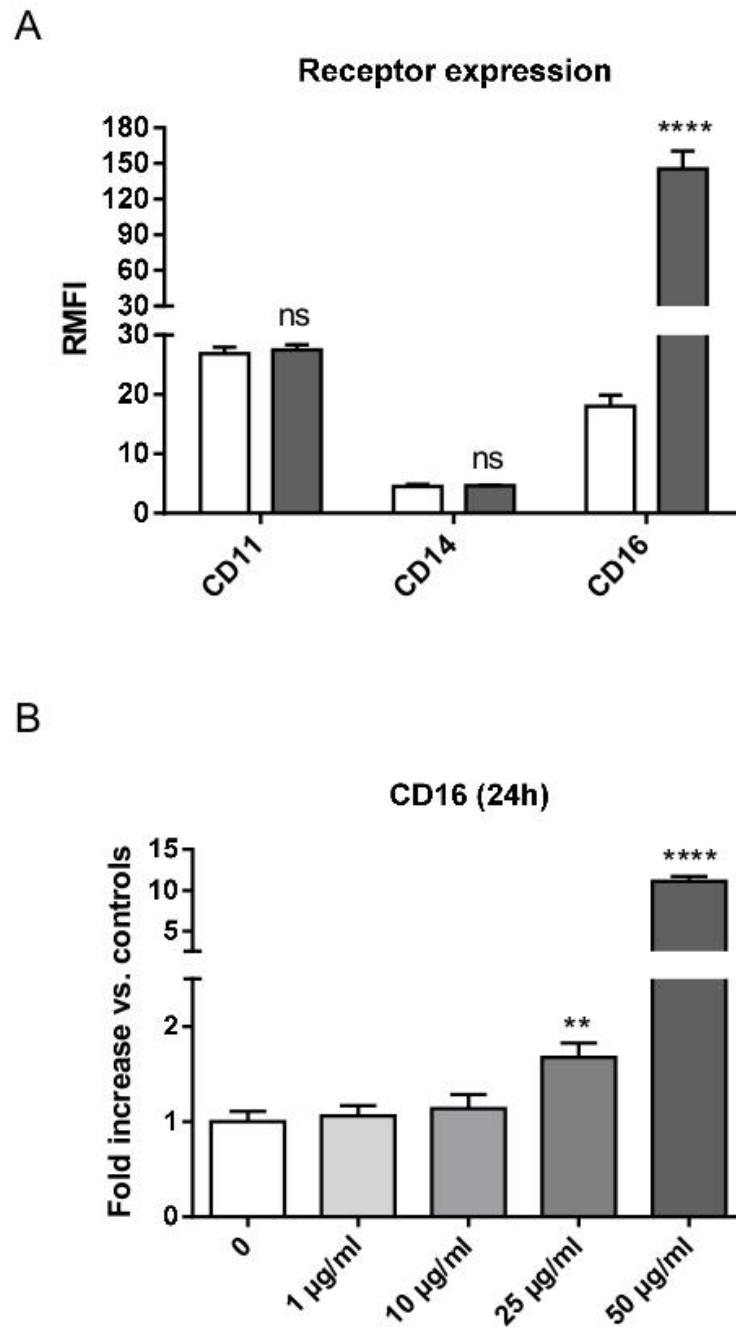


Fig. 3 Characterization of THP-1 surface inflammatory receptors.

A) Relative mean fluorescence intensity (RMFI) of CD11b, CD14 and CD16 receptors in untreated (white bars) and 50 µg/ml CeO₂ NPs treated (dark grey bars) THP-1 for 24 h. B) Bars represent CD16 expression normalized vs. control on the THP-1 cell surface after increasing concentration of CeO₂ NPs. Statistical significance vs. control (untreated) was analysed by Student t test ** P< 0.01, ****P<0.0001.

Fig 4

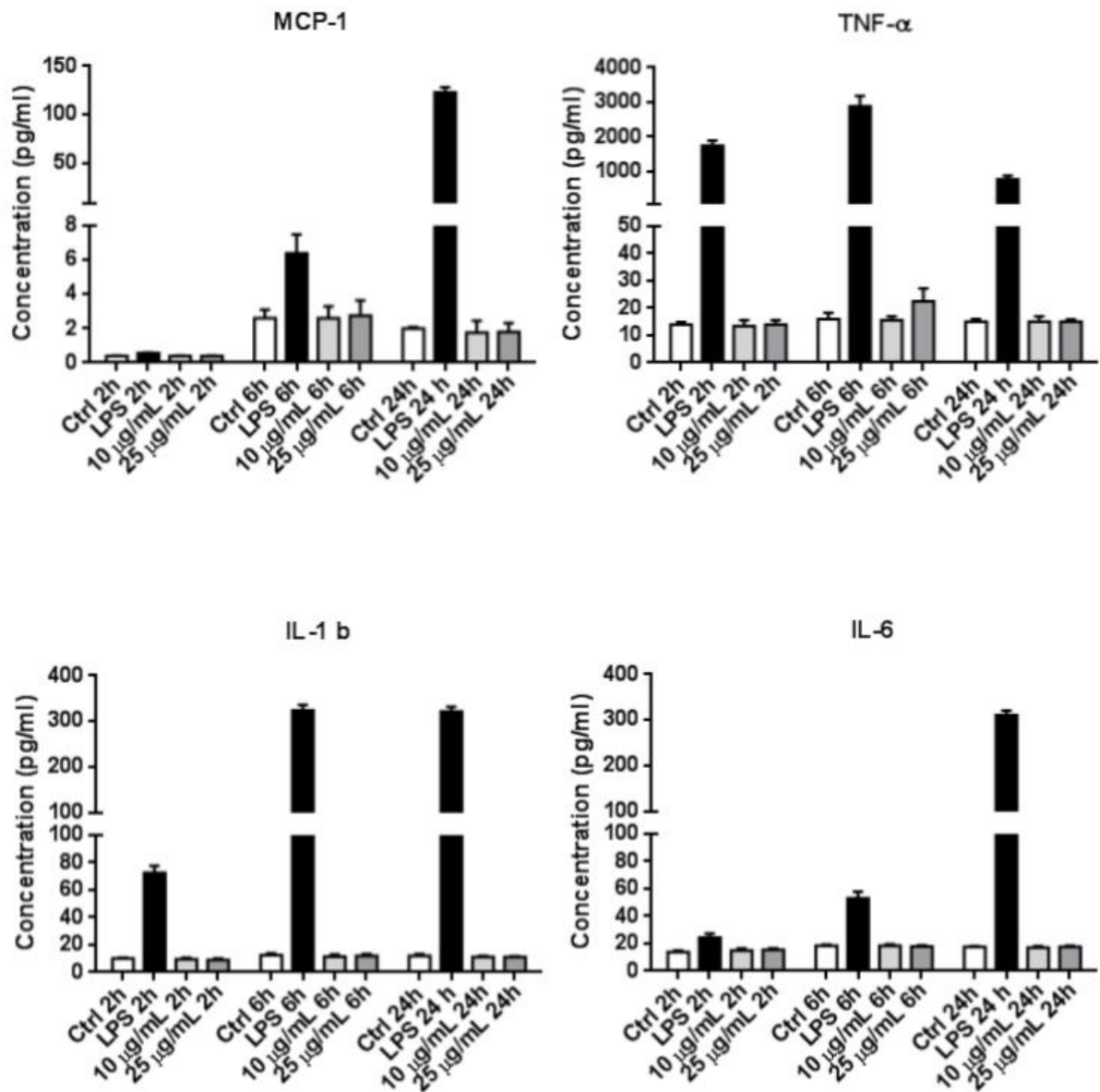


Fig. 4 Cytokine release into the medium by CeO₂ nanoparticle treated THP-1.

Bar graph showing CCR2 (A) and CCR5 (B) expression in THP-1 conditioned for 2 h with the supernatants of THP-1 untreated (white bar) and treated (grey bar) with 50 $\mu\text{g/ml}$ CeO₂ NPs for 24 h. Data are expressed as means \pm SEM of five experiments. One-way ANOVA has been performed and no statistical difference between each CeO₂ NPs concentration treatment and controls was observed. MCP-1 (C), TNF- α (D), IL-1 β (E) and IL-6 (F) release measured by Elisa assays after 2, 6 and 24 h THP-1 exposure to 10 (light grey bars) and 25 $\mu\text{g/ml}$ (dark grey bars) CeO₂ NPs. Lipopolysaccharide (LPS) 100 ng/ml (black bars) was used as positive control. One-way ANOVA has been performed and no statistical difference between each CeO₂ NPs concentration treatment and controls was observed.

Fig 5

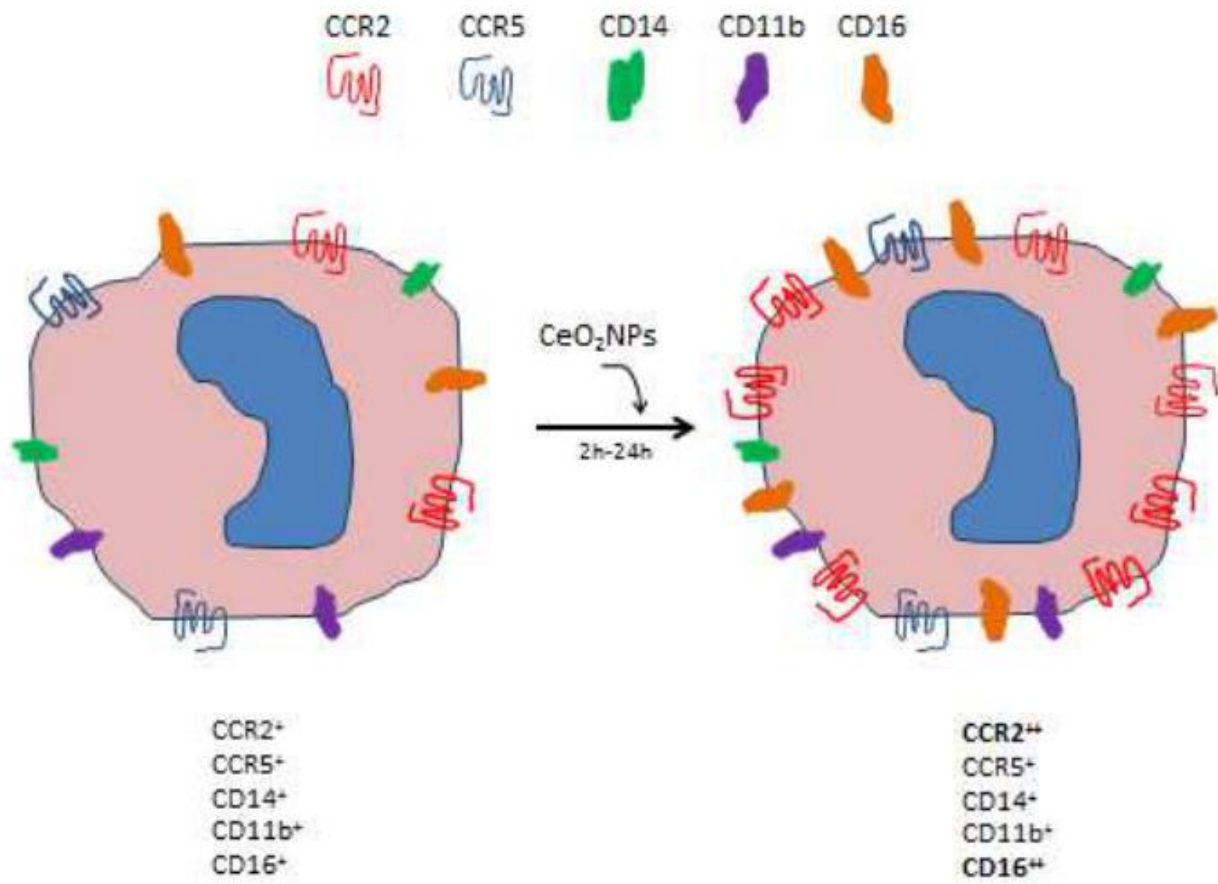
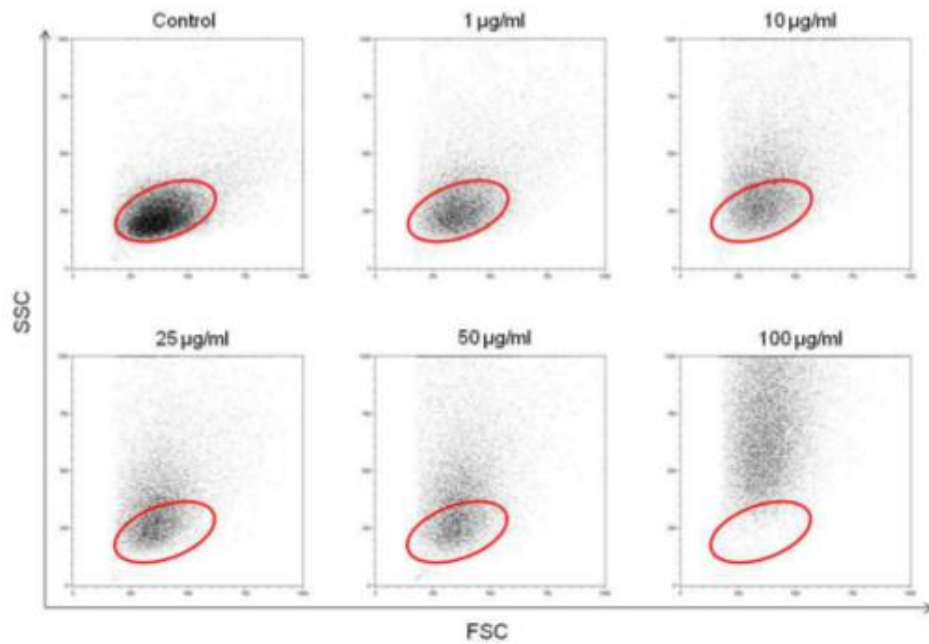


Fig. 5 THP-1 pro-inflammatory receptor profile before and after exposure to CeO₂ NPs

THP-1 monocytes expression of pro-inflammatory receptors CCR2, CCR5, CD14, CD11b and CD16. CeO₂NPs administration induce early up-regulation of CCR2 and CD16 lasting 24 h.

Fig S1
A

B

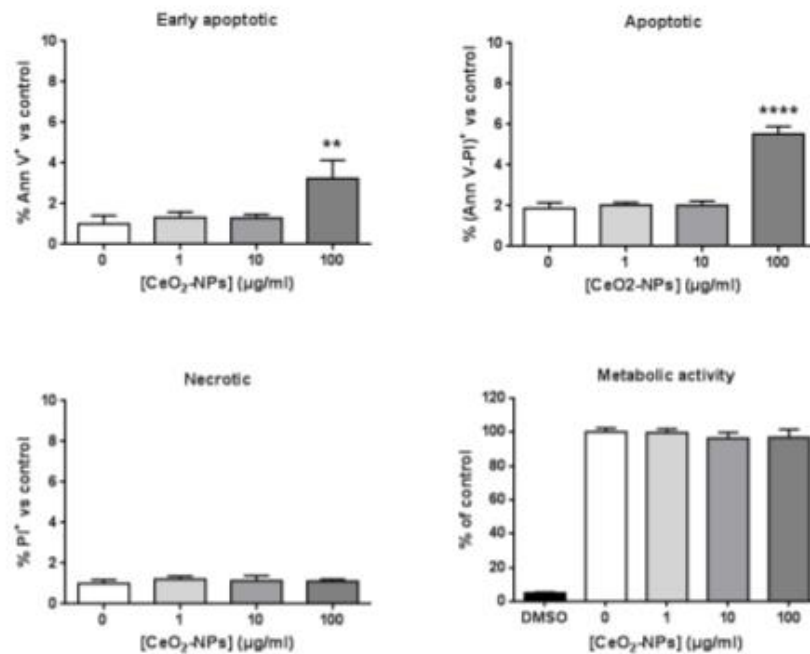


Fig. S1 Monocyte viability after CeO₂ NPs exposure.

THP-1 were treated with different doses (1, 10, 25, 50, 100 µg/ml) of CeO₂ NPs for 24 h and analyzed by flow cytometry.

A) Dot plots showing Forward vs. Side scattering (in the red circle the monocyte population area of control cells).

B) Cell death (apoptosis and necrosis) measurement by Annexin/PI staining (left panel) and metabolic activity by WST-8 assay (right panel). 5% DMSO was used as positive control. Results are presented as % of control. Data are expressed as means ± SEM of three experiments. One-way ANOVA, Bonferroni post hoc test ****P<0.0001.

Fig S2

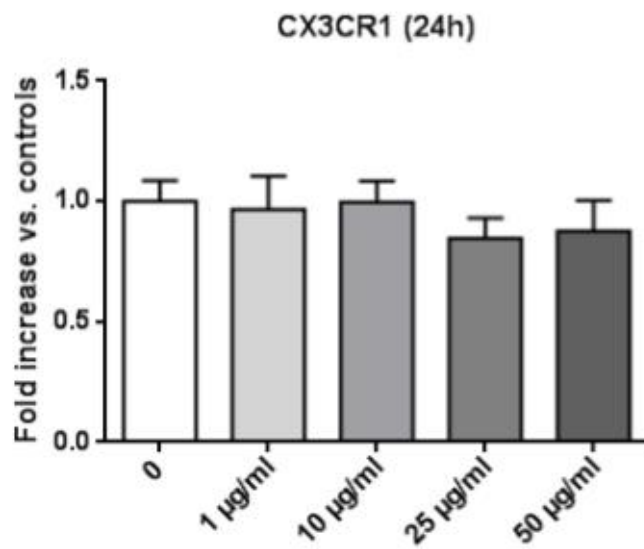


Fig. S2 Fold increase of CX3CR1 expression after 24 h treatment with 1, 10, 25, 50 µg/ml CeO₂ NPs. Student t test vs. control not significant.

Fig S3

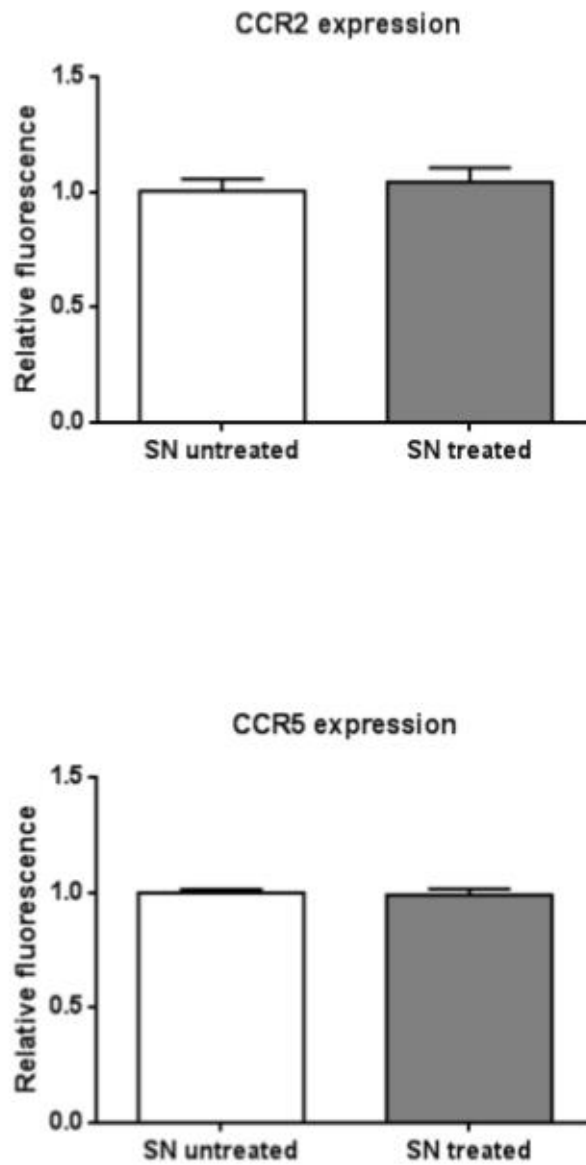


Fig. S3 CCR2 and CCR5 surface expression in THP-1 cells cultured in medium collected from monocytes previously treated with 50 $\mu\text{g}/\text{ml}$ CeO_2 NPs.