Cerium dioxide nanoparticles selectively modulate C-C chemokine receptor 2 expression

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Cerium dioxide nanoparticles (CeO_2 NPs) are widely used for industrial purposes as catalysts and have potential therapeutic applications for inflammatory processes, since they are scavengers of reactive oxygen species (ROS) for the coexistence of Ce^{3+}/ Ce^{4+} oxidation states [1]. However, health effects of CeO_2 NPs are still a matter of debate, because of controversial pro and anti-inflammatory results after *in vitro* and *in vivo* CeO_2 NPs treatment [2,3]. A critical role in the inflammatory process as modulators of the influx of monocytes to specific tissue and organ targets has been established for chemokine receptors, in particular for chemokine (C-C motif) receptor 2 (CCR2).

[1] Celardo I, Traversa E, Ghibelli L. *Cerium oxide nanoparticles: a promise for applications in therapy*, J Exp Ther Oncol., 9(1), (2011) 47-51.

[2] Hirst SM, Karakoti AS, Tyler RD, Sriranganathan N, Seal S, Reilly CM. *Anti-inflammatory properties of cerium oxide nanoparticles.* Small. 5(24), (2009) 2848-56.

[3] Ma JY, Mercer RR, Barger M, Schwegler-Berry D, Scabilloni J, Ma JK, Castranova V. *Induction of pulmonary fibrosis by cerium oxide nanoparticles.* Toxicol Appl Pharmacol. 262(3), (2012) 255-64.

AIM of the STUDY

We investigated the immune effects induced by non-cytotoxic concentrations of CeO_2 nanoparticles in human monocytes.

1. CeO_2 NPs do not affect monocyte viability

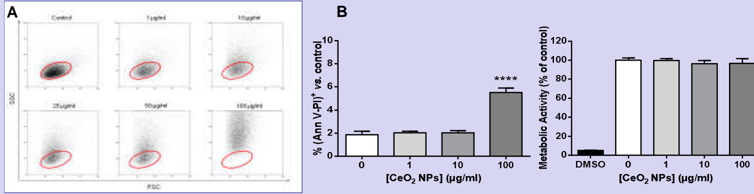


Fig. 1 Monocyte viability after CeO_2 NPs exposure. THP-1 were treated with different doses (1, 10, 25, 50, 100 µg/ml) of CeO_2 NPs for 24 h and analyzed by flow cytometry. **A**) Dot plots showing Forward *vs.* Side scattering (in the red circle the monocyte population area of control cells). **B**) 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.

2. CCR2 is significantly over-expressed after CeO_2 NPs treatment

C-C chemokine receptor type 2 (CCR2/CD192) is a G protein-coupled receptor expressed on the surface of blood leukocytes, macrophages and microglia. Its highly affine ligand is monocyte chemoattractant protein-1 (MCP-1), a chemokine which specifically mediates monocyte infiltration in inflammatory diseases (i.e. rheumatoid arthritis, tumors).

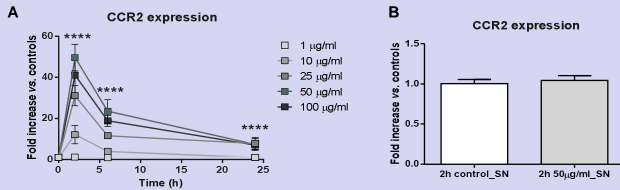


Fig. 2 CCR2 expression after CeO_2 treatment. A) Fold increase of CCR2 expression in THP-1 treated for 2, 6 and 24 h with increasing CeO_2 NPs concentrations and analyzed by flow cytometry. **B**) Bar graph showing CCR2 expression in THP-1 conditioned for 2 h with the surnatants of THP-1 untreated (white bar) and treated (grey bar) with 50 µg/ml CeO_2 NPs for 24 h. Data are expressed as means ± SEM of five experiments. All data are statistically significant by one-way ANOVA, Bonferroni *post hoc* test ****P< 0.0001 (not significant 1 µg/ml all time points and 10 µg/ml at 24h).

3. CeO_2 NPs treatment enhances MCP-1 mediated chemotaxis and CCR2 internalization

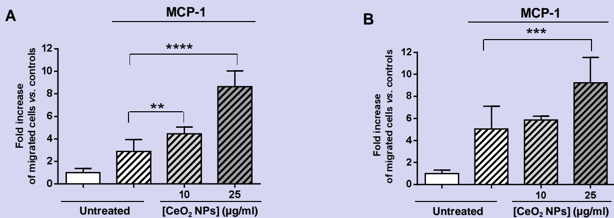
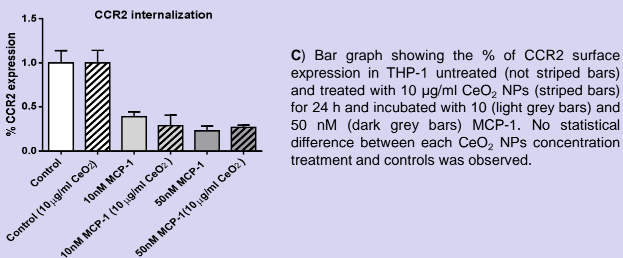


Fig. 3 CCR2 functional activity after CeO_2 NPs treatment. THP-1 were cultured for **A**) 2 h and **B**) for 24 h with the indicated concentrations of CeO_2 NPs. At the end of the treatment, cells were collected, washed and incubated in the bottom part of a chemotaxis chamber, where in the upper part 10 nM MCP-1 in chemotaxis medium (RPMI + 0.1% BSA) for 30' at 37°C, 5% CO_2 was placed. 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 CeO_2 NPs (striped bars) for 24 h and incubated with 10 (light grey bars) and 50 nM (dark grey bars) MCP-1. No statistical difference between each CeO_2 NPs concentration treatment and controls was observed.

5. CeO_2 NPs do not promote cytokine release *in vitro*

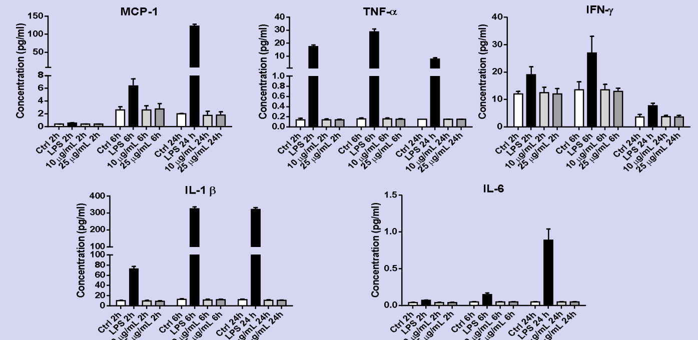


Fig. 4 Cytokine release into the medium by THP-1. MCP-1, TNF-α, IFN-γ, IL-1β and IL-6 release measured by Elisa assays after 2, 6 and 24 h THP-1 exposure to 10 (light grey bars) and 25 µg/ml (dark grey bars) CeO_2 NPs. Lipopolysaccharide (LPS) 100 ng/ml (black bars) was used as positive control. One-way ANOVA has been performed and no statistical difference between each CeO_2 NPs concentration treatment and controls was observed.

6. CCR5 expression is not affected by CeO_2 NPs treatment

Another important chemokine receptor involved in the immune and inflammatory responses to infections is C-C chemokine receptor type 5 (CCR5/CD195). CCR5 is predominantly expressed on T cells, macrophages, dendritic cells and microglia.

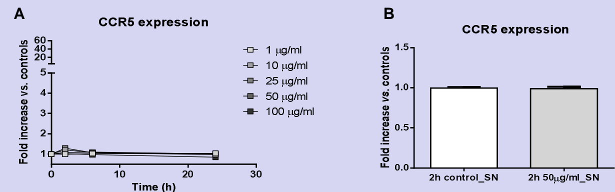


Fig. 5 CCR5 expression after CeO_2 NPs treatment. A) Fold increase of CCR5 expression in THP-1 treated for 2, 6 and 24 h with increasing CeO_2 NPs concentrations and analyzed by flow cytometry. **B**) Bar graph showing CCR5 expression in THP-1 conditioned for 2 h with the surnatants of THP-1 untreated (white bar) and treated (grey bar) with 50 µg/ml CeO_2 NPs for 24 h. Data are expressed as means ± SEM of five experiments. One-way ANOVA has been performed and no statistical difference between each CeO_2 NPs concentration treatment and controls was observed.

7. Inflammatory profile of monocytes after CeO_2 NPs treatment

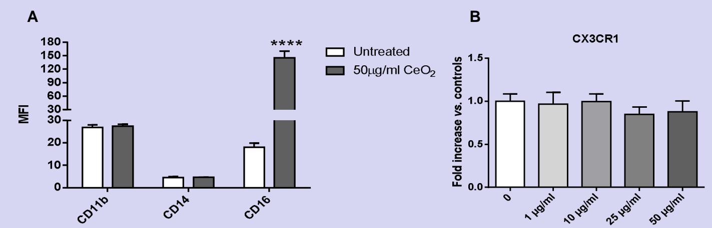


Fig. 6 Characterization of monocyte receptor profile. A) Mean fluorescence intensity of CD11b, CD14 and CD16 receptors in untreated (white bars) and 50 µg/ml CeO_2 NPs treated (dark grey bars) THP-1 for 24 h. **B**) Fold increase of CX3CR1 expression after 24 h treatment with 1, 10, 25, 50 µg/ml CeO_2 NPs. Student t test ****P<0.0001.

CONCLUSIONS

Our *in vitro* data define a monocyte restricted pro-inflammatory response to CeO_2 nanoparticles. Specific up-regulation of CCR2 receptor and enhanced capability to promote chemotaxis are the major pro-inflammatory outcomes of CeO_2 NPs, without any impairment of the receptor function as suggested by the internalization results. The over-expression of CD16 is not coordinated by CX3CR1 down-regulation on the same cells as in a typical inflammatory phenotype. As well, no inflammatory cytokines are released following exposure to CeO_2 NPs.