Anti-inflammatory effect of gold nanoparticles supported on metal oxides

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Gold (Au) can be deposited as nanoparticles (NPs) smaller than 10 nm in diameter on a variety of metal oxide (MOx) NPs. Au/MOx have high catalytic performance and selective oxidation capacity which could have implications in terms of biological activity, and more specifically in modulation of the inflammatory reaction. Therefore, the aim of this study was to examine the effect of Au/TiO2, Au/ZrO2 and Au/CeO2 on viability, phagocytic capacity and inflammatory profile (TNF-α and IL-1β secretion) of murine macrophages. The most important result of this study is an anti-inflammatory effect of Au/MOx depending on the MOx nature with particle internalization and no alteration of cell viability and phagocytosis. The effect was dependent on the MOx NPs chemical nature (Au/TiO2 > Au/ZrO2 > Au/CeO2 if we consider the number of cytokines whose concentration was reduced by the NPs), and on the inflammatory mediator considered. The effect of Au/TiO2 NPs was not related to Au NPs size (at least in the case of Au/TiO2 NPs in the range of 3–8 nm). To the best of our knowledge, this is the first demonstration of an anti-inflammatory effect of Au/MOx.

Physicochemical properties of Au/MOx. Physicochemical properties of Au/MOx are summarized in Table 1. Sizes of primary particles of MOx (TiO2, ZrO2, and CeO2) were in the range of 7–26 nm but MOx NPs are generally agglomerated to form secondary micrometer-sized (2.4–9.4 μm) particles. Actual gold loading amounts of Au/MOx were ca. 1 wt% for all samples. The average sizes of Au NPs on TiO2 and ZrO2 prepared by...
Table 1. Physicochemical properties of Au NPs supported on metal oxides. a Specific surface area of metal oxide was calculated from N₂ adsorption isotherms using the Brunauer–Emmett–Teller (BET) method. b Primary particle size of metal oxide was estimated from the specific surface area. c Secondary particle size of metal oxide after the deposition of Au NPs was measured by dynamic laser scattering. d Actual loading amount of Au was determined by inductively coupled plasma–atomic emission spectrometry (ICP-AES) or atomic absorption spectrometry (AAS). e Average particle size of Au NPs on metal oxides were estimated by HAADF-STEM. f Majority of Au species was considered to be atomically dispersed on CeO₂ and could not be detected due to a limitation of resolution and low contrast between heavy Ce and Au atoms by TEM.

|          | Specific surface area /m²/g | Primary particle size of metal oxide/nm | Secondary particle size of metal oxide/μm | Au loading/wt% | Au particle size/nm |
|----------|-----------------------------|----------------------------------------|-------------------------------------------|----------------|---------------------|
| Au₃/TiO₂ | 54                          | 26                                     | 9.4                                       | 0.96           | 3.3 ± 1.4           |
| Au₈/TiO₂ | 54                          | 26                                     | 9.4                                       | 1.18           | 7.8 ± 2.7           |
| Au/ZrO₂  | 94                          | 11                                     | 3.6                                       | 0.97           | 3.3 ± 1.8           |
| Au/CeO₂  | 114                         | 7.3                                    | 2.4                                       | 0.96           | No data             |

deposition–precipitation were both of 3.3 nm. To examine the size effect of Au NPs, Au NPs with an average size of 8 nm were deposited on TiO₂ by solid grinding. High-angle annular dark-field scanning transmission electron microscopic (HAADF-STEM) images of Au/MOX are shown in Fig. 1. For Au/TiO₂ and Au/ZrO₂, Au NPs were highly dispersed on MOx NPs. On CeO₂, the most of Au species is atomically dispersed but only small amount Au NPs with ca. 20 in diameter was observed (Fig. 1d).

**No cytotoxic effect of Au/TiO₂ NPs on mouse macrophages.** Since Au/TiO₂ has been extensively studied in the field of catalysis, we have first compared biological effects of Au/TiO₂ samples having different size of Au NPs versus TiO₂ NPs on mouse peritoneal macrophages. Au NPs with two different mean diameters (3 and 8 nm) supported on pristine TiO₂ NPs were termed Au₃/TiO₂ and Au₈/TiO₂, respectively. No cytotoxicity was observed by either 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) or lactate dehydrogenase (LDH) release assays for TiO₂ and Au/TiO₂ NPs regardless of the concentration (1 to 100 μg ml⁻¹), the exposure time (24 and 48 h) and the size of Au NPs (Fig. 2).

In addition to cytotoxicity evaluation, we examined whether Au/TiO₂ NPs modified the phagocytic capacity of macrophages (an important function of these cells) as compared to TiO₂ NPs. This was performed by quantifying phagocytosis of fluorescent beads after cells incubation with TiO₂ and Au/TiO₂ NPs (50 μg ml⁻¹, 6 h incubation). The results of these experiments showed both TiO₂ and Au/TiO₂ NPs significantly increased the phagocytosis of fluorescent beads by peritoneal macrophages, but the differences between TiO₂ and Au/TiO₂ NPs was not observed. This result shows that Au NPs did not modulate phagocytic capacity of macrophages (an important function of these cells).

Macrophages incubation with TiO₂ and Au/TiO₂ NPs significantly and similarly increased the phagocytosis of fluorescent beads by peritoneal macrophages (Fig. S2).

**TiO₂ and Au/TiO₂ NPs are internalized in mouse peritoneal macrophages.** Cellular uptake and intracellular morphology upon 24 h exposure to TiO₂ and Au/TiO₂ NPs were investigated in ultra-thin sections of resin-embedded cells using TEM.

TiO₂ NPs and Au/TiO₂ NPs (50 μg ml⁻¹) were internalized in cells. The prevalent localization of NPs agglomerates was observed in cell vacuoles (Fig. S3). In accordance with the previous paragraph showing a lack of cytotoxicity, no morphologic sign of cell damage was observed.

**No inflammation induced by Au/TiO₂ NPs.** Incubation of mouse peritoneal macrophages with TiO₂ NPs (50 μg ml⁻¹, 6 h), induced a significant increase in the concentration of TNF-α and IL-1β, two main pro-inflammatory cytokines, in cell culture medium (Fig. 4). In contrast, the deposition of Au NPs onto TiO₂ greatly reduced the concentration of both TNF-α and IL-1β compared to pristine TiO₂, and this effect was independent on the size of Au NPs.

**Au/TiO₂ NPs attenuate lipopolysaccharide (LPS)-induced inflammation.** Having demonstrated that Au/TiO₂ NPs did not elicit an inflammatory reaction, we questioned whether Au/TiO₂ NPs could blunt inflammation induced in a model of pathophysiological relevance, such as macrophages exposure to *Escherichia Coli* LPS. Since no difference of anti-inflammatory effect was observed between Au₃/TiO₂ and Au₈/TiO₂, we investigated the effects of Au₃/TiO₂ NPs (referred to as Au/TiO₂ in the following experiments). Mouse peritoneal macrophages were incubated with culture media for 6 h (control condition) or LPS for 2 h, and then the following 4 h with LPS alone or LPS plus TiO₂ or Au/TiO₂. Taking into account the results presented in the previous paragraph, TNF-α and IL-1β cell culture supernatant concentrations were measured as a surrogate of the inflammatory reaction. As expected, incubation of cells with LPS for 2 + 4 h induced a significant increase as compared to the control condition (p < 0.05, Fig. 5a,b). Incubation of LPS followed by TiO₂ NPs did not significantly modify the effect of LPS alone. By contrast, this last effect was significantly attenuated by macrophage incubation with Au/TiO₂ NPs (p < 0.05, Fig. 5a,b). These results show that Au/TiO₂ can attenuate LPS induced inflammation, even when cells are incubated with these NPs after incubation with LPS.
Effects of Au/ZrO$_2$ and Au/CeO$_2$ NPs on LPS-induced inflammation. After showing the anti-inflammatory effect of Au/TiO$_2$ NPs, we investigated if this effect was related to the chemical nature of the MOx NPs. To answer this question, we examined the effect of Au NPs deposited on either ZrO$_2$ or CeO$_2$ NPs on mouse peritoneal macrophages. Physicochemical characterization of these Au/MOx are provided in Table 1. No effect of these NPs on macrophages viability was observed (Fig. 3).
Figure 2. Cytotoxicity of Au nanoparticles (NPs) with two different diameters (3 and 8 nm respectively) supported on pristine TiO$_2$ (Au3/TiO$_2$ and Au8/TiO$_2$ respectively) and TiO$_2$ NPs alone on mouse peritoneal macrophages. Cell viability was measured using the MTT assay (a,b) and LDH relargage (c,d) after 24 and 48 h incubation (panels a and c, and b and d, respectively). Data are expressed as percentage of control condition (without NPs, 100% is the reference value) and are represented as mean ± standard error of the mean (SEM) of 6 independent experiments. Positive controls are doxorubicine (DOXO, 1, 5 and 10 µg ml$^{-1}$) and TritonX-100 (10 vol. %). * significantly different from control ($p < 0.05$).

Figure 3. Cytotoxicity of Au nanoparticles (NPs) supported on TiO$_2$, ZrO$_2$ and CeO$_2$ NPs and TiO$_2$, ZrO$_2$ and CeO$_2$ NPs alone on mouse peritoneal macrophages. Cell viability was measured using the WST-1 assay after 24 h incubation. Data are expressed as percentage of control condition (without NPs, 100% is the reference value) and are represented as mean ± SEM of 6 independent experiments. Positive control is doxorubicine (DOXO, 1, 5 and 10 µg ml$^{-1}$) * significantly different from control ($p < 0.05$).
previously. Since the pristine CeO$_2$ NPs possess antioxidant properties, the effect of Au deposited on CeO$_2$ was studies showed opposite results. For example, Ng et al.\cite{16} showed that incubation of human bronchial epithelial

cells with 50 µg ml$^{-1}$ of 20 nm Au NPs activated NF-κB in bronchial epithelial cells. Close results were shown in other conditions\cite{10,11}. To investigate this issue, we measured the effect of Au/MOx NPs on intracellular reactive oxygen species (ROS) concentration. These experiments showed that ROS production induced by Au/TiO$_2$ and Au/ZrO$_2$ NPs was smaller than that of TiO$_2$ and ZrO$_2$, respectively, demonstrating an antioxidant effect of Au/TiO$_2$ and Au/ZrO$_2$ (Table 2), in accordance with their (partial) anti-inflammatory effect. This effect was more pronounced in the case of Au/ZrO$_2$ compared to Au/TiO$_2$ (mean reduction of 32% vs 11% compared to ZrO$_2$ and TiO$_2$ respectively). This antioxidant effect could be involved in the anti-inflammatory effect described previously. Interestingly, the level of ROS in cells exposed to CeO$_2$ was lower as compared to those in cells exposed to TiO$_2$ and ZrO$_2$, which is in accordance with antioxidant properties of CeO$_2$ described previously\cite{12}. Since the pristine CeO$_2$ NPs possess antioxidant properties, the effect of Au deposited on CeO$_2$ was probably limited.

**Discussion**

The most important result of this study is an anti-inflammatory effect of Au/MOx NPs depending on the MOx nature with particle internalization but no alteration of cell viability or phagocytic capacity of Au/MOx NPs as compared to MOx NPs (Figs. 2 and 3). The effect of Au/TiO$_2$ NPs was not related to Au NPs size (at least in the case of Au/TiO$_2$ NPs in the range of 3–8 nm) (Fig. 4) and was dependent on the MOx NPs chemical nature (Au/TiO$_2$ > Au/ZrO$_2$ > Au/CeO$_2$ if we consider the number of cytokines whose concentration was reduced by the NPs) and on the inflammatory mediator considered (Fig. 5). To the best of our knowledge, this is the first demonstration that Au/MOx presents anti-inflammatory properties.

The effect of Au/MOx could be related to anti-inflammatory properties of Au NPs per se. Indeed, gold colloids have been thought to cure various diseases for many centuries, and recent studies have demonstrated that this effect is mediated by inhibition of NF-κB activation\cite{13}. Different studies showed a similar effect in the case of Au NPs in vitro and in vivo, this effect being usually mediated by antioxidant properties\cite{14-16}. However, other studies showed opposite results. For example, Ng et al.\cite{16} showed that incubation of human bronchial epithelial cells with 50 µg ml$^{-1}$ of 20 nm Au NPs activated NF-κB in bronchial epithelial cells. Close results were shown in other cell types\cite{17,18}. Therefore, an anti-inflammatory effect of Au NPs per se seems not a univocal mechanism explaining the anti-inflammatory effect of Au/MOx NPs. Moreover, most of the studies showing the effect of Au NPs on inflammation have been conducted at much higher concentrations than the cells were exposed to in this study. Considering that Au NPs account for only 1% of the mass of Au/MOx, simply attributing the present results to the biological activity of Au NPs is unlikely.

Adsorption of TNF-α and IL-1β on Au/MOx surface could also explain their different anti-inflammatory activity. Indeed, NPs, including Au NPs, can adsorb different molecules on their surface, resulting in their inactivation and reduction in their concentration in the medium\cite{19}. However, this mechanism seems unlikely in the present study, since we did not observe any significant difference in TNF-α and IL-1β levels when these cytokines were incubated with TiO$_2$ or ZrO$_2$ NPs and their respective Au counterparts (Fig. 6).

Finally, the differences in the anti-inflammatory effect of Au/MOx NPs could be related to their different antioxidant properties. However, the anti-inflammatory effect of Au/MOx NPs was not good consistent with the

| Nanoparticle | Mean values ± SEM | Statistical significance |
|--------------|------------------|-------------------------|
| Au/TiO$_2$   | 12,068 ± 499     | *                       |
| TiO$_2$      | 13,547 ± 340     |                         |
| Au/ZrO$_2$   | 9170 ± 109       | **                      |
| ZrO$_2$      | 13,538 ± 129     |                         |
| Au/CeO$_2$   | 12,743 ± 340     |                         |
| CeO$_2$      | 12,583 ± 255     | #                       |

Table 2. Reactive oxygen species (ROS) production in RAW 264.7 cells after expositions to different NPs. The final concentration of NPs was 50 µg ml$^{-1}$. Amounts of ROS production were evaluated by the DCFH-DA (n = 7). *p < 0.05 vs TiO$_2$. **p < 0.01 vs ZrO$_2$. #p < 0.05 vs TiO$_2$ and ZrO$_2$. 

Concerning LPS-induced inflammation, no effect was observed with Au/CeO$_2$ NPs, whereas Au/ZrO$_2$ NPs attenuated LPS-induced IL-1β production without any effect on TNF-α concentration (Fig. 5a,b). Therefore, we can conclude that Au/MOx possess an anti-inflammatory effect which depends on the nature of the MOx NPs and on the inflammatory mediator examined.

**Is the anti-inflammatory effect of Au/MOx NP related to cytokines adsorption on their surface?** We investigated the mechanisms underlying the anti-inflammatory effect of Au/MOx. Since MOx NPs adsorb different proteins on their surfaces\cite{9}, we then investigated if the decreased concentration of LPS-induced TNF-α and IL-1β concentration observed with Au/TiO$_2$ and Au/ZrO$_2$ (only for IL-1β in this last case) was related to adsorption of these cytokines on these Au/MOx. The results of these experiments showed that incubation of each cytokine with the different Au/MOx did not change its respective concentration when compared to the MOx NPs without Au (Fig. 6). This result excludes cytokines adsorption as a mechanism explaining the anti-inflammatory effect of Au/TiO$_2$ and Au/ZrO$_2$.

**Is the anti-inflammatory effect of Au/MOx related to their antioxidant properties?** We then examined if the anti-inflammatory effect of Au/TiO$_2$ and Au/ZrO$_2$ was related to antioxidant properties, as demonstrated in other conditions\cite{10,11}. To investigate this issue, we measured the effect of Au/MOx NPs on intracellular reactive oxygen species (ROS) concentration. These experiments showed that intracellular ROS production induced by Au/TiO$_2$ and Au/ZrO$_2$ NPs was smaller than that of TiO$_2$ and ZrO$_2$, respectively, demonstrating an antioxidant effect of Au/TiO$_2$ and Au/ZrO$_2$ (Table 2), in accordance with their (partial) anti-inflammatory effect. This effect was more pronounced in the case of Au/ZrO$_2$ compared to Au/TiO$_2$ (mean reduction of 32% vs 11% compared to ZrO$_2$ and TiO$_2$ respectively). This antioxidant effect could be involved in the anti-inflammatory effect described previously. Interestingly, the level of ROS in cells exposed to CeO$_2$ was lower as compared to those in cells exposed to TiO$_2$ and ZrO$_2$, which is in accordance with antioxidant properties of CeO$_2$ described previously\cite{12}. Since the pristine CeO$_2$ NPs possess antioxidant properties, the effect of Au deposited on CeO$_2$ was probably limited.
Figure 4. Effect of Au3/TiO2, Au8/TiO2, and TiO2 NPs (50 µg ml⁻¹, 6 h incubation) on TNF-α and IL-1β secretion by mouse peritoneal macrophages (a,b). Data are expressed as picograms/ml in cell culture medium and are represented as mean ± SEM of 6 independent experiments. #Significantly different from control (p < 0.01).

Figure 5. Effect of Au deposited on different metal oxide NPs on LPS induced TNF-α and IL-1β secretion by mouse peritoneal macrophages (a,b). Cells were incubated with culture media for 6 h (control condition, C) or LPS for 2 h, and then the following 4 h with LPS alone or LPS plus the different MOx or Au/MOx NPs (50 µg ml⁻¹). *Significantly different from C (p < 0.05), **significantly different from LPS 2 h—TiO2 4 h (p < 0.05), &significantly different from LPS 2 h—ZrO2 4 h (p < 0.05).
intracellular antioxidant properties, since both Au/TiO₂ and Au/ZrO₂ NPs reduced intracellular ROS concentration whereas only Au/TiO₂ attenuated both TNF-α and IL-1β induction (Fig. 5). In concordance with different studies, CeO₂ NPs exerted an antioxidant effect as compared to TiO₂ and ZrO₂ NPs (Table 1), but this effect was not accompanied by an anti-inflammatory effect. On the other hand, the absence of an anti-inflammatory effect of Au/CeO₂ was paralleled by an absence of an antioxidant effect of CeO₂ NPs. Collectively, these data suggest that, in contrast with our initial hypothesis, an antioxidant effect is not a mechanism globally explaining the anti-inflammatory effect of Au/TiO₂ and Au/ZrO₂. Indeed, an antioxidant effect could potentially explain the effect of Au/TiO₂ NPs but not the one of Au/ZrO₂ NPs, because Au/ZrO₂ NPs had showed an antioxidant effect similar to Au/TiO₂ but attenuated only IL-1β induction. Consequently, the anti-oxidant effect of Au/ZrO₂ appears to relate on pathways inhibiting IL-1β but not TNF-α expression.

Numerous mechanisms have been reported to control the production and activity of IL-1β, including the processing of the 31-kDa, inactive IL-1β precursor into the bioactive, 17-kDa cytokine via intracellular protein complexes termed the inflammasomes. The most intensely studied inflammasome is the NLRP3 inflammasome. Various stimuli activate the NLRP3 inflammasome: bacterial structures such as muramyl dipeptide or LPS, bacterial RNA, β-glucan, double-stranded RNA, etc. The release of ROS has been reported to mediate NLRP3 inflammasome activation by various stimuli, including LPS, but this has been surrounded by controversy. Other proposed mechanisms responsible for NLRP3 inflammasome activation are: (i) translocation to mitochondria, (ii) release of mitochondrial DNA or cardiolipin, (iii) release of lysosomal cathepsins into the cytoplasm, and (iv) calcium-dependent phospholipase 2 activation. Interference with one or several mechanisms could explain attenuation of LPS-induced IL-1β expression by Au/ZrO₂ NPs. However, investigating these possibilities needs further studies.

Figure 6. TNF-α and IL-1β adsorption on Au deposited on different MOx NPs. MOx or Au/MOx NPs (50 µg ml⁻¹) were incubated during 6 h with TNF-α (150 pg ml⁻¹) and IL-1β (100 pg ml⁻¹) in culture media, then the cytokines were quantified in the media after centrifugation.
This study presents four main limitations. First, as MOx NPs are made of different materials, the cells will be exposed to a different amount of particles. However, we consider this possibility unlikely because Au content in the different Au/MOx NPs was very low (around 1 wt%). Second, we used two different murine macrophages populations: primary cultures of peritoneal macrophages and the RAW 264.7 macrophages cell line. We chose to use primary cultures of peritoneal macrophages because a large body of literature combining both in vitro approaches using peritoneal macrophages, and in vivo experiments in mice, demonstrated the relevance of the findings obtained in peritoneal macrophages, and their potential translation to what may occur in vivo. Moreover, we wanted to be at most in accordance with the 3R's guidelines, and for this reason we performed the experiments with the murine macrophage cell line RAW 264.7. Indeed, as demonstrated by Maurya and coworkers, RAW264.7 cells behave similarly to primary cultures of peritoneal macrophages cells in some ways and can be used as a good model for inflammation- and immune function-related kinetic studies. RAW 264.7 behave different from peritoneal macrophages in other aspects of lipid metabolism and phenotypes used as models for various disorders such as atherosclerosis, but these types of responses were not concerned in the present study. Third, we only measured two cytokines as a surrogate of inflammatory markers. Even if these cytokines are broadly representative of the inflammatory reaction and are one of the very early ones in the inflammatory cascade, and we investigate primary murine macrophages (a key cell involved in inflammation), a wider analysis of mediators of this reaction is necessary to validate the present results. Fourth, we investigated only three MOx NPs supports. Although we choose supports widely characterized in terms of catalytic properties when combined with Au NPs, investigating a broader panel of MOx could allow a better characterizing the anti-inflammatory properties of Au/MOx NPs.

In conclusion, despite of the above-mentioned limitations, this study showed that Au/MOx possess anti-inflammatory properties in macrophages and the effect depended on the kind of MOx NPs. Au/TiO2 showed anti-inflammatory effect for both TNF-α and IL-1β. Although Au/ZrO2 was ineffective to reduce the concentration of TNF-α, the anti-inflammatory effect for IL-1β was more pronounced for Au/ZrO2, as compared to Au/TiO2. In addition, Au/TiO2 and Au/ZrO2 exhibited improved antioxidant properties in RAW 264.7 compared to the pristine TiO2 and ZrO2 NPs. Although the relationships between antioxidant properties and anti-inflammatory effect of Au/MOx NPs and the mechanism are still unclear, our finding, anti-inflammatory effect of Au/MOx NPs could have potential applications in the clinical setting, since it does not interfere with physiological parameters of these cells such as viability and phagocytosis.

Methods
Preparation of Au nanoparticles supported on metal oxides (Au/MOx). Materials. TiO2 (AEROSOL P25) was supplied from Nippon Aerosil Co., Ltd. ZrO2 (RC-100) and CeO2 (high purity fine cerium oxide) were purchased from Daiichi Kigenso Kagaku Kogyo Co., Ltd. Tetrachloroauric acid (HAuCl4·4H2O) and dimethyl gold(III)(acetylacetonate), Me2Au(acac), were purchased from Tanaka Kikinzoku Kogyo K.K. and Tri Chemical Laboratories Inc., respectively. Au/ZrO2 and Au/CeO2 prepared by deposition–precipitation were purchased from Haruta Gold Inc. All reagents and materials were used as received.

Characterization of Au/MOx. Average particle size of metal oxides after the deposition of Au NPs were measured by laser diffraction/scattering particle size distribution analyzer using a HORIBA, Partica LA-950V2. Specific surface area of metal oxides was estimated from the N2 adsorption measurement was carried out using a SHIMADZU, Tristar 3000. The samples were pretreated at 200 °C for 2 h under vacuum, and then the N2 adsorption isotherms were obtained at −196 °C. The Brunauer–Emmet–Teller (BET) method was used to calculate the specific surface area. Loading amount of Au on metal oxides were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Thermo Fisher Scientific, iCAP6500) or atomic absorption spectrometry (AAS, SHIMADZU, AA-6200). The samples were treated in aqua regia to dissolve Au NPs, filtered, and then diluted to measure. Average size of Au NPs supported on metal oxides were estimated by high-angle annular dark-field scanning transmission electron microscopic (HAADF-STEM) observation using a JEOL, JEM-3200FS.

Preparation of Au/MOx. Au NPs with two different mean diameters (3 and 8 nm) supported on pristine TiO2 NPs were prepared, termed Au3/TiO2 and Au8/TiO2, respectively. Au3/TiO2 was prepared by deposition–precipitation. An aqueous solution of HAuCl4 (1 mM, 50 mL) was warmed to 70 °C, and the pH was adjusted to 6 by adding NaOH aqueous solution. TiO2 (1.0 g) was added to the solution and the suspension was stirred at 70 °C for 1 h. The suspension was centrifuged, washed with water at 40 °C for five times, and then filtered. The resultant powder was dried in air at 100 °C overnight and calcined at 300 °C for 4 h to reduce Au(III) to Au(0). Au/ZrO2 and Au/CeO2 were also prepared by deposition–precipitation as well as Au3/TiO2. Au8/TiO2 was prepared by solid grinding. TiO2 (1.0 g) and Me2Au(acac) (15 mg) were ground in an agate mortar in air at room temperature for 20 min. The mixture was calcined at 300 °C for 2 h to give Au8/TiO2.

Physicochemical properties of the obtained Au/MOx are summarized in Table 1. The primary particle sizes of Au/TiO2, Au/ZrO2, and Au/CeO2 were 26 nm, 11 nm, and 7.3 nm respectively, estimated from their specific surface areas of the MOx NPs. The secondary particle sizes of Au/TiO2, Au/ZrO2, and Au/CeO2 were 9.4 µm, 3.6 µm, and 2.4 µm, respectively, determined by light scattering. The actual Au loadings for Au3/TiO2, Au8/TiO2, Au/ZrO2, and Au/CeO2 were 0.96, 1.18, 0.97, and 0.96 wt%, respectively. The mean diameters of Au NPs were estimated by HAADF-STEM observation to be 3.3 ± 1.4 nm for Au3/TiO2, 7.8 ± 2.7 nm for Au8/TiO2, and 3.3 ± 1.8 nm for Au/ZrO2 (Fig. 1). In the case of Au/CeO2, it was difficult to observe Au NPs because Au was mostly deposited as single atoms and the atomic number of Ce is close to that of Au.
The different NPs were evaluated for LPS contamination (Limulus Amebocyte Lysate test), and the observed absorbance at 405 nm was below detection limits demonstrating no LPS contamination.

Cells isolation and incubation with nanoparticles. Peritoneal macrophages and macrophages from the RAW 264.7 cell line were used in the different experiments.

Mice peritoneal macrophages were prepared as follows. C57BL/6 mice were injected intraperitoneally with 1 ml of 3% Brewer thioglycollate (Sigma-Aldrich, B2551). After 96 h, peritoneal cells were harvested by lavage with 0.67% phosphate buffered saline (PBS). After a soft centrifugation, cells were maintained in Dulbecco’s modified Eagle medium (DMEM) 4.5 g l⁻¹ glucose supplemented with 10% fetal calf serum (FCS) and 1% penicillin and 1% streptomycin. Then mice peritoneal macrophagic cells were exposed for 6–48 h to 1–100 µg ml⁻¹ particles of Au/TiO₂, Au/ZrO₂, Au/CeO₂, and MOx alone.

The RAW 264.7 cells were cultured using a medium having the same composition as above.

The viability of cells was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) assay, and the quantification of the release of lactate dehydrogenase (LDH).

Inflammatory responses. Inflammatory response was evaluated by the quantification of cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β in cell supernatant, measured by enzyme-linked immunosorbent assay (ELISA).

Measurement of oxidative stress by DCFH-DA assay. Endogenous ROS were quantified by oxidation of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) into 2′,7′‐dichlorofluorescin (Sigma, Saint Quentin Falavier, France). Briefly, cells were cultivated in six‐well culture plates and treated with 50 µg ml⁻¹ NPs. Cells were also treated with 250 µM H₂O₂ as a positive control (data not shown). Cells were incubated with 20 µM DCFH-DA for 30 min at 37 °C and fluorescence recorded for 90 min. Results were expressed as the mean ratio of fluorescence recorded every 15 min during the 90 min period.

Measurement of TNF-α and IL-1β adsorption to NPs. Adsorption of TNF-α and IL-1β adsorption to NPs was performed by ELISA as described previously described³⁹.

Statistical analysis. JASP software (version 0.11.1, https://jasp-stats.org/) was used to analyze quantitative data. Non-parametric ANOVA was used to compare multiple groups. Paired comparisons with Mann–Whitney test were performed if the differences using ANOVA were statistically significantly different. Data were presented as mean values ± standard error of the mean (SEM), and results were considered statistically significant if p < 0.05.

Study approval for animal experiments. The Institutional Animal Care and Use Committee approved experimental procedures on mice (APAFIS authorization #14914-2018042515599016).

All methods were carried out in accordance with relevant guidelines and regulations and are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 12 February 2021; Accepted: 2 November 2021 Published online: 30 November 2021

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Acknowledgements
This work is financially supported by JSPS KAKENHI Grant Number JP19K15410.

Author contributions
T.F. prepared and analyzed NPs, performed chemical experiments and critically reviewed the manuscript. T.F., M.Z. and D.E. performed biological experiments. T.M., M.H., and S.L. critically reviewed the manuscript and worked on its final elaboration. J.B. designed the study and wrote the first and final drafts of the manuscript. All authors approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-02419-4.

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