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Effects of Gold Nanoparticles and Gold Anti-Arthritic Compounds on Inflammation Marker Expression in Macrophages

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Effects of Gold Nanoparticles and Gold Anti-arthritic Compounds on Inflammation Marker Expression in Macrophages

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Abstract

The ability of aurothiomalate and auranofin to alter the production of a number of cellular mediators of inflammation by RAW264.7 macrophages, was compared to each other, and that of gold nanoparticles (Au NPs). Addition of auranofin was found to have a pronounced ability to lower the production of reactive nitrogen and oxygen species (RNS and ROS, respectively), as well as Interleukin-10 (IL-10) and Tumour Necrosis Factor (TNF), by macrophages that were subsequently treated with lipopolysaccharide (LPS) to stimulate production of the mediators. In contrast, prior treatment of the cells with either aurothiomalate or Au NPs had either little or no significant effect on production of RNS and ROS. Treatment of the macrophages with Au NPs had a small effect on production of TNF by cells that were subsequently stimulated with LPS, however, the effect was much smaller than that elicited by auranofin. Similarly, aurothiomalate had a small, but significant effect on production of IL-10. Varying the size of the Au NPs, or the identity of the protective sheath surrounding the nanoparticles, did not have a significant effect on the production of RNS or ROS by LPS-stimulated macrophages. The results of some of these investigations are discussed in the light of other studies reported in the literature. In addition, results obtained by scanning electron microscopy and energy dispersive X-ray spectroscopy are presented which provide evidence for the accumulation of gold within macrophages exposed to Au NPs.

Key words: gold; bioinorganic chemistry; nanomedicine

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that typically occurs as inflammation around the synovial joints of the body, and causes discomfort and difficulty in movement. The prevalence rate of RA in the developed world is estimated to be approximately 0.5 – 1%,[1] with the disease usually presenting between ages 45 and 65,[2] and occurring more frequently in women.[3] Following a series of trials in the 1920's and 1930's,[4–7] and confirmation in subsequent publications,[8–11] injectable gold(I) compounds such as sodium aurothiomalate became a popular choice for treatment of the disease. Several decades later the gold(I) phosphine complex auranofin was developed as a treatment for RA. Auranofin was not as effective as earlier gold drugs, but could be administered orally, and was found to have fewer or less severe side-effects.[12] Today the clinical use of gold(I) complexes for the treatment of RA has largely been superseded by other drugs, such as methotrexate, tumour necrosis factor (TNF) inhibitors, and "biologics" such as infliximab and etanercept.[1,13–15] While gold complexes are still used occasionally for treatment of RA,[16,17] this is generally only for refractory forms of the disease that are unresponsive to other medications.

There has also been a significant amount of recent effort focussed on the potential of gold nanoparticles (Au NPs) for a wide range of purposes in biology and medicine, including the treatment of RA.[18] One such study was published in 1997, and involved a group of ten patients suffering from a chronic form of the disease, who exhibited minimal response to several existing therapies, including gold(I) drugs.[19] The patients were then given an oral formulation of Au NPs called Aurasol, which was synthesised by the citrate method with several undisclosed "proprietary modifications." Within one week of commencing treatment, patients showed a pronounced reduction in tenderness and swelling symptoms, which continued with further treatment. Statistically significant drops in the levels of several inflammation markers were observed by the end of the study.

A subsequent study examined the effects of citrate-stabilised Au NPs on two groups of rats in which polyarthritis had been induced by injections of *Mycobacterium tuberculosis*, collagen, or the saturated alkane, pristane.[20] Rats treated with either aurothiomalate or Au NPs exhibited milder symptoms than the group of control rats, when arthritis was induced with the bacterium. In contrast, only rats treated with Au NPs showed improvements in their condition when symptoms of arthritis were induced by administration of collagen or pristane.

The above studies highlight the potential for Au NPs to be used for the treatment of RA, and potentially other inflammatory diseases. However, many questions remain concerning how Au NPs may exert anti-arthritic effects, and their effectiveness in comparison to that of the traditional gold(I) drugs. In order to address these questions, we recently compared the cytotoxicity, extent of uptake, and distribution of several gold(I) compounds in RAW264.7 macrophages, with that of Au NPs.[21] The latter were less cytotoxic than any of the gold complexes examined, despite being incorporated to a significantly greater extent by the cells. The results of experiments conducted using synchrotron radiation X-ray fluorescence spectroscopy indicated that gold tended to accumulate in specific areas of the macrophages, regardless of what the latter were initially treated with. However, it was not possible to determine whether or not the gold was simply bound to the external surface of the macrophages, or had been internalised. Mass spectrometry was also used to examine whether or not Au NPs could bind to thioredoxin reductase, an enzyme involved in regulating the redox activity of macrophages.[22–24] These experiments were conducted in order to determine if anti-arthritic activity may arise in part because of changes to the activity of this enzyme. While all gold(I) compounds were found to bind readily to thioredoxin reductase, no such evidence was found when experiments were conducted with Au NPs. There is therefore still much that remains to be learnt about how both gold(I) compounds and Au NPs exert anti-arthritic effects.

Previously there have been a number of *in vitro* studies that examined the effects of Au NPs upon expression of key inflammation markers.[25–31] Despite this, none of these prior investigations systematically compared the effects of Au NPs to that of clinically used gold(I) compounds, in order to draw conclusions about the likely mechanism(s) of action of the Au NPs. We report here the results of the first such systematic study, which explored the effects of both types of gold reagents on the levels of reactive oxygen and nitrogen species, tumour necrosis factor (TNF) and interleukin 10 (IL-10) produced by RAW264.7 macrophages. In the experiments presented here, the macrophages were treated with the lipopolysaccharide (LPS) in order to stimulate production of the above inflammation markers. LPS is an toll-like receptor (TLR) 4 agonist, that has been used for this purpose in a number of similar studies.[25,27,29,32–37] The Au NPs used in the experiments described herein were synthesised by the "classical" method,[38] involving reduction of gold(III) chloride by sodium citrate, which then acts as the stabilising agent for the nanoparticles. In addition to the above investigations, we present the results of further studies into the uptake of Au NPs by RAW264.7 macrophages, conducted using scanning electron microscopy (SEM) in order to determine if gold is simply bound to the surface of the cells, or becomes internalised.

Results and Discussion

Cellular Uptake of Au NPs by Field Emission Scanning Electron Microscopy

In order to determine whether or not gold is incorporated into macrophages exposed to Au NPs, field emission scanning electron microscopy (FESEM) was employed. Figure 1 shows a series of FESEM images of an identical group of macrophages that had been treated with a solution containing 60 μM Au NPs. Each image was obtained using a different accelerating voltage. Since the use of higher voltages results in electrons with greater penetrating power, it was possible to detect gold that was deeper below the surface of the cells. Heavy metal deposits are easily identified by FESEM owing to their pronounced electron scattering properties, resulting in their appearance as white spots.[39] Confirmation that these spots were in fact gold was provided by comparing the FESEM image of a cluster of macrophages treated with Au NPs (Supp Figure 1a), to a gold elemental map of the same cells, obtained using energy dispersive X-ray (EDX) spectroscopy (Supp Figure 1b). The gold elemental map contains clear hot spots corresponding to regions of the cells where there are higher concentrations of gold. Furthermore the overlay of the SEM micrograph and EDX gold map (Supp Figure 1c) shows that the hot spots in the latter, correspond to the distinct white spots in the former, providing strong support that there is a high gold content in the regions.

The SEM image shown in Figure 1a was collected at the highest accelerating voltage used (25 kV), and shows three cells with many white spots most likely corresponding to Au NPs. Most of the spots appear somewhat diffuse, or out of focus. These are believed to derive from gold that is located below the membrane surface. In contrast, more sharply defined spots, such as that highlighted by the red arrow, are likely to correspond to surface-bound gold. Consistent with this conclusion, the latter spots remained visible in all other images obtained using lower accelerating voltages. In contrast, when the accelerating voltage was decreased, the number of diffuse, or out of focus spots arising from gold particles present below the surface of the cell decreased. Figure 1 therefore provides evidence that supports the conclusion that much of the gold present in citrate stabilised Au NPs becomes internalised by macrophages, and does not simply bind to the cell surface. The results here are generally consistent with those of an earlier study involving smaller (10 nm) Au NPs, with a similar outer stabilising layer to those examined in the current study.[40]

Nitric Oxide Release

The free radical nitric oxide (NO) is an important signalling molecule for a number of biological processes including vasodilation, neurotransmission and immune system regulation.[18] However, in RA it has been shown to act as a pro-inflammatory mediator.[19] This is illustrated by studies which showed that NO production is linked to generation of inflammatory cytokines, enhanced bone resorption,[18] diminished bone proliferation,[18] and chondrocyte apoptosis.[20] NO is a very short-lived species ($t_{1/2}$ = 6 s) and consequently exhibits low bioavailability.[41] Therefore the amount of NO released in the following studies was inferred by determining the concentration of its major decay product, the nitrite ion (NO_2^-), using the Griess assay.[42]

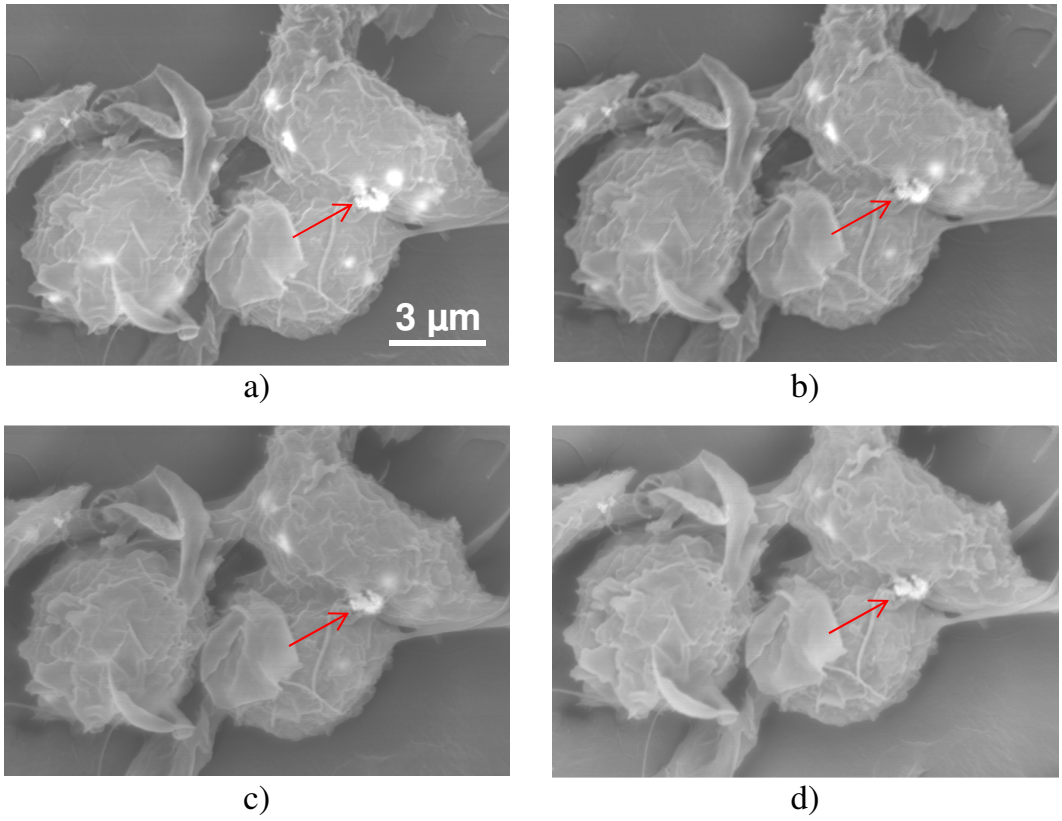

Fig. 1. FESEM images of RAW264.7 macrophages treated with 60 µM Au NPs at 37 °C under an atmosphere of 5% CO_2 for 24 h. The images are of the same cells collected at ×4300 magnification and were obtained with accelerating voltages of: **a)** 25 kV; **b)** 18 kV; **c)** 15 kV; and **d)** 10 kV.

Initial experiments showed that treating RAW264.7 macrophages with aurothiomalate, auranofin, Au NPs or their respective solvents (vehicles), did not significantly induce NO_2^- formation (Fig. 2a). In contrast, treating the same macrophages with LPS induced formation of significant amounts of nitrite ion. These results meant it was possible to look at the effect of pre-treatment of the macrophages with Au NPs, or gold compounds, on the amounts of NO_2^- produced by subsequent treatment of the cells with LPS. The results of these experiments are presented in Fig. 2b.

Pre-incubation of the macrophages with 2.5 µM aurothiomalate did not result in a statistically significant reduction in formation of NO_2^-, compared to treating the cells with the corresponding vehicle (incomplete medium). When the concentration of aurothiomalate was increased to 60 µM, a drop in the production of NO_2^- was observed; however, the magnitude of the decrease was not sufficiently large for it to be deemed statistically significant. In contrast, when the cells were pre-treated with 2.5 µM auranofin, almost complete inhibition of NO_2^- production was observed after subsequent addition of LPS. Pre-incubation of the macrophages with Au NPs at both concentrations examined resulted in small (~10%), statistically insignificant decreases in the concentration of NO_2^-, compared to what was observed when the cells were first treated with the Au NPs vehicle.

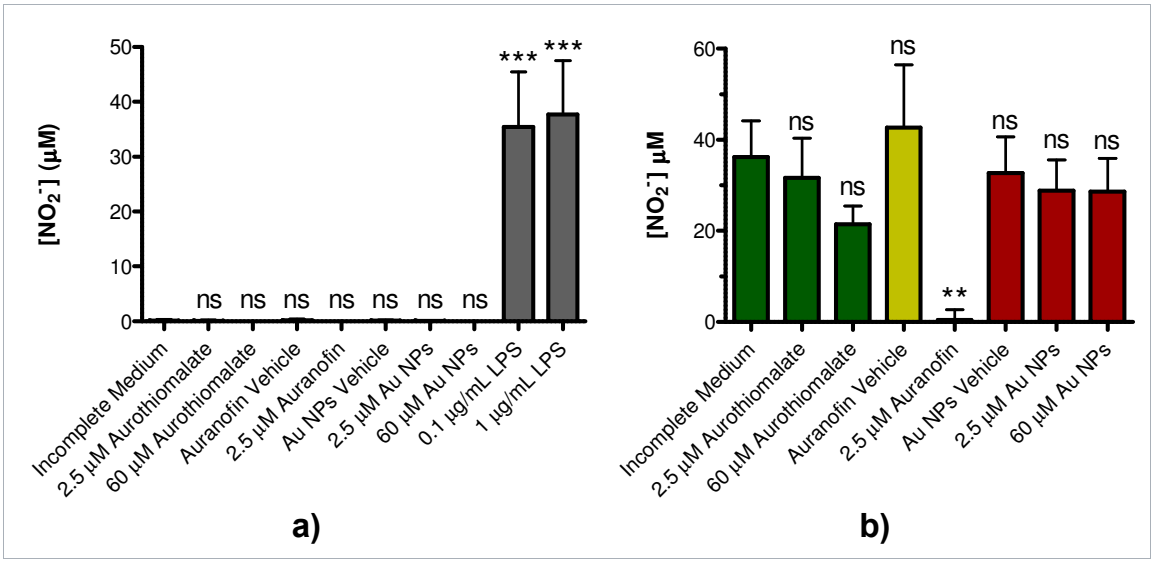

Fig 2. Effect of gold compounds or Au NPs, on the extent of nitrite production by RAW264.7 macrophages. **a)** Cells incubated with gold compounds, Au NPs or LPS at 37 °C under an atmosphere of 5% CO_2 for 24 h (control experiments); **b)** Cells pre-incubated with gold compounds or Au NPs at 37 °C under an atmosphere of 5% CO_2 for 4 h, then 0.1 µg/mL LPS added, and the cells incubated under the same conditions for a further 20 h. Nitrite concentration was then measured by the Griess assay. The error bars represent one standard deviation calculated from triplicate experiments. ** = statistically significant ($P < 0.01$) compared to corresponding vehicle; *** = statistically significant ($P < 0.001$) compared to corresponding vehicle; ns = not significant compared to corresponding vehicle ($P > 0.05$).

The results obtained with auranofin are in good agreement with those obtained in other studies using RAW264.7 cells, where the amount of LPS-induced nitrite was measured after first treating the cells with 3 or 20 µM gold complex.[34,36] In contrast, previous studies also showed that pre-treatment with aurothiomalate resulted in moderate to large decreases in NO_2^- production for RAW264.7 cells activated by LPS,[43] as well as for murine H4 chondrocytes activated by IL-1β,[44] and non-activated peritoneal macrophages isolated from mice.[45] Whilst the results obtained using aurothiomalate in the current study may not be statistically different from those obtained with its vehicle, there appears to be a general trend which suggests that pre-incubation with even higher concentrations of this gold complex might lead to statistically relevant decreases in NO_2^- production.

The absence of a significant effect of pre-treatment with Au NPs on the production of NO_2^-, is in contrast to results published by Ma *et. al.*[27] The latter authors used a very similar methodology to that followed in the current work. However, they observed statistically significant, concentration-dependent reductions in NO_2^- production when the macrophages were pre-treated using solutions containing 10, 20 and 40 µg Au/mL of Au NPs (equivalent to 51, 102 and 203 µM Au, respectively). Since the latter solutions contained much higher concentrations of gold compared to those used in the current study, it was decided to repeat our initial experiment using Au NP pre-treatment solutions with higher concentrations. A small drop in NO_2^- production was observed for macrophages that were pre-treated with solutions containing 400 or 600 µM Au NPs (Supp Figure 2). However, the reduction in NO_2^- levels was still not statistically significant compared to the macrophage sample treated with the Au NPs vehicle.

The results obtained in the current study using Au NP solutions are therefore in contrast to those reported previously.[27] The need for additional research to be carried out in this area is further highlighted by the results of a number of other published studies. For example, in one study pre-treatment of RAW264.7 macrophages with PEG-coated Au NPs caused a small increase in NO_2^- production, after the cells were subsequently treated with 0.1 µg/mL LPS.[29] In another investigation, pre-treatment of NR8383 rat macrophages with Au NPs stabilised using citrate or dihydrolipoic acid, caused no significant change in the amount of NO_2^- produced, after the cells were exposed to LPS.[28] The results of this last study mirror closely those presented here. This may be attributable to the Au NPs in both cases being stabilised using low molecular mass compounds, whereas in some of the other studies the nanoparticles had a protective polymeric coating.[27,29] The latter may have endowed the Au NPs with very different physical and chemical properties, that could have affected their ability to inhibit LPS-induced formation of NO_2^-.

Reactive Oxygen Species Formation

The term reactive oxygen species (ROS) refers to a group of compounds containing oxygen that are involved in cell signalling and homeostasis, and includes O_2, O_2^-, H_2O_2 and •OH.[46] Oxidative stress occurs when these molecules are produced in increased quantities, and can lead to significant damage to deoxyribonucleic acid

(DNA), proteins and lipids.[47] In addition, ROS can degrade extracellular matrix materials such as collagens and proteoglycans,[48] which directly leads to joint degradation and inflammation. In patients with RA, oxidative stress is observed in the synovial fluid along with a decrease in the activity of anti-oxidant enzymes.[49] This provides support for the conclusion that production of ROS is one of the main mechanisms of pathogenesis of the disease.[50]

The production of ROS *in vitro* was determined by measuring the extent of cellular conversion of the dye 2',7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) to its fluorescent form, 2',7'-dichlorofluorescein (DCF), using flow cytometry.[51,52] Addition of different concentrations of the gold compounds, or Au NPs, to the macrophages failed to induce ROS production. In contrast, addition of LPS to the cells did result in production of significant amounts of ROS (Fig. 3a). These results therefore mirrored those obtained when measuring NO production, described above. Furthermore, they enabled the following experiments to be performed, in which the effects of pre-incubation with auranofin, aurothiomalate, or Au NPs, on production of ROS by RAW264.7 cells subsequently treated with LPS, to be compared. The results of these experiments are presented in Fig. 3b, and were normalised with respect to those obtained when the macrophages were treated with incomplete medium only.

Treatment of the macrophages with 2.5 µM aurothiomalate had little effect on the production of ROS. However, when the concentration of aurothiomalate was increased to 60 µM, a 44% drop in ROS production was observed. As a result of the large standard deviation associated with this result, it was not statistically significant in the ANOVA analysis performed on this subset of samples. This is most likely due to the relatively large errors associated with the results for these as well as other samples, which are not uncommon for experiments conducted using the DCF assay. The results, nonetheless, suggest that treatment with aurothiomalate can decrease the amount of ROS produced by LPS-induced macrophages.

Treatment of the cells with the auranofin vehicle, consisting of 1% (v/v) dimethylsulfoxide (DMSO)/99% (v/v) incomplete medium, caused a significant (62%, $P < 0.05$) drop in ROS production relative to that caused by pre-treatment with incomplete medium. This is most likely a result of the DMSO present in the auranofin vehicle, as the former is a known ROS scavenger.[53] A similar conclusion was reached in a previous study performed using neuroblastoma cells and the DCF assay.[54]

Figure 3b shows that pre-treatment with 2.5 µM auranofin led to almost complete loss of ROS formation ($P < 0.05$), suggesting that this compound may have a much stronger effect on production of ROS than aurothiomalate. While its effect on total ROS production does not appear to have been measured previously, auranofin has been shown to inhibit the formation of the superoxide ion (O_2^-) in a number of studies using phagocytes,[55] polymorphonuclear leucocytes,[56–58] and neutrophils.[59] In addition, auranofin has been shown to quench highly reactive singlet oxygen.[60]

It is also worth noting that the results presented here using aurothiomalate are consistent with literature reports, which show this gold compound has a weaker inhibitory effect on the formation of superoxide than auranofin.[55–58] It has been suggested that one mechanism by which these two compounds produce their inhibitory effects is through activation of the heterodimeric transcription factor Nrf2/small maf, that upregulates anti-oxidant proteins.[61] In addition, it has been reported that both auranofin and aurothiomalate can suppress the pro-inflammatory transcription factor NF-κβ.[62]

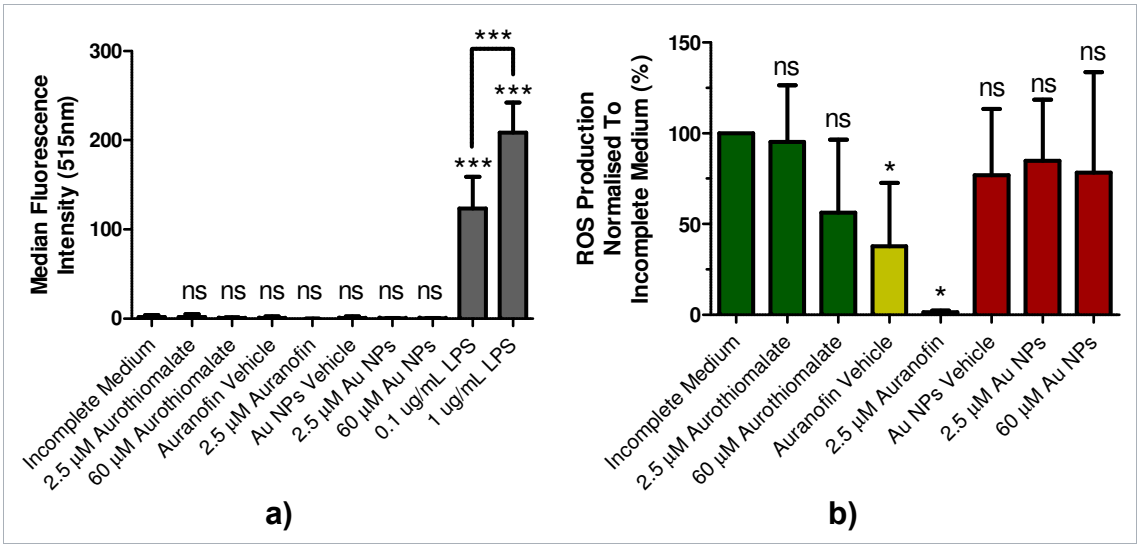

Fig. 3. Effect of gold compounds or Au NPs on ROS production by RAW264.7 macrophages. **a)** Cells incubated with gold compounds, Au NPs or LPS at 37 °C under an atmosphere of 5% CO_2 for 24 h (control experiments); **b)** Cells pre-incubated with gold compounds or Au NPs at 37 °C under an atmosphere of 5% CO_2 for 4 h, then 0.1 µg/mL LPS added, and

incubation continued under the same conditions for a further 20 h. ROS was measured by the DCF assay and presented as normalised MFI values at 515 nm, relative to incomplete medium. The error bars represent one standard deviation calculated from triplicate experiments. * = statistically significant ($P < 0.05$) compared to the corresponding vehicle; *** = statistically significant ($P < 0.001$) compared to the corresponding vehicle; ns = not significant compared to the corresponding vehicle ($P > 0.05$).

When the macrophages were pre-treated with the Au NPs vehicle, or solutions containing 2.5 µM or 60 µM Au NPs, there was perhaps a minor decrease in the amount of ROS produced, compared to cells pre-treated with incomplete medium. However, further analysis of the results revealed that the effects were not statistically significant. The lack of a significant effect of Au NPs on production of ROS appears to contradict the findings of a number of other researchers. For example, one study showed that Au NPs bought from BBI (Cardiff, UK) could reduce ROS formation by human peripheral blood mononuclear cells (PBMCs).[30] A luminol-dependent chemiluminescence assay was used to measure ROS production in this study, which also showed that Au NPs with smaller diameters were more effective at inhibiting ROS formation. One key difference between this study and that described here, is that ROS formation was induced using zymosan,[30] and not LPS. This is potentially important, as zymosan is a TLR2 ligand, whilst LPS is a TLR4 ligand. Therefore their inflammatory effects may be induced through separate pathways, which in turn, the Au NPs may interact differently with.

Au NPs synthesised by reaction of $[AuCl_4]^-$ with the soil bacterium *Bacillus licheniformis* have also been shown to inhibit ROS formation and lipid peroxidation in diabetic mice.[31] In this study, treatment of the mice with Au NPs caused upregulation of the anti-oxidant molecule glutathione, and the anti-oxidant proteins superoxide dismutase and glutathione peroxidase. It was concluded that the greater abundance of these proteins may have been the cause of the lower ROS levels observed.[31] In another study ROS production was not changed when RAW264.7 cells were treated with poly-L-lysine-coated Au NPs for a 24 h period.[25] However, exposure to a 100 µM solution of the Au NPs for 48 h did result in a significant reduction.[25]

Despite the above literature studies suggesting that various types of Au NPs can inhibit ROS formation in cells, there are a number of other investigations, which did not involve the use of an inflammation-stimulating molecule, which showed they have the opposite effect. For example, the DCF assay was used to show that myeloid leukaemia and liver carcinoma cell lines treated with 30, 50 and 90 nm Au NPs purchased from CymitQuímica (Barcelona, Spain) exhibited an increase in total ROS production, coupled with strongly decreased glutathione levels and superoxide dismutase activity.[63] In addition, Au NPs (1.4 nm diameter) stabilised by triphenylphosphine monosulfate caused significant ROS formation in cervix carcinoma cells, with the effect becoming more pronounced with longer exposure periods.[64] Similarly, cervix carcinoma cells exposed to various types of 2 nm Au NPs with organic stabilizers of different lengths, showed high levels of ROS production, according to measurements performed using the DCF assay.[65]

The variety of results in the literature highlights the likely importance of variations in the size and chemical composition of Au NPs on their biological activity. No literature studies appear to have looked at the effect of citrate stabilised Au NPs on ROS formation, making the results presented here distinct from those in the literature. The absence of a significant effect on ROS production, together with the minimal influence on NO production, suggests that the Au NPs studied here either do not have significant anti-inflammatory properties, or that they exert their effects through a different mechanism or mechanisms. In order to test the latter hypothesis, the effects of the Au NPs (and gold compounds) on the production of the inflammation cytokines TNF and IL-10 were studied.

Tumour Necrosis Factor Release

High levels of TNF in synovial fluid are a characteristic feature of RA, and are strongly linked to progression of the disease. This is due to pro-inflammatory role of TNF, which stimulates the release of a large number of other inflammatory mediators.[66] In addition to cytokine release, TNF can signal oxidative burst,[67] stimulate production of prostaglandins,[68] and induce synthesis of matrix metalloproteinases.[69,70]

Treatment of RAW264.7 macrophages with either 0.1 or 1 µg/mL LPS caused release of much larger amounts (~80 times) of TNF than that elicited by any of the gold compounds or Au NPs (Fig. 4a). For example, treatment of the cells with 60 µM aurothiomalate only resulted in production of 1.04 ng/mL of TNF, which was not significantly different ($P > 0.05$) compared to the amount of this cytokine produced when the macrophages were treated with incomplete medium (0.177 ng/mL). Similarly, treatment of the macrophages with auranofin or Au NPs failed to result in statistically significant increases in TNF production. These results indicate that the gold compounds and Au NPs do not induce production of significant amounts of this cytokine.

Figure 4b shows the effects of pre-incubation of RAW264.7 macrophages with aurothiomalate, auranofin or Au NPs, on TNF production by the cells after treatment with 0.1 µg/mL LPS. Pre-incubation with 2.5 µM aurothiomalate resulted in no significant effects on the amounts of TNF produced. When the concentration of aurothiomalate was increased to 60 µM, a statistically insignificant ($P > 0.05$) stimulatory effect was observed.

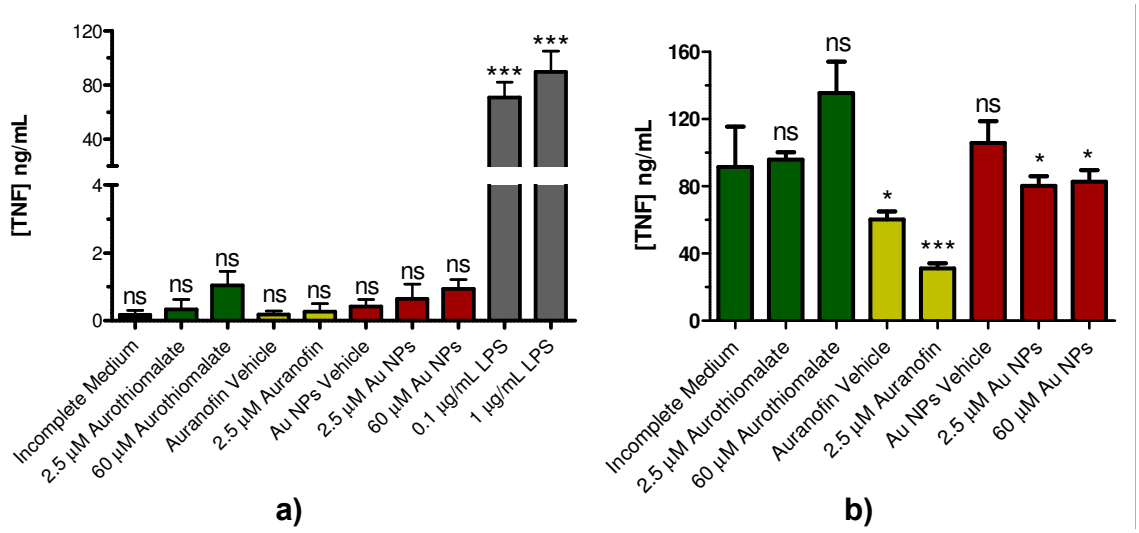

Fig. 4. Effect of gold compounds or Au NPs on TNF production by RAW264.7 macrophages. **a)** Cells treated with gold compounds, Au NPs, or LPS and incubated at 37 °C under an atmosphere of 5% CO_2 for 24 h (control experiments); **b)** cells pre-incubated with gold compounds or Au NPs at 37 °C under an atmosphere of 5% CO_2 for 4 h, then 0.1 µg/mL LPS added, and incubation continued under the same conditions for a further 20 h. TNF was measured by ELISA. The error bars represent one standard deviation calculated from triplicate experiments. * = statistically significant ($P < 0.05$) compared to corresponding vehicle; *** = statistically significant ($P < 0.001$) compared to corresponding vehicle; ns = not significant compared to corresponding vehicle ($P > 0.05$).

The above results contradict some reports in the literature that showed aurothiomalate has a small inhibitory effect on LPS-induced TNF production in human PBMCs.[71–73] A possible explanation for this difference could be that the experiments described here were performed using a murine macrophage cell line, as opposed to PBMCs isolated directly from humans[72,73] or mice.[71] It could also arise from differences in methodology, such as differences in the length of the pre-incubation period, and use of different LPS concentrations.[71–73] It should also be noted, however, that within the literature there are reports that suggest that aurothiomalate has no effect on production of TNF. For example, one study that also investigated human PBMCs (from RA patients) showed that aurothiomalate does not alter LPS-stimulated TNF production,[74] whilst another reported that the compound does not affect TNF mRNA levels in LPS-stimulated murine macrophages.[32]

Figure 4b also shows that pre-treatment of the macrophages with 2.5 µM auranofin resulted in a significant decrease (~50%, $P < 0.001$) in TNF production, compared to the auranofin vehicle. This is consistent with the outcomes from a previous study that showed there was inhibition of LPS-induced TNF formation in PBMCs following treatment with auranofin.[32,71] Figure 4b also indicates that pre-treatment using the auranofin vehicle, which contains 1% DMSO, may have had a significant ($P < 0.05$) inhibitory effect on TNF formation. A similar effect of DMSO upon TNF formation has previously been demonstrated for murine alveolar and peritoneal macrophages,[53] and is likely a result of inhibition of the transcription factor NF-κβ, which is involved in production of TNF and IL-10.[75]

Pre-treatment using either 2.5 or 60 µM Au NPs resulted in small decreases in LPS-induced TNF production, that were statistically significant ($P < 0.05$) when compared to the amount of cytokine produced when the pre-treatment was performed using the Au NPs vehicle. These results therefore appear to contrast those reported by Ma *et. al*,[27] who observed that Au NPs had no effect on LPS-induced TNF production. They are also inconsistent with results obtained from a study in which RAW264.7 macrophages were pre-treated with Au NPs coated with poly-*N*-vinylpyrrolidone, prior to the addition of LPS.[76] This once again highlights the importance that the composition of the nanoparticle shell may play in determining overall biological activity.

Interleukin-10 Release

It was also of interest to investigate whether the gold compounds and Au NPs might up-regulate anti-inflammatory activity, as opposed to inhibiting the production of pro-inflammatory cytokines. In order to investigate this possibility, experiments were performed using IL-10. This cytokine has been shown to suppress production of the pro-inflammatory mediators IL-1α, IL-6 and IL-8,[77] as well as TNF and NO in macrophages.[78]

Figure 5a shows the amounts of IL-10 produced by RAW264.7 cells exposed to aurothiomalate, auranofin, Au NPs or LPS. The results obtained indicate that none of the above gold reagents promote an anti-inflammatory process involving IL-10. This was not entirely surprising in the case of auranofin, as the results presented above showed this compound has a significant ability to suppress production of a variety of pro-inflammatory

cytokines. Furthermore aurothiomalate is likely to behave similarly to auranofin, due to similarities in their uptake mechanisms and metabolites. Treatment of the macrophages with LPS, at both 0.1 and 1 µg/mL, resulted in expression of large amounts of IL-10. This is most likely due to LPS directly inducing production of pro-inflammatory mediators such as TNF, which when present at sufficient levels acts as the signal for initiation of an anti-inflammatory response involving IL-10 and other cytokines. This is consistent with previous work which showed that after 12 – 24 h treatment with LPS, bone marrow derived macrophages up-regulate production of IL-10 to suppress formation of the pro-inflammatory cytokine IL-1β.[79]

Whilst the results presented in Fig. 5a show that none of the gold compounds or Au NPs had a significant ability to induce IL-10 production in macrophages, the possibility remains that they may affect IL-10 production caused by other stimuli. In order to investigate this hypothesis, a series of experiments were performed to examine whether pre-treatment of macrophages with gold compounds or Au NPs, has an effect on the amount of IL-10 produced upon subsequent treatment with LPS. Figure 5b shows the results of these experiments.

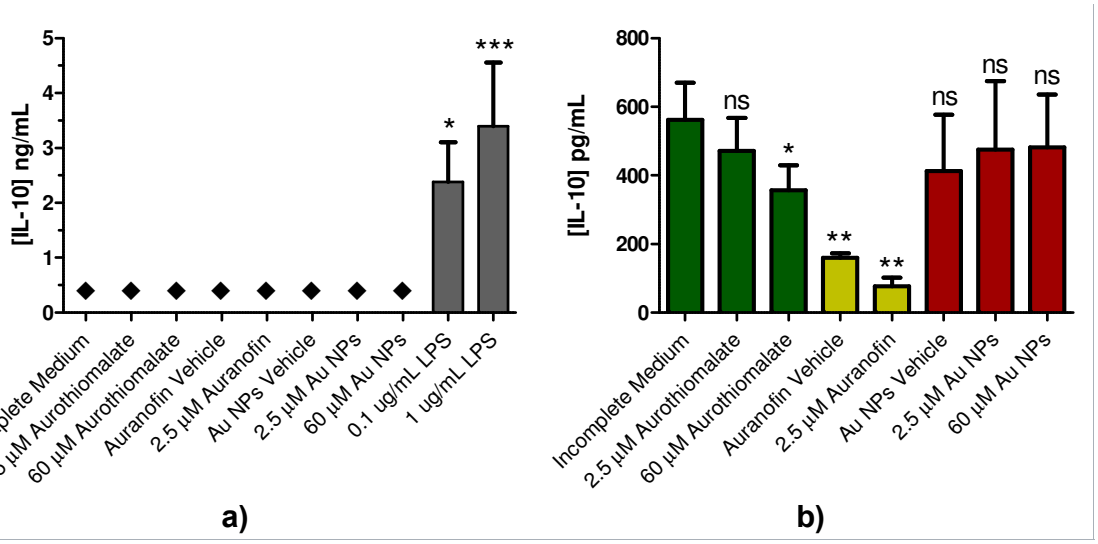

Fig. 5. Effect of gold compounds or Au NPs on IL-10 production by RAW264.7 macrophages. **a)** Cells treated at 37 °C under an atmosphere of 5% CO_2 for 24 h (control experiments); **b)** cells pre-incubated with gold compounds or Au NPs at 37 °C under an atmosphere of 5% CO_2 for 4 h, then 0.1 µg/mL LPS added, and incubation continued under the same conditions for a further 20 h. IL-10 was measured by ELISA. The error bars represent one standard deviation calculated from triplicate experiments. ◆ = below detection limit; * = statistically significant ($P < 0.05$) compared to corresponding vehicle; ** = statistically significant ($P < 0.01$) compared to corresponding vehicle; *** = statistically significant ($P < 0.001$) compared to the corresponding vehicle; ns = not significant compared to corresponding vehicle ($P > 0.05$).

Pre-treatment of the macrophages with the auranofin vehicle resulted in a significant decrease ($P < 0.01$) in IL-10 levels compared to treatment with incomplete medium. When solutions containing auranofin itself were used, an even larger decrease in cytokine production was observed. This decrease was statistically significant compared to that caused by the auranofin vehicle alone, indicating that the DMSO present in the latter was not solely responsible for the decrease in IL-10 levels, and that the gold complex also had an effect. This result on its own is contrary to what would be expected from a compound that is used as a treatment for RA. However, it can be explained after consideration of results presented earlier. Previously it was noted that addition of auranofin caused a significant reduction in the amount of LPS-induced TNF, NO and ROS produced by RAW264.7 macrophages. The decrease in levels of each of these pro-inflammatory cytokines may therefore have been a signal to the cell that lower amounts of IL-10 are required.

Figure 5b shows that pre-treatment of the macrophages with 60 µM aurothiomalate resulted in a small, but statistically significant, decrease in the amount of IL-10 produced. This may reflect the slightly lower levels of production of NO and ROS noted earlier in experiments involving high concentrations of this gold compound. In contrast, addition of aurothiomalate may have had the opposite effect on TNF levels, although the results obtained were once again not deemed to be statistically relevant. Further work is therefore required to provide a definitive answer to this question. Pre-treatment with Au NPs did not cause a statistically significant change to the amount of IL-10 produced, once again indicating a lack of interaction between the nanoparticles and the macrophages. There is therefore still further work that is required in order to fully understand the mechanism of the anti-arthritic action of Au NPs.

Studies with Commercial Au NPs

There was no significant anti- or pro-inflammatory behaviour observed for macrophages exposed to the Au NPs prepared for this study. This suggests the anti-arthritic activity noted previously for Au NPs might arise by mechanisms involving other mediators of inflammation. Alternatively the above results might be a consequence of differences in the size and/or composition between the nanoparticles used in the current work, and those that formed part of the earlier investigations. In order to explore the latter possibility, it was decided to repeat some of the above studies into the effects on ROS and NO, using a range of commercial Au NPs with different sizes (2 nm, 20 nm and 100 nm), or stabilised using different media (PBS or citrate buffer). The ability of these commercial Au NPs to affect the formation of the above species in LPS-stimulated macrophages was compared to that of the citrate-stabilised Au NPs synthesised for this project, as well as auranofin as a positive control (not shown). Figure 6 shows the results of these experiments.

None of the commercial Au NPs resulted in a statistically significant decrease in the amount of NO_2^- produced, compared to that elicited by the Au NPs vehicle. However, it is worth noting that the 2 nm BBI Au NPs did produce a 20% reduction in the amount of NO_2^-. This indicates that regardless of the size or the identity of the stabilising shell, none of the Au NPs showed a pronounced ability to alter the amount of LPS-induced NO_2^- produced. This included, once again, the citrate-stabilised Au NPs prepared for this project.

The above results therefore suggest that varying the size and/or identity of the surrounding stabilising layer does not alter the ability of Au NPs to affect production of NO in macrophages that are subsequently stimulated with LPS. However, in a separate experiment in which macrophages were incubated with the different types of Au NPs in the absence of LPS, it was discovered the citrate-buffered Sigma Au NPs did increase basal cellular NO_2^- production (Supplementary Figure 3). This result therefore does provide support for the view that changes to the size and/or composition of Au NPs can have a significant impact on their biological activity.

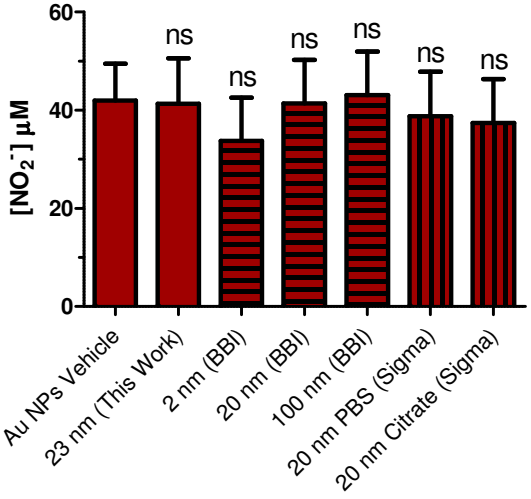

Fig. 6. Effect of pre-incubation with commercial Au NPs on LPS-induced nitrite production by RAW264.7 macrophages. Cells were pre-incubated with Au NPs at 37 °C and under an atmosphere of 5% CO_2 for 4 h, and then 0.1 µg/mL LPS added and incubation continued under the same conditions for a further 20 h. Nitrite concentration was measured by the Griess assay. All Au NP treatment solutions contained 40 µM gold, with the exception of 2 nm (BBI) Au NPs, which contained 9 µM gold, and the Au NP solutions produced as part of this study, which contained 60 µM gold. The error bars represent one standard deviation calculated from triplicate experiments. ns = not significant compared to corresponding vehicle.

Figure 7 shows the effects of the commercial Au NPs on ROS production in LPS-stimulated macrophages. None of the commercial Au NPs produced statistically significant changes in the amount of LPS-induced ROS formation, as was also the case for the Au NPs synthesised for this project. Addition of the citrate-buffered Au NPs obtained from Sigma, and the Au NPs synthesised for this project, had similar effects on the amount of ROS produced by the macrophages. Furthermore in both cases the amount of ROS detected was perhaps higher than that observed when the Au NPs vehicle was used, although the magnitude of the changes was not statistically significant. Therefore the results obtained in this experiment are in general in good agreement with those presented earlier in Fig. 3b.

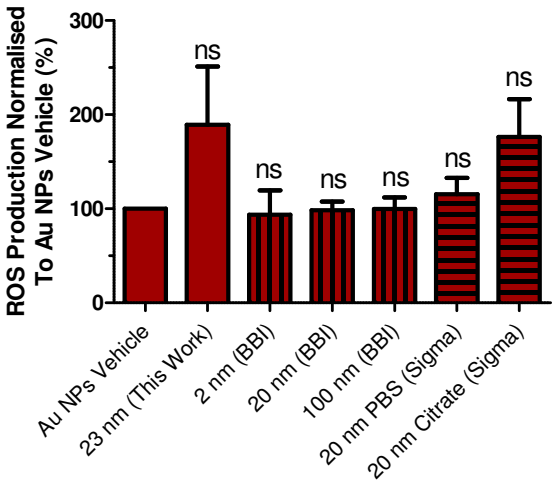

Fig. 7. Effect of pre-incubation with commercial Au NPs on LPS-induced ROS production by RAW264.7 macrophages. Cells were pre-incubated with Au NPs at 37 °C under an atmosphere of 5% CO_2 for 4 h, and then 0.1 µg/mL LPS added and incubation continued under the same conditions for a further 20 h. ROS was measured by the DCF assay and presented as normalised MFI values at 515 nm, relative to Au NPs vehicle. All Au NP treatment solutions contained 40 µM gold, with the exception of the 2 nm (BBI) Au NPs, which contained 9 µM gold and the Au NPs produced as part of this study, which contained 60 µM gold. The error bars represent one standard deviation calculated from triplicate experiments. ns = not significant compared to corresponding vehicle ($P > 0.05$).

Conclusions

Auranofin was shown to have powerful inhibitory effects on production of the pro-inflammatory mediators NO, ROS, and TNF, in macrophages activated using LPS. In contrast, aurothiomalate had a much weaker inhibitory effect on the production of all three mediators. These results are in agreement with those available in the literature, which indicate that auranofin is in general a stronger inhibitor of the production of inflammation mediators than aurothiomalate.

Scanning electron microscopy showed evidence of internalisation of citrate stabilized Au NPs. Despite evidence showing substantial gold uptake in a previous study,[21] and the results of electron microscopy presented here which showed gold is incorporated into macrophages treated with Au NPs, the latter did not show a pronounced ability to alter inflammatory marker expression. Both the citrate-stabilised Au NPs produced as part of this work, and a number of commercial nanoparticles, had no significant effect on the LPS-stimulated production of NO or ROS, or on induction of the anti-inflammatory cytokine IL-10. The citrate-stabilised Au NPs produced as part of this work did, however, have a small inhibitory effect on the production of the pro-inflammatory cytokine TNF. It would therefore be interesting to see in future work if the use of higher concentrations of the same Au NPs, or any of the commercial nanoparticles, elicited more significant effects.

There are several possible explanations for the general lack of biological activity exhibited by the Au NPs. First, Au NPs may interact with cells by a mechanism or mechanisms that do not involve the inflammation mediators investigated here. Secondly, the conditions under which the *in vitro* studies presented here were performed may be a poor approximation for *in vivo* conditions. In particular some key inflammation processes which the Au NPs might either interfere or enhance may be absent. Finally, the Au NPs used may not have produced any substantial changes to macrophage function owing to the absence of mechanisms available *in vivo* for activating the relatively inert Au(0) atoms. Further work is therefore required to confirm that Au NPs are efficacious anti-arthritic agents *in vivo*, and if so, how those effects are elicited.

Experimental

Materials and General Methods

All aqueous solutions were prepared using Milli Q™ water (18.2 MΩ, Millipore, Billerica, USA) unless otherwise specified. Sodium aurothiomalate, sodium aurothioglucose, auranofin, gold(III) chloride (99.9%), sodium citrate, sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride (NED), sodium nitrite and lipopolysaccharide (*Escherichia coli* serotype 055:B5) were obtained from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide pellets (98%), sodium chloride, potassium chloride, potassium dihydrogen phosphate,

sodium hydrogen phosphate, polysorbate 20 (TWEEN) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (all ACS grade) were obtained from Amresco (Solon, USA). DMSO (99.98%) was obtained from Fisher Scientific (Waltham, USA). Au NPs were purchased from BBI (product codes EM.GC2, EM.GC20 and EM.GC100; diameters 2, 20 and 100 nm, respectively) and Sigma-Aldrich (product codes 741965 and 753610; both have diameters of 20 nm, stabilised in citrate buffer and PBS, respectively).
Mouse IL-10 and mouse TNF enzyme-linked immunosorbent assay (ELISA) kits (ELISA MAX™ Deluxe) were obtained from BioLegend (San Diego, USA). Invitrogen (Grand Island, USA) was used to obtain $DCFH_2$-DA. Enzo Life Sciences (Plymouth Meeting, USA) was used to obtain 7-aminoactinomycin D (7AAD). Foetal bovine serum (FBS, heat-inactivated) was obtained from Bovogen Biologicals (East Keilor, Australia). GlutaMAX™ and Roswell Park Memorial Institute-1640 (RPMI-1640) cell culture medium powder were obtained from Life Technologies (Carlsbad, USA).

Synthesis and Characterisation of Gold Nanoparticles

The synthesis and characterisation of Au NPs was carried out as described previously.[21] Milli-Q water (30 mL) was heated to near boiling and a solution of $HAuCl_4.3H_2O$ (10.00 mM in water, 2.000 mL) was added. This was followed by a solution of tri-sodium citrate (20.00 mM, 4.000 mL), resulting in the solution changing colour to red. The solution was subsequently heated for one hour, cooled to room temperature, and then transferred to a volumetric flask (50.00 mL) to which Milli-Q water was added to produce a solution with a final concentration of 400 µM Au(0), assuming complete conversion of Au(III). We have previously reported on the properties of these nanoparticles, as established using field emission scanning electron microscopy (FESEM) and other techniques.[21] FESEM showed that the nanoparticles were spherical in shape, and had diameters of between 10 and 20 nm. Consistent with this, the results of particle size analysis showed that the nanoparticles had an average diameter of 23 ± 7 nm. A single broad plasmon resonance band centred at 522 nm was present in the absorption spectrum of the nanoparticles, as expected.
More concentrated solutions of Au NPs required for nitric oxide formation experiments were prepared by centrifuging the above preparation of Au NPs (11000×g, 10 min), removing the top aqueous layer, and re-suspending the pellet containing the nanoparticles in a smaller volume of water.

Cell Culture

The adherent mouse RAW264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, USA) as frozen permanents. The cells were maintained in RPMI-1640 medium containing 10% (v/v) FBS and 2 mM GlutaMAX™ (complete medium) at 37 °C and 5% CO_2/95% air in a Revco (Twinsburg, USA) Ultima incubator. Cells were subcultured at 90% confluence, and used between 3 and 30 passages. Cells were lifted from the surface of flasks by gentle scraping. Treatment solutions containing sodium aurothiomalate were prepared in RPMI-1640 medium containing 2 mM GlutaMAX™ (incomplete medium). Control cells were also treated with incomplete medium. Treatment solutions containing auranofin were prepared by first dissolving the metal complex in DMSO, and then diluting to the desired auranofin concentration using incomplete medium. The final concentration of DMSO in auranofin treatment solutions was 1% (v/v). Treatment solutions containing gold nanoparticles were spiked with a small volume of concentrated Dulbecco's phosphate-buffered saline (10×D-PBS, composition defined in 'Buffer Solutions' below) to counteract disturbance of isotonicity due to dilution of the incomplete medium. These treatment solutions were prepared using 1.5% (v/v) 10×D-PBS, 15% (v/v) water or Au NPs solution, and 83.5% (v/v) incomplete medium. For example, a 500 µL treatment solution contained 7.5 µL 10×PBS, 75 µL of Au NPs and 417.5 µL of treatment medium. The concentration of Au NPs in treatment solutions prepared from commercial nanoparticle solutions was 40 µM. The only exception to this was stock solutions containing 2 nm Au NPs, which were originally obtained from BBI. In this case, the final concentration of the stock solution was 9 µM.
Cell treatments for all experiments were performed using the following, similar steps. RAW264.7 cells were seeded into a 24-well plate (5×10^5/1.000 mL/well) and incubated overnight. The medium was removed by aspiration and the cells were washed three times with incomplete medium (1 mL). The relevant vehicle (500 µL/well) containing LPS (a positive control treatment), gold compounds or Au NPs was added and incubated with the cells for 24 h. In other experiments, cells in incomplete medium (500 µL/well) were pre-incubated with gold compounds or Au NPs for 4 h, after which LPS (100 ng/mL) was added and the cells were incubated for a further 20 h.

Buffer Solutions

Dulbecco's phosphate-buffered saline (D-PBS) consisted of KCl (2.67 mM), KH_2PO_4 (1.47 mM), NaCl (138 mM) and Na_2HPO_4 (8.10 mM) at pH 7.4. 10×D-PBS contained KCl (26.7 mM), KH_2PO_4 (14.7 mM), NaCl

(1.38 M) and Na_2HPO_4 (81.0 mM) at pH 7.4. Enzyme-linked immunosorbent assay (ELISA) wash buffer contained 0.5% v/v TWEEN/D-PBS. Incomplete NaCl medium contained KCl (5 mM), HEPES (10 mM) and NaCl (145 mM) at pH 7.4, while complete NaCl medium was prepared by adding glucose (5 mM) to incomplete NaCl medium.

Scanning Electron Microscopy of Cells

Samples were prepared on 15 mm diameter Nunc™ coverslips, which were initially washed with ethanol (70% v/v) and then complete medium, before being placed in a 60-mm cell culture dish. RAW264.7 cells in complete medium (3×10^6/5.00 mL/dish) were seeded into the dishes and incubated overnight. After confirming that the cells had grown to a sufficient confluence on the coverslips, the medium was removed and the cells were washed twice with incomplete medium to remove FBS. Treatment solutions (5.00 mL) containing the appropriate concentrations of Au NPs were then added to the dishes prior to incubation for 24 h. Following this period the coverslips were removed from the dishes, washed in PBS twice and then placed into glutaraldehyde solution (2% v/v in PBS) for 2 h at room temperature. At the end of this period, the coverslips were washed twice with PBS, then dehydrated by washing twice with the following ethanol solutions: 30% (v/v), 50% (v/v), 70% (v/v); 10 min/solution), and then refrigerating the coverslips overnight in 70% (v/v) ethanol. The coverslips were cut to size, and then further dehydrated by adding the following ethanol solutions (once each): 70% (v/v), 80% (v/v), 90% (v/v), 100% (v/v); 10 min/solution).
At this point, the samples were dried by critical point drying (CPD) using an EM CPD030 instrument (Leica, Wetzlar, Germany). Briefly, the samples were submerged in ethanol at 8 °C in the chamber of the instrument, and liquid CO_2 added until the chamber was full. After 15 min, the chamber was partially drained and refilled with liquid CO_2, which was repeated three times. The chamber was then heated to 40 °C and CO_2 gas slowly removed to dry the samples. At this point, the samples were mounted in a sample holder, coated with 10 nm of platinum and analysed using a JEOL JSM-6490LV SEM instrument for general images and a JEOL JSM-7500FA FESEM instrument for variable accelerating voltage and electron dispersive X-ray (EDX) images.

Nitric Oxide Formation

Prior to commencement of an assay, a stock solution of sulfanilamide was prepared by dissolving 1.0 g in 100 mL of 5% (v/v) HCl. In addition, a stock solution of NED was prepared by dissolving the dihydrochloride salt (0.1 g) in Milli-Q H_2O (100 mL). Macrophages were first treated with gold compounds or Au NPs as described above, after which the resulting solutions were removed by aspiration and centrifuged (300×*g*, 5 min) to obtain cell-free supernatants. Triplicate aliquots (50 µL) of each supernatant or freshly prepared $NaNO_2$ standards (0 – 100 µM, in 10 µM increments) were added to the wells of a 96-well plate. The sulfanilamide stock solution (25 µL) was added to each well using an 8-channel micropipette, followed by NED stock solution (25 µL). The plate was then incubated at room temperature for 10 min. Wells containing nitrite changed to a pink colour. The absorbance at 540 nm (A_{540}) was measured using a Molecular Devices (Sunnyvale, USA) SpectraMAX Plus 384 microplate reader. The mean absorbance of the triplicates for each sample or standard were calculated and the concentration of nitrite in each sample was interpolated from a linear standard curve (0 – 100 µM, 11 points, 10 µM increments) using GraphPad Prism version 5.03 (GraphPad Software, Inc., La Jolla, USA). The experiment was performed three times, with the errors reported being the standard error of the mean for each replicate experiment.

Reactive Oxygen Species Formation

Stock solutions containing $DCFH_2$-DA were dissolved in DMSO to obtain a concentration of 5 mM and were stored frozen at -20 °C in 30 µL aliquots. Experiments were performed in minimal light to prevent photodecay of the fluorescent dyes. After macrophages were treated with gold compounds or Au NPs, the treatment solution was removed by aspiration and the cells were washed three times with incomplete NaCl medium (1 mL). $DCFH_2$-DA (500 µL, 10 µM in complete NaCl medium) or DMSO for autofluorescence control samples (500 µL, 0.2% in complete NaCl medium) was added to the wells and the plate was incubated for 30 min. The cells were then washed three times with incomplete NaCl medium (1 mL) before being harvested by gentle scraping in complete NaCl medium (500 µL). The cell suspensions were then transferred to 6 mL round bottom polystyrene tubes and centrifuged (300×*g*, 5 min) to produce a pellet. The supernatant was decanted and the resuspended cells (<50 µL) were incubated with 7AAD (1 µL, 1 mg/mL) at room temperature for 5 min. Complete NaCl medium (300 µL) was added and the suspension analysed using a Becton Dickinson (San Jose, USA) LSR II flow cytometer. The samples were excited using a blue laser (488 nm) with emissions measured at 515 nm and 695 nm corresponding to DCF and 7AAD fluorescence, respectively. A total of 10000 events were

counted for each sample. Instrumental fluorescence compensation was applied in some experiments to account for 'bleeding' of one high intensity fluorescent marker into the other.
The data was analysed using FlowJo (Tree Star, Ashland, USA), with ROS production reported as the median fluorescence intensity at 515 nm for viable cells. Viable cells were gated initially on the basis of forward scatter and side scatter, and secondly on the basis of fluorescence at 695 nm, to exclude cells with 7AAD uptake (non-viable) cells. The median fluorescence intensity (MFI) at 515 nm for DMSO-treated autofluorescence control samples was subtracted from that of the relevant sample treated with $DCFH_2$-DA to determine the amount of ROS production following each treatment. The experiment was performed five times with the errors reported as the standard error of the mean. For the LPS-induced ROS production experiment, final median fluorescence intensity values were normalised to the control sample (incomplete medium only) and reported as percentages.

IL-10 and TNF Release

The extent of production of the cytokines IL-10 and TNF was assessed by ELISAs performed using kits obtained from BioLegend. These kits contained all the buffers, standards, antibodies and high-binding 96-well plates required to perform the assays, with the exception of the wash buffer. The protocol for performing the ELISA assays was provided by the manufacturer. The plates were washed by first inverting them, blotting them dry using paper towel, and then adding approximately 300 µL of wash buffer. At the end of the procedure, the absorbance of each well was measured at 450 nm and 570 nm using a Molecular Devices SpectraMAX Plus 384 microplate reader. The concentration of cytokine in each sample was interpolated from a standard curve (0 – 2000 pg/mL for IL-10, 0 – 500 pg/mL for TNF; 8 points per curve, 2 fold increments), which was transformed to a log scale on both axes using GraphPad Prism version 5.03. Each experiment was performed three times, with the errors reported as the standard error of the mean for each replicate experiment.

Statistical Analyses

Statistical significance was tested by using either a one-way ANOVA with a Tukey post-test set at 95% confidence intervals, or a two-tailed unpaired *t*-test with a 95% confidence interval. The tests were applied on subgroups of samples based on what vehicle was used. Specifically, both concentrations of aurothiomalate-treated samples were sub-grouped with their vehicle (incomplete medium) and tested by one-way ANOVA, whilst both concentrations of Au NP-treated samples were sub-grouped with the Au NPs vehicle and also tested by one-way ANOVA. Samples treated with 2.5 µM auranofin were grouped with the auranofin vehicle treatment and tested by *t*-test. The auranofin and Au NPs vehicles were both grouped with incomplete medium and tested by one-way ANOVA. As such, significance reported for a sample refers to the comparison with the relevant vehicle. All statistical analyses were performed using GraphPad Prism version 5.03.

Supplementary Material

EDX and FESEM images of RAW264.7 cells treated with 60 mM Au NPs; figures showing effects of Au NPs on production of nitrite by RAW264.7 macrophages.

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Conflicts of Interest

The authors declare that there are no conflicts of interest associated with the work report within this manuscript, or with the manuscript itself.

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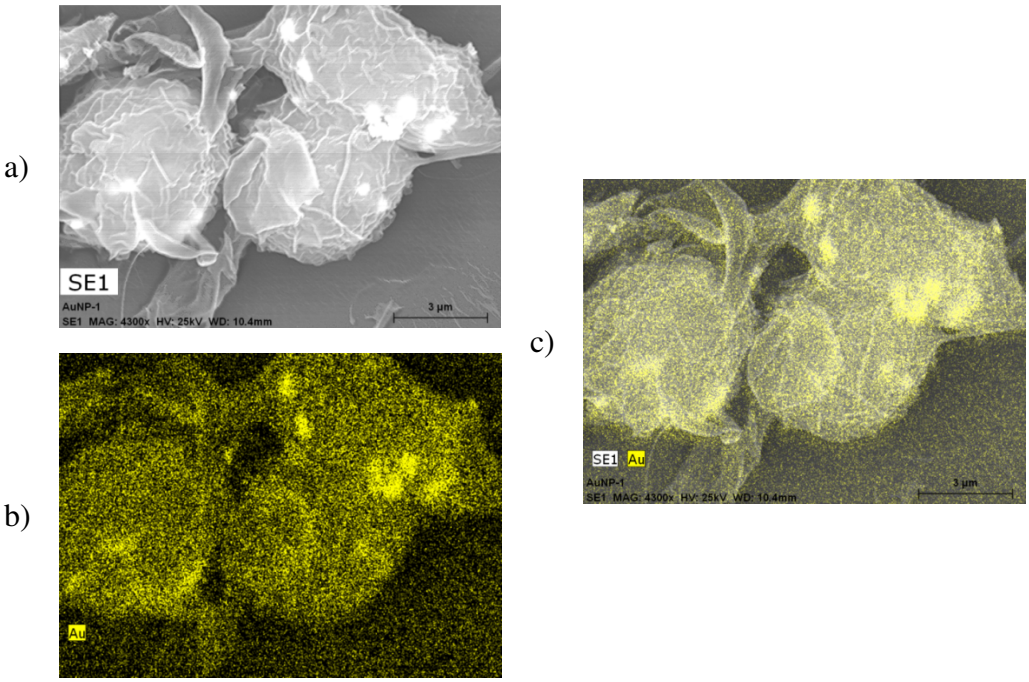
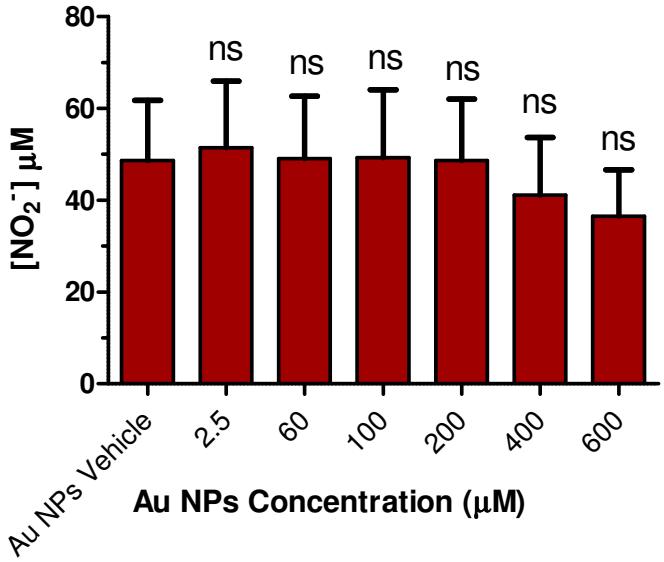
Supplementary Material

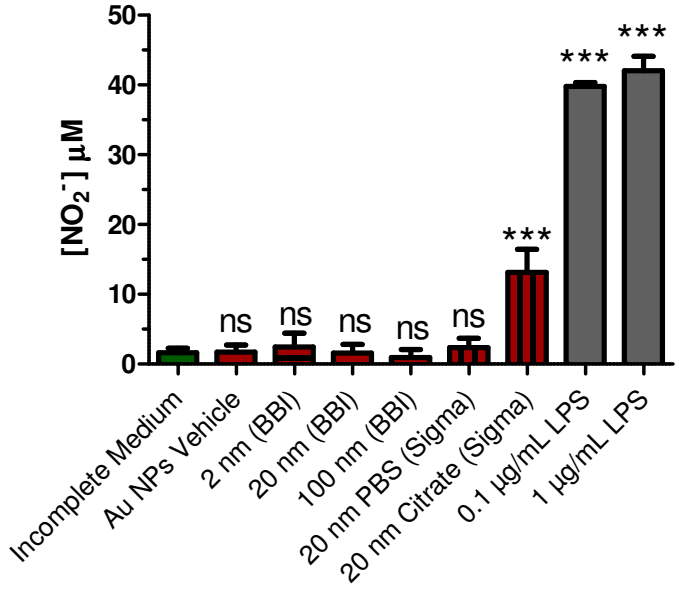


Supplementary Figure 1. EDX and FESEM images of RAW264.7 cells treated with 60 µM Au NPs at 37˚C under an atmosphere of 5% CO_2 for 24 h. Images were collected at 4300x magnification with an accelerating voltage of 25 kV. EDX maps were collected for 90 min: (a) FESEM image: (b) EDX map for gold; (c) overlap of (a) and (b).



Supplementary Figure 2. Effect of pre-incubation with Au NPs on LPS-induced nitrite production by RAW264.7 macrophages. Cells were pre-incubated with various concentrations of Au NPs at 37 °C under an atmosphere of 5% CO_2 for 4 h, then 0.1 µg/mL LPS added, and the cells incubated under the same conditions for a further 20 h. Nitrite

concentration was then measured by the Griess assay. The error bars represent one standard deviation calculated from triplicate experiments. ns = not significant compared to Au NPs vehicle ($P > 0.05$).



Supplementary Figure 3: Effect of addition of commercial Au NPs on nitrite production by RAW264.7 macrophages. Cells were incubated with the Au NPs at 37 °C under an atmosphere of 5% CO_2 for 24 h, and the nitrite concentration was then measured by the Griess assay. All Au NP treatment solutions contained 40 µM gold, with the exception of the 2 nm (BBI) Au NPs, which contained 9 µM gold. The error bars represent one standard deviation calculated from triplicate experiments. *** = statistically significant ($P < 0.001$) compared to corresponding vehicle; ns = not significant compared to corresponding vehicle ($P > 0.05$).