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In vitro macrophage response to nanometer-size chromium oxide particles

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Abstract: An increasing number of studies have reported adverse tissue reactions around metal-on-metal (MM) hip implants. However, the origin and mechanisms of these reactions remain unclear. Moreover, the biological effects of nanometer-size chromium oxide particles, the predominant type of wear particles produced by MM implants, remain mostly unknown. The purpose of this study was to analyze the cytotoxic effects of clinically relevant nanometer-size chromium oxide particles on macrophage response *in vitro*. J774.A1 macrophages were cultured with either 60 nm or 700 nm commercially available Cr_2O_3 particles at different concentrations. Two different particle sizes were analyzed to evaluate potential volume effects. Cell mortality was analyzed by light microscopy, flow cytometry (annexin V-fluorescein isothiocyanate and propidium iodide assay), and using a cell death detection enzyme-linked immunosorbant assay (ELISA). Tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1 alpha (MIP-1α) release was measured by ELISA, and gene expression was analyzed by quantitative real-time PCR. Results showed that, at high concentrations, Cr_2O_3 particles of both sizes can be cytotoxic, inducing significant decreases in total cell numbers and increases in necrosis. Results also suggested that these effects were dependent on particle volume. However, TNF-α, MCP-1, and MIP-1α cytokine release and gene expression remained low. Overall, this study demonstrates that nanometer-size particles of Cr_2O_3, a stable form of chromium oxide ceramic, have rather low cytotoxic effects on macrophages. Therefore, these particles may not be the main culprit in the initiation of the inflammatory reaction in MM periprosthetic tissues. However, other parameters (e.g., potential intracellular damage) remain to be investigated. J Biomed Mater Res Part B: Appl Biomater, 102B: 149–159, 2014.

Key Words: hip implants, macrophages, wear particles, chromium oxide, cytotoxicity

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INTRODUCTION

Metal-on-metal (MM) hip implants were reintroduced over two decades ago because of their lower volumetric wear and expected lower wear particle-induced periprosthetic osteolysis, which has been recognized as the prominent cause of conventional metal-on-polyethylene (MPE) implant failure. Since the mid-1980's, over one million MM hip implants have been implanted worldwide. However, a major cause for concern is the greater amount of metal products (wear particles and ions) released by MM compared to MPE implants, both from the bearing and the modular interfaces. An increasing number of studies have reported adverse tissue reactions to these metal products, which can compromise the functionality and survivorship of the implants[1–3]. These reactions include periprosthetic osteolysis, hypersensitivity and destructive soft tissue masses (referred to as pseudotumors). However, the mechanisms by which MM wear particles and ions lead to these reactions remain unclear.

Macrophages have been shown to be the primary cell type in inflamed periprosthetic tissues[4]. Although wear particle-induced inflammatory response in periprosthetic tissues from failed MM implants has been reported to be lower than that in tissues surrounding conventional MPE implants[4], macrophages and cytokines associated with bone resorption and implant loosening can still be found in MM periprosthetic tissues[4–6]. Features consistent with inflammatory reactions to metal wear, such as macrophages with wear particles, have also been observed in pseudotumor cases, in addition to features consistent with metal hypersensitivity reactions such as lymphocyte aggregates[3]. Finally, macrophages can also act as antigen-presenting cells in

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type-IV hypersensitivity reactions driven by T-lymphocytes[7]. Therefore, it is critical to investigate the effects of MM wear particles and ions on macrophages[8].

Despite their lower volumetric wear compared to conventional MPE bearings, MM implants have been reported to generate a large number of nanometer-size particles, and specific characterization studies have shown that round to oval chromium oxide particles were the predominant type[9–13]. In addition, other studies have shown higher levels of blood Co and Cr ions from the CoCrMo alloy used in MM implants, compared to pre-operative values and to levels in patients with conventional MPE bearings[14–16]. The effects of metal ions on macrophage mortality and cytokine release have been previously documented[17–19]. However, most *in vitro and in vivo* studies on the biological effects of MM wear particles have been conducted with CoCrMo particles[20–25], and little is known about the effects of nanometer-size chromium oxide particles. One study measuring the effects of nanometer-size chromium particles (likely chromium oxide particles due to the propensity of nanometer-size chromium particles to oxidize instantaneously) reported no significant decrease in macrophage numbers *in vitro*[8]. However, the results of that study showed a substantial decrease in cell number due to these particles. The specific type of cell mortality (apoptosis and necrosis) and cytokine release were not investigated.

Wear particles have been shown to induce cell mortality by apoptosis or necrosis, depending on their composition[26,27]. Cell apoptosis and necrosis are two distinct modes of cell death, and both have been observed in periprosthetic tissues[28,29]. Apoptosis is an active form of programmed cell death, sometimes referred to as "cell suicide"[29]. As the cell membrane maintains its integrity throughout the process, the release of mediators (cytokines) into the extracellular environment is limited. In contrast to apoptosis, necrosis is a passive uncontrolled form of cell death that occurs due to tissue injury and in acute inflammatory response[29]. A notable difference between apoptosis and necrosis is their association with inflammation. While necrosis is known to cause and participate in inflammatory response, a less-well defined association exists between apoptosis and inflammation[29]. Interestingly, ultra-high molecular weight polyethylene (UHMWPE) particles have been shown to induce necrosis whereas Al_2O_3 ceramic particles have been reported to induce primarily apoptosis[27]. However, the effects of chromium oxide particles, another form of stable ceramic, on apoptosis and necrosis have not been studied.

Previous reports have shown that local and systemic trafficking of inflammatory cells to the site of particle generation was mediated by chemotactic cytokines (chemokines) including monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1α)[30,31]. Cytokines such as tumor necrosis factor alpha (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6) as well as chemokines (e.g., MCP-1 and MIP-1α) have been found in periprosthetic tissues of failed implants[32–35]. *In vitro*, macrophages and monocytes have also been shown to release multiple cytokines, including: TNF-α after incubation with bone cement, high density polyethylene, Al_2O_3, Ti_6Al_4V, CoCrMo, and Ti particles[22,36–39]; MCP-1 after incubation with Ti_6Al_4V and Al_2O_3 particles[22,40]; and MIP-1α after incubation with Ti_6Al_4V and bone cement particles[33]. Both MCP-1 and MIP-1α chemokines are involved in the innate and adaptive immune responses[41]. Indeed, MCP-1 regulates the migration and accumulation of monocytes/macrophages, dendritic cells, natural killer (NK), and memory T lymphocytes, while MIP-1α is primarily chemotactic for B lymphocytes, $CD8^+$ T lymphocytes and NK cells[41]. Hence, the analysis of the effects of MM wear particles on macrophage secretion of these chemokines is of particular interest to better understand the role of these particles in the overall immune response surrounding MM implants.

Therefore, the purpose of the present study was to analyze the effects of clinically relevant nanometer-size chromium oxide particles on macrophage response *in vitro*, in particular cell mortality [apoptosis and necrosis using flow cytometry and a cell death detection enzyme-linked immunosorbant assay (ELISA)], as well as cytokine release and gene expression (by ELISA and quantitative real-time PCR, respectively). Particles of two different sizes were analyzed to evaluate potential volume effects.

MATERIALS AND METHODS

Cells and particles

J774A.1 mouse macrophages (ATCC, Manasas, VA) were used in the present study because of their morphological similarities with macrophages found at the bone-cement interface[42]. The cell line was maintained at 37°C (in a humidified atmosphere of 95% air and 5% CO_2) in complete growth medium composed of Dulbecco's Modified Eagle Medium (DMEM) (Wisent, St. Bruno, QC) supplemented with heat-inactivated fetal bovine serum (Wisent; premium grade) to a final concentration of 5% (v/v). For experiments, cells were harvested by scraping followed by a 3–4 h "recovery" incubation at 37°C. They were then washed and exposed to commercially available round Cr_2O_3 particles of two different sizes [60 nm (Catalog No. 634239; Sigma-Aldrich, St. Louis, MO); and 700 nm (Catalog No. 192085000; Acros, Geel, Belgium)], at different concentrations (0–3.5 million particles/macrophage for the 60 nm particles and 0–2000 particles/macrophage for the 700 nm particles), as described below in the individual tests. The mass of particles required to produce a desired concentration was calculated based on chromium oxide density ($\rho = 5.21$ g/cm^3) and considering that the particles were spherical. Macrophages incubated without particles served as negative controls, and macrophages incubated with lipopolysaccharide (LPS) (Sigma-Aldrich) were used as positive controls for cytokine quantification, as previously described[39].

Particles were sterilized for 3 h in sterile-filtered (0.20 μm) 70% (v/v) ethanol, dispersed by a 15-min incubation in an ultrasonic bath (35 kHz), and washed 3 times in phosphate buffered saline (Wisent) (particles were dispersed by 10 min of sonication between each wash). The washed particles were then resuspended in complete growth medium, dispersed by 10 min of sonication, and serially diluted as detailed below in the individual tests. Particles tested negatively for endotoxins using the ToxinSensor™ Chromogenic LAL Endotoxin Assay kit (GenScript, Piscataway, NJ).

Particle engulfment and cell mortality

For each experimental condition, 0.5 million macrophages were resuspended in 1 mL of complete growth medium containing the particles at different concentrations: 0.5, 1.5, 2.5, and 3.5 million particles per macrophage (60 nm Cr_2O_3 particles); and 500, 1000, 1500, and 2000 particles per macrophage (700 nm Cr_2O_3 particles). Incubations were performed at 37°C, in capped 5-mL untreated polystyrene culture tubes, with constant rotation (8 rpm) to maintain the particles in suspension.

Particle engulfment after a 24 h incubation was confirmed by light microscopy and by analyzing changes in side scatter (reflecting cell granularity) using flow cytometry, as previously described[36]. The sample supernatants were frozen and stored at –80°C for subsequent cytokine quantification by ELISA.

For photomicrography, the cell suspensions were transferred into poly-D-lysine-coated 35-mm glass-bottom culture dishes (MatTek corporation, Ashland, MA) at the end of the 24 h incubation. Micrographs were taken with a Nikon Digital Sight DS-Ri1 camera mounted on a Nikon Eclipse Ti inverted microscope system using NIS Elements Basic Research imaging software version 3.20.01 and a 60X plan apochromatic water immersion objective (Nikon Instruments, Melville, NY).

Cell mortality was analyzed after a 20–24 h incubation by counting total cell numbers (viable and dead) using an improved Neubauer hemocytometer, and by quantifying apoptosis and necrosis using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) assay (TACS® Annexin V-FITC kit; Trevigen, Gaithersburg, MD), and a cell death detection ELISA (Cell Death Detection ELISA^{Plus}; Roche Diagnostics, Indianapolis, IN), as previously described[19]. Cell mortality was further measured in kinetic experiments by counting total cell numbers after 6, 12, and 24 h incubations.

The annexin V-FITC and PI assay was used to differentiate early apoptosis from late apoptosis/necrosis, whereas the cell death detection ELISA was used to differentiate late apoptosis from necrosis. For the ELISA, the specific enrichment of mono- and oligo-nucleosomes in cell lysates (reflecting the level of late apoptosis), and in culture supernatants (reflecting the level of necrosis), is presented as an enrichment factor (EF) for each experimental condition (with EF being the ratio of mono- and oligo-nucleosome levels in lysates or culture supernatants of cells with Cr_2O_3 particles at each concentration over the mono- and oligo-nucleosome levels in lysates or culture supernatants of cells alone).

Flow cytometry was performed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, CA). Percentages of positively and negatively stained cells (annexin V-FITC and PI assay) were determined using CXP Analysis software version 2.2 (Beckman Coulter, Brea, CA). Absorbance measurements for the ELISA were performed using a Synergy™ 4 hybrid microplate reader (Biotek, Winooski, VT).

Cytokine release

Levels of TNF-α, MCP-1, and MIP-1α in the culture supernatants from the cell mortality experiments were measured by ELISA with the following kits: Mouse TNF-α Quantikine® ELISA, Mouse CCL2/JE/MCP-1 Quantikine® ELISA, and Mouse CCL3/MIP-1α Quantikine® ELISA (R&D Systems, Minneapolis, MN). Culture supernatants were thawed and gently mixed prior to analysis. The nominal minimum concentrations of TNF-α, MCP-1, and MIP-1α detectable by ELISA were less than 5.1, 2, and 1.5 pg/mL, respectively, and the kits were specific enough to avoid cross-reactivity with other cytokines (R&D Systems).

Quantitative real-time PCR

The kinetics of TNF-α, MCP-1, and MIP-1α gene expression were analyzed by quantitative real-time PCR (qPCR). For each experimental condition, one million macrophages were suspended in 2 mL of complete growth medium containing 0.5 million or 3.5 million particles per macrophage (60 nm Cr_2O_3 particles). Cells with no particles served as negative controls, and cells with LPS (1 μg/mL) were used as positive controls (data not shown). The cells were incubated at 37°C, in capped 5-mL untreated polystyrene culture tubes, with constant rotation (8 rpm) to maintain the particles in suspension.

At the start of the incubation, and after 1, 3, 6, and 9 h, the cells from triplicate samples were collected by centrifugation (6 min at 150×g) and lysed in a total volume of 900 μL of QIAzol® Lysis Reagent (Qiagen, Mississauga, ON). The lysates were immediately frozen and stored at –80°C for <3 months. Total RNA was extracted from freshly thawed lysates using an RNeasy® Plus Universal total RNA purification kit (Qiagen). RNA purity and quantity were assessed by measuring absorbance at 230, 260, and 280 nm using a microvolume spectrophotometer (NanoDrop 2000; Thermo Scientific, Wilmington, DE). RNA integrity was verified by electrophoresis using an Experion™ Automated Electrophoresis System with the Experion™ RNA StdSens Analysis kit (Bio-Rad, Hercules, CA). The average RNA quality indicator (RQI) value was 10.0 ± 0.1. Extracted RNA was stored at –80°C for <3 weeks. cDNA was synthesized by reverse transcription using the iScript™ cDNA Synthesis kit (Bio-Rad) and 250 ng of freshly thawed RNA template per 25-μL reaction. qPCR was performed with a qPCR system (CFX96™ Real-Time PCR System; Bio-Rad) using SsoFast™ EvaGreen® Supermix (Bio-Rad) and 1 μL of cDNA template per 20-μL reaction containing forward and reverse primers at a final concentration of 400 n*M* each. The following cycling conditions were used: 2 min at 98°C (polymerase activation step) followed by 40 two-step cycles of 5 s at 98°C (denaturation) and 5 s at 55°C (annealing/extension). For melt curve analysis, temperature was increased from 65 to 95°C in 0.5°C increments. The identity of the qPCR product (amplicon) for each gene was verified (by size) using agarose gel electrophoresis [1.2% (w/v) PCR agarose (Bio-Rad)] with SYBR® safe DNA stain (Invitrogen, Burlington, ON) and GeneRuler™ Ultra Low Range DNA Ladder (Fermentas, Waltham, MA). The gene expression of TNF-α, MCP-1, and MIP-1α was analyzed using CFX Manager™ software version 3.0 (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB) were used as reference genes. Primers (Sigma

TABLE I. Oligonucleotide Primers Designed for qPCR

Target mRNA	Primer Sequence (5′→3′)	Direction	T_m (°C)	Amplicon Size (bp)	Efficiency (%)
TNF-α	CCTCTCATCAGTTCTATGGCC	Forward	58.5	95	99.5
	TGGTGGTTTGCTACGACG	Reverse	58.5		
MCP-1	CAACCACCTCAAGCACTTCTGTAG	Forward	57.4	75	99.3
	TTAAGGCATCACAGTCCGAGTCAC	Reverse	58.4		
MIP-1α	TTCCTGACTAAGAGAAACCGGCAG	Forward	58.3	96	99.0
	AGACTCTCAGGCATTCAGTTCCAG	Reverse	58.1		
GAPDH	TCTCCCTCACAATTTCCATCCCAG	Forward	58.3	100	99.0
	GGGTGCAGCGAACTTTATTGATGG	Reverse	58.4		
ACTB	TTCCAGCCTTCCTTCTTGGGTATG	Forward	58.5	94	99.3
	GGTCTTTACGGATGTCAACGTCAC	Reverse	57.5		

Efficiency (E) was calculated [$E = (10^{-1/slope} - 1) \times 100\%$] from the slope of qPCR standard curves generated by a serial dilution of cDNA prepared from J774.A1 cells.

T_m, melting temperature.

Genosys, St-Louis, MO) were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and PrimerQuest (Integrated DNA Technologies, Coralville, IA) (Table I). Gene expression, normalized to the reference genes, is presented relative to the expression of the gene in the control (cells with no particles) at the zero time point.

Statistical analysis

Particle engulfment and cell mortality experiments (cell counts, annexin V-FITC and PI assay, and cell death detection ELISA) were repeated four to six times and samples in each experiment were analyzed in triplicate. Cytokine ELISAs and qPCR experiments were repeated three times and samples in each experiment were analyzed in triplicate. Statistical analysis of cell mortality and cytokine ELISA data was performed using a two-way analysis of variance (ANOVA). Statistically significant interactions between the two independent variables were observed but attributed to the small number of technical replicates. Statistical analysis of the qPCR data was performed using a one-way ANOVA for each independent variable (time and particle concentration). A p-value <0.05 was considered significant.

RESULTS

Particle engulfment

Macrophages incubated with 60 nm or 700 nm Cr_2O_3 particles were examined under light microscopy. Figure 1 clearly shows Cr_2O_3 particles (of both sizes) located inside the cytoplasm of the cells. Engulfment of the particles was confirmed by flow cytometry (increase in side scatter) for both particle sizes, at all concentrations analyzed (data not shown).

Total cell number

Cell counts revealed a particle concentration-dependent decrease in total cell numbers for macrophages cultured with either 60 nm or 700 nm Cr_2O_3 particles. A significant decrease was observed at all concentrations analyzed, up to 67% with 3.5 million particles per macrophage for the 60 nm particles [Figure 2(A)], and up to 69% with 2000 particles per macrophage for the 700 nm particles [Figure 2(B)] at 24 h ($p < 0.005$ for each condition vs. control). When combining particle size and number into a volume, the comparison of the effects of the 60 nm and 700 nm Cr_2O_3 particles revealed that the total cell numbers may have been dependent upon the overall particle volume [Figure 2(C)].

Kinetic experiments revealed that, after only 6 h of incubation, 60 nm Cr_2O_3 particles induced a significant decrease in total cell number of up to 11%, 23%, 57%, and 59% with 0.5 million, 1.5 million, 2.5 million, and 3.5 million particles per macrophage, respectively ($p < 0.01$ for each condition vs. control), as shown in Figure 3. Between 6 and

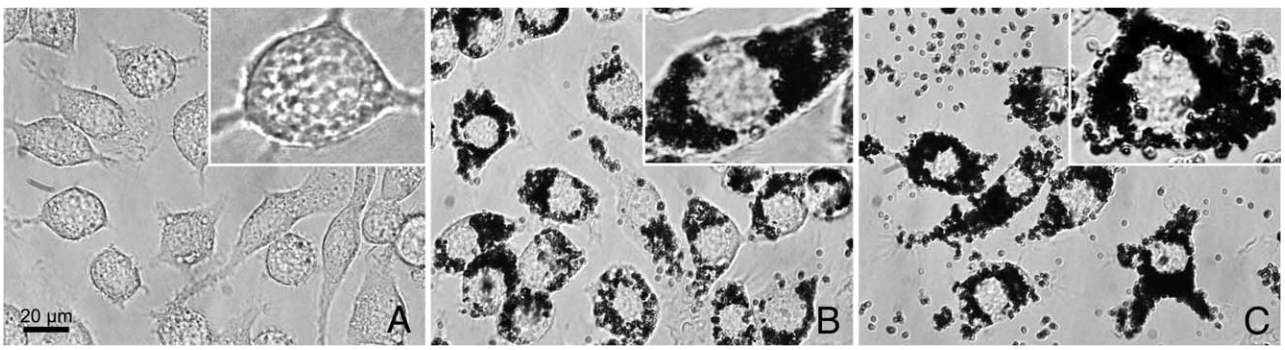

FIGURE 1. Photomicrographs of J774A.1 macrophages after a 24 h incubation with: (A) no particles (negative control); (B) 60 nm Cr_2O_3 particles (0.5 million per cell); and (C) 700 nm Cr_2O_3 particles (500 per cell). Inserts show an enlarged micrograph of a cell. The cell suspensions were transferred into poly-D-lysine-coated glass-bottom culture dishes for photomicrography.

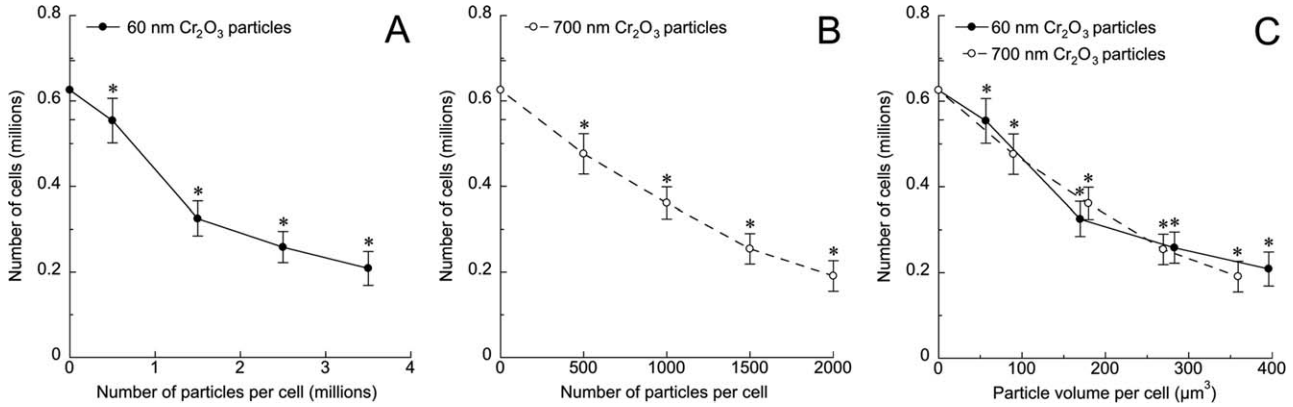

FIGURE 2. Effects of 60 nm and 700 nm Cr_2O_3 particles on the total number of J774A.1 macrophages after 20–24 h incubations. (A) and (B) show the effect of the number of particles per cell, and (C) shows the overall effect of particle volume. An asterisk (*) indicates a significant difference ($p < 0.005$) between an experimental condition and the control (no particles). The macrophages were incubated with different concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the size, number, and sphericity of the particles. Total cell numbers were determined by hemocytometry. Data are presented as means ± pooled SD of six independent experiments performed in triplicates.

12 h of incubation, total cell numbers further decreased significantly with 1.5 million, 2.5 million and 3.5 million Cr_2O_3 particles per macrophage, and increased significantly with 0.5 million particles per macrophage ($p < 0.05$). No significant change occurred between 12 and 24 h with concentrations of 1.5 million, 2.5 million and 3.5 million particles per macrophage. In contrast, total cell numbers in control samples (cells without added particles) and with 0.5 million Cr_2O_3 particles per macrophage increased significantly ($p < 0.001$) during the same time period.

Apoptosis versus necrosis

Flow cytometry. Neither the 60 nm nor 700 nm Cr_2O_3 particles induced a significant increase in early apoptosis (annexin V-FITC positive/PI negative cells) at any particle concentration [Figure 4(A,B)]. When combining particle size and number into a volume, the comparison of the effects of the 60 nm and 700 nm Cr_2O_3 particles suggested a dependence upon the overall particle volume [Figure 4(C)].

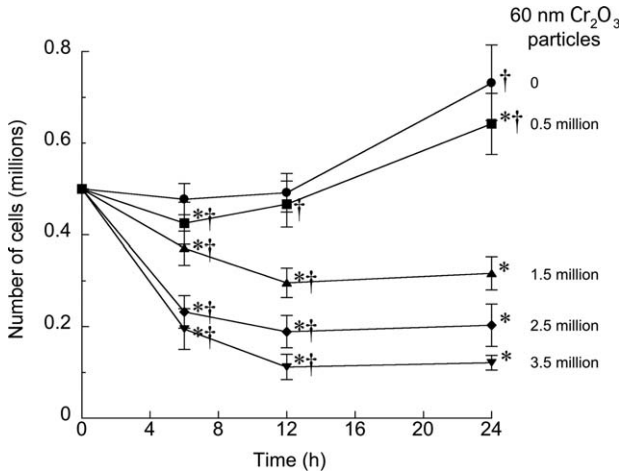

FIGURE 3. Effects of 60 nm Cr_2O_3 particles on total numbers of J774A.1 macrophages after 6 h, 12 h, and 24 h incubations. An asterisk (*) indicates a significant difference ($p < 0.02$) between an experimental condition and the control (no particles) at a given time point, and an obelisk (†) indicates a significant difference ($p < 0.05$) between two consecutive time points at a given particle concentration. The macrophages were incubated with different concentrations of 60 nm Cr_2O_3 particles. Total cell numbers were determined by hemocytometry at each time point. Data are presented as means ± pooled SD of three independent experiments performed in triplicates.

Percentages of late apoptotic and necrotic cells (PI positive cells), increased significantly with both 60 nm and 700 nm Cr_2O_3 particles up to a maximum of 16% with 2.5 million particles per macrophage for the 60 nm particles, and approximately 19% with 1500 particles per macrophage for the 700 nm particles ($p < 0.001$ for each condition vs. control) [Figure 5(A,B), respectively]. Interestingly, percentages appeared to plateau at the highest concentrations of 60 nm particles, and even decrease significantly with 2000 particles per macrophage for the 700 nm particles ($p < 0.002$ for 1500 vs. 2000 particles per cell).

As observed for the total cell numbers and the percentages of early apoptotic cells, the effects of 60 nm and 700 nm Cr_2O_3 particles on the percentages of late apoptotic and necrotic cells (PI positive cells) appeared to be dependent upon the overall particle volume [Figure 5(C)].

Cell death detection ELISA. Late apoptosis and necrosis induced by the Cr_2O_3 particles were differentiated using a cell death detection ELISA.

Levels of late apoptosis, indicated by the enrichment of mono- and oligo-nucleosomes in cell lysates, remained low for both sizes of Cr_2O_3 particles. The 60 nm Cr_2O_3 particles did not induce a significant increase in the level of late apoptosis, except with 3.5 million particles per macrophage (mono- and oligo-nucleosome EF of 1.9; $p = 0.006$) [Figure 6(A)]. Similarly, the 700 nm Cr_2O_3 particles did not induce a significant increase in late apoptosis, except with 2000 particles per macrophage (mono- and oligo-nucleosome EF of 1.7; $p = 0.002$) [Figure 6(B)].

Both the 60 nm and the 700 nm Cr_2O_3 particles induced significant increases in necrosis, indicated by the enrichment of mono- and oligo-nucleosomes in culture

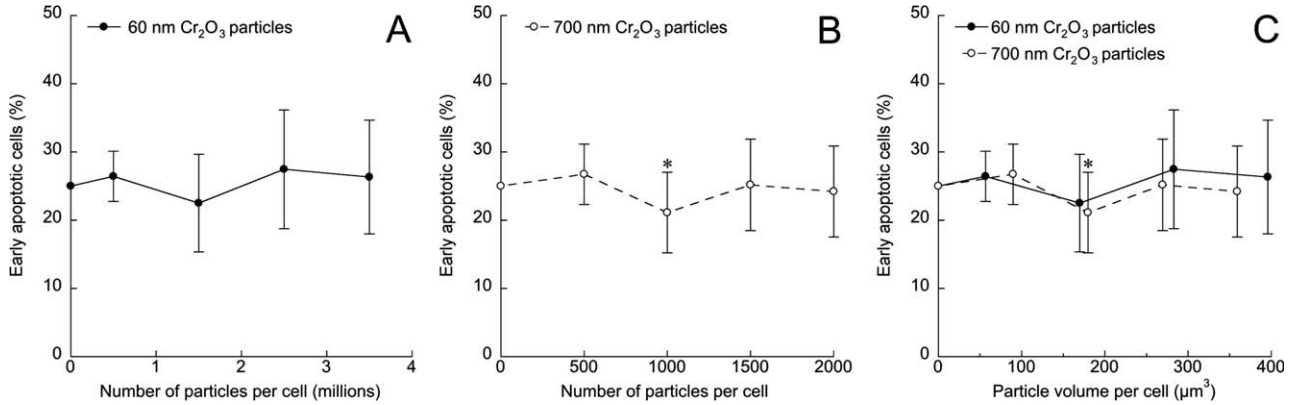

FIGURE 4. Effects of 60 nm and 700 nm Cr_2O_3 particles on early apoptosis of J774A.1 macrophages after 20–24 h incubations, as determined using flow cytometry (annexin V-FITC and PI assay). (A) and (B) show the effects of the number of particles per cell, and (C) shows the overall effect of particle volume. An asterisk (*) indicates a significant difference ($p < 0.05$) between an experimental condition and the control (no particles). The macrophages were incubated with different concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the size, number, and sphericity of the particles. Data are presented as means ± pooled SD of six independent experiments performed in triplicates.

supernatants, at all particle concentrations ($p < 0.002$). As shown in Figure 6(A,B), the mono- and oligo-nucleosome EF reached a maximum of 5.7 with 3.5 million particles per macrophage for the 60 nm Cr_2O_3 particles and a maximum of 6.5 with 2000 particles per macrophage for the 700 nm Cr_2O_3 particles ($p < 0.002$).

In agreement with the results presented above (total cell numbers and flow cytometry), the effects of 60 nm and 700 nm Cr_2O_3 particles appeared to be dependent upon the overall particle volume, both for late apoptosis [Figure 6(C)] and necrosis [Figure 6(D)]. Overall, particles of both sizes induced primarily necrotic (as opposed to late apoptotic) cell death at all concentrations.

Cytokine release and gene expression

ELISA. Incubation of the macrophages with 60 nm or 700 nm Cr_2O_3 particles did not induce a significant increase in the concentrations of TNF-α [Figure 7(A)], MCP-1 [Figure 7(B)], or MIP-1α [Figure 7(C)] at any of the particle concentrations analyzed. In contrast, stimulation of the macrophages with LPS (positive control) increased TNF-α, MCP-1, and MIP-1α concentrations approximately 17 fold, 2 fold, and 12 fold, respectively (data not shown). A small but significant decrease in the concentrations was observed in some conditions (especially for TNF-α), but overall, concentrations remained similar to those of the controls. Finally, for each cytokine, a potential volume effect was observed.

qPCR. Incubation of the macrophages with 60 nm Cr_2O_3 particles did not induce a significant increase in the gene expression of TNF-α, MCP-1, and MIP-1α after up to 9 h of incubation (Figure 8). Furthermore, the effect of particle concentration at each time point (3, 6, and 9 h) was not significant. These results are in agreement with the observation that secretion of these cytokine and

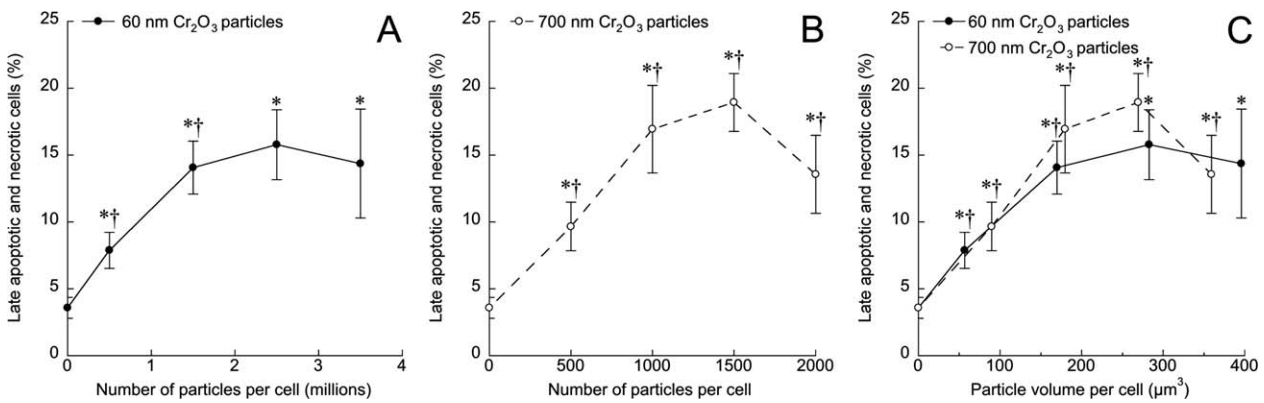

FIGURE 5. Effects of 60 nm and 700 nm Cr_2O_3 particles on late apoptosis and necrosis (PI-positive cells) of J774A.1 macrophages after 20–24 h incubations, as determined using flow cytometry (annexin V-FITC and PI assay). (A) and (B) show the effects of the number of particles per cell, and (C) shows the overall effect of particle volume. An asterisk (*) indicates a significant difference ($p < 0.001$) between an experimental condition and the control (no particles), and an obelisk (†) indicates a significant difference ($p < 0.03$) between two consecutive particle concentrations (Figures A and B) or volumes (Figure C). The macrophages were incubated with different concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the size, number, and sphericity of the particles. Data are presented as means ± pooled SD of four independent experiments performed in triplicates.

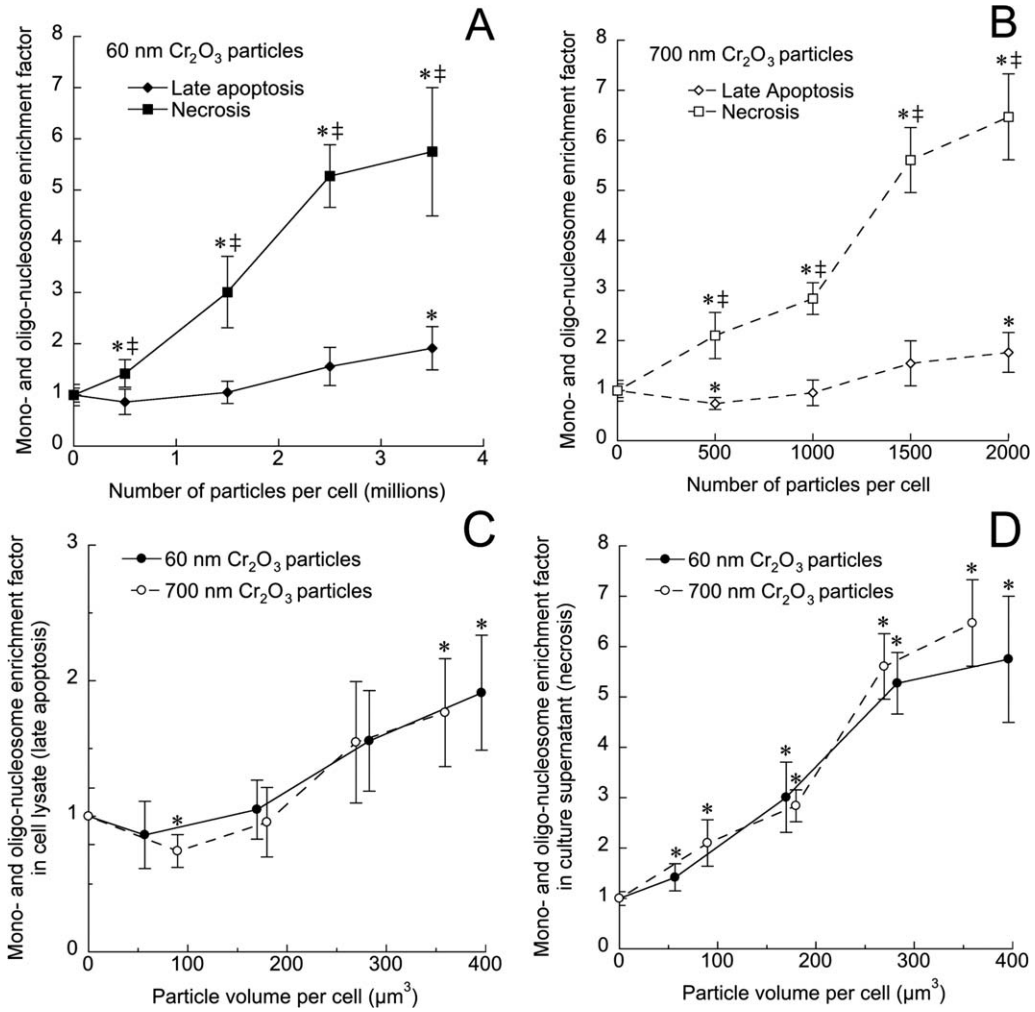

FIGURE 6. Effects of 60 nm and 700 nm Cr_2O_3 particles on late apoptosis and necrosis of J774A.1 macrophages after 20–24 h incubations, as determined using a cell death detection ELISA. (A) and (B) show the effects of the number of particles per cell, and (C) and (D) show the overall effect of particle volume. An asterisk (*) indicates a significant difference ($p < 0.01$) between an experimental condition and its respective control (no particles), and a double dagger (‡) indicates a significant difference ($p < 0.05$) between the enrichment factors (EF) for late apoptosis and necrosis, for a given particle concentration (Figures A and B). The macrophages were incubated with the indicated concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the size, number, and sphericity of the particles. The EF is defined as the ratio of mono- and oligo-nucleosomes levels in lysates (late apoptosis) or culture supernatants (necrosis) of cells with Cr_2O_3 particles at each concentration over the mono- and oligo-nucleosomes levels in lysates or culture supernatants of cells alone. Data are presented as means ± pooled SD of four independent experiments performed in triplicates.

chemokines, as measured by ELISA, was not increased significantly after 20 to 24 h incubation with the particles.

DISCUSSION

Although nanometer-size chromium oxide particles are the predominant type of particles generated by MM implants[9–13],

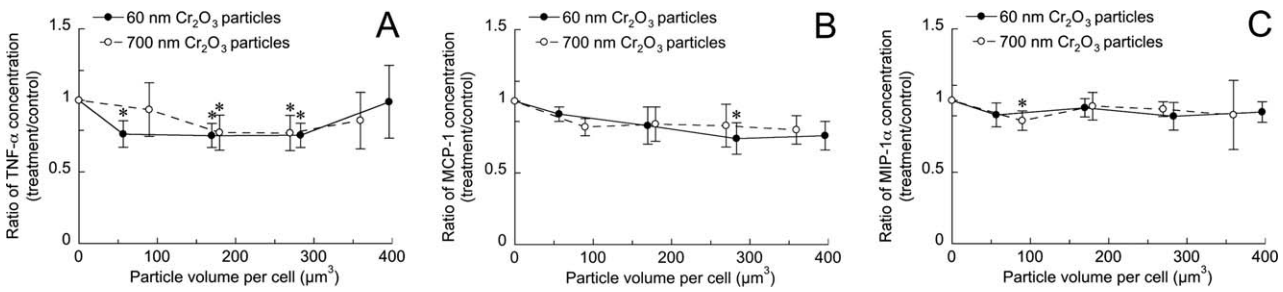

FIGURE 7. Effects of 60 nm and 700 nm Cr_2O_3 particle volume on (A) TNF-α, (B) MCP-1, and (C) MIP-1α release by J774A.1 macrophages after 20–24 h incubations. An asterisk (*) indicates a significant difference ($p < 0.05$) between an experimental condition and the control (no particles). The macrophages were incubated with different concentrations of 60 nm or 700 nm Cr_2O_3 particles. Particle volumes were calculated based on the size, number, and sphericity of the particles. Data are presented as means ± pooled SD of three independent experiments performed in triplicates.

the biological response to these particles has not been described specifically. Therefore, the present study aimed to determine the cytotoxic effects of clinically-relevant Cr_2O_3 particles on cultured macrophages by measuring cell mortality (decrease in cell number, apoptosis, and necrosis) as well as the release and gene expression of inflammatory cytokine (TNF-α) and chemokines (MCP-1 and MIP-1α) after incubation with particles of two different sizes.

Commercially available 60 nm round Cr_2O_3 particles were used in this study. These particles were considered clinically relevant because their characteristics are consistent with the size, shape, and composition of particles isolated from MM periprosthetic tissues *in vivo*[9,11–13], and particles generated by hip simulators *in vitro*[10,11]. Larger round Cr_2O_3 particles (700 nm) were also used to analyze the effects of particle size and overall particle volume by comparison with the 60 nm particles. Particle concentrations were selected to study a large range of cytotoxic effects on macrophages. Although these concentrations probably exceeded those expected *in vivo*[43], macrophages *in vitro* require higher stimulating concentrations of an exogenous agent than *in vivo* macrophages, which are subject to many other stimulating factors. Indeed, *in vitro* studies have demonstrated that prestimulated cells respond to a greater extent than non-prestimulated cells[44,45].

Particle engulfment (likely through pinocytosis rather than phagocytosis given the very small size of the particles used in the present study) was confirmed using light microscopy and flow cytometry. Light microscopy analysis clearly showed particles of both sizes inside the cytoplasm of the cells, demonstrating that particles had been ingested by the macrophages. In addition, changes in cell granularity (reflected by increases in side scatter measured by flow cytometry) further supported the observations made by light microscopy.

With both particle sizes, cell mortality results showed a significant decrease in total cell numbers as well as a significant, although very mild, increase in late apoptosis, and a moderate increase in necrosis with increasing particle concentrations. The detection of late apoptosis (even at low levels) was surprising given the non-significant levels of early apoptosis measured by flow cytometry using the annexin V-FITC and PI assay. Nevertheless, caution is required when interpreting annexin V-FITC and PI assay results considering the incomplete separation that was observed between the annexin V-FITC negative and positive cell populations. Indeed, this assay is based on the translocation of phosphatidylserine (PS) to the outer layer of the cell plasma membrane at the early stage of apoptosis[46]. This PS relocation is a general feature of apoptosis and is independent of the initiating stimulus[47]. The incomplete separation between annexin

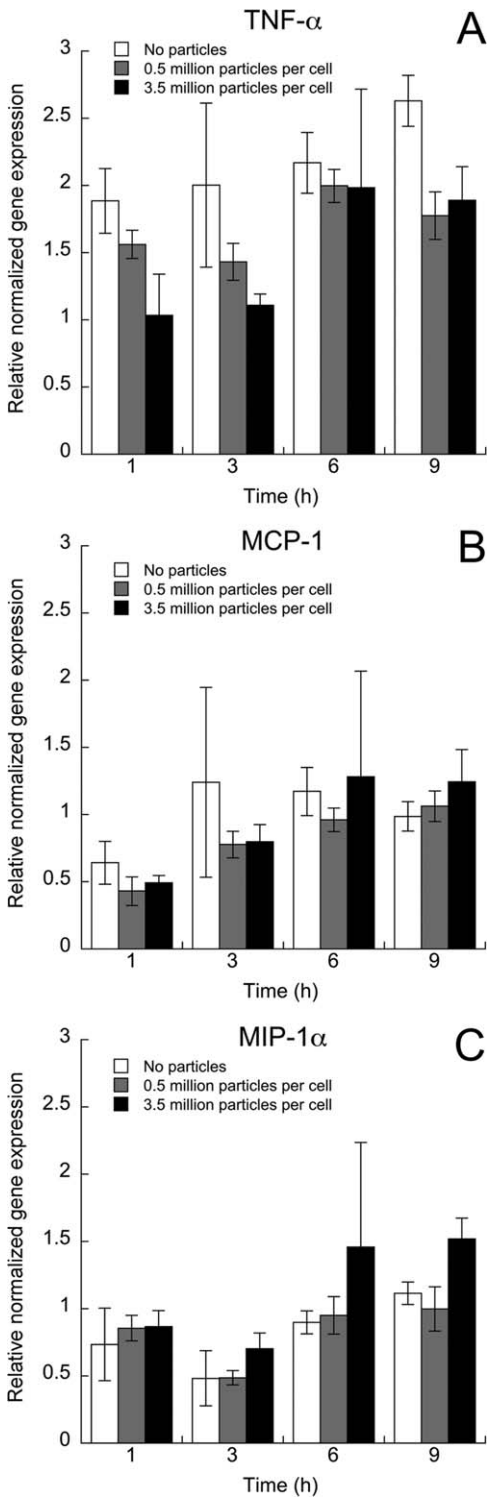

FIGURE 8.

FIGURE 8. Effects of 60 nm Cr_2O_3 particles on the relative normalized gene expression of (A) TNF-α, (B) MCP-1, and (C) MIP-1α in J774A.1 macrophages after 1 h, 3 h, 6 h, and 9 h incubations. The macrophages were incubated with two different concentrations of 60 nm Cr_2O_3 particles (0.5 and 3.5 million particles per cell). Gene expression, normalized to the reference genes, is presented relative to the expression of the gene in non-stimulated cells at the zero time point. Data are presented as means ± pooled SD of four independent experiments performed in triplicates at all time points with the following exceptions: for TNF-α and MCP-1, the four experiments were performed in duplicate (as opposed to triplicates) at 9 h, and for MIP-1α, three experiments (as opposed to 4) were performed in triplicates, at all time points.

V-FITC negative and positive cells in the present study suggests that PS translocation to the outer leaflet does not occur extensively in J774A.1 macrophages, as noted in a previous study[19]. It is therefore possible that the percentages of early apoptotic cells may have been underestimated. It should also be noted that the overall percentages of late apoptotic and necrotic cells may also have been underestimated. Indeed, flow cytometry results provide a quantitative analysis of the cells remaining in suspension, without taking into account cells that may have lysed completely (some necrotic cells) or been phagocytosed by others (apoptotic bodies). In this regard, the cell death detection ELISA results are probably more representative of the initial population of cells incubated with the particles since they included not only DNA information from the late apoptotic and necrotic cells observed by flow cytometry, but also DNA information from the cells that may have lysed completely[19].

Despite the presence of low levels of apoptosis, necrosis remained the predominant mode of cell death, as demonstrated by cell death detection ELISA. As previously mentioned, necrosis is a mode of cell death which has been associated with an inflammatory response[29]. Surprisingly, however, TNF-α release did not increase significantly (and even decreased slightly in some conditions), and the MCP-1 and MIP-1α chemokine levels also remained low. These low levels of cytokines may be explained by the rapid cell mortality preventing cytokine production. Indeed, the kinetic studies revealed that total cell numbers were reduced by more than half within 6 h of incubation with the highest particle concentrations. The absence of cytokine production was further confirmed at the gene level, with no significant upregulation of the gene expression for any of the cytokines studied.

When combining particle size and number into an overall particle volume, macrophages appeared to respond similarly to equivalent volumes of 60 nm and 700 nm Cr_2O_3 particles per cell. This suggests that the volume of Cr_2O_3 particles, rather than absolute particle size or number, dominated the macrophage response. These results are in agreement with previous studies using poly(methyl methacrylate) (PMMA)[48] and titanium particles[49], which reported the effects of particle size, surface area and volume on the overall macrophage response. In addition, using UHMWPE particles, Green et al.[50] reported that 0.24 μm particles at a ratio of 10 μm^3 per cell stimulated murine peritoneal macrophages to generate bone resorbing activity, while larger particles (0.45 and 1.71 μm) were active at a ratio of 100 μm^3 per cell, and 7.62 and 88 μm particles were inactive at all the doses analyzed. The authors concluded that particle size and volumetric concentration were critical factors for macrophage-mediated bone resorption. Finally, in a study analyzing the effects of size, concentration, and composition of ceramic (Al_2O_3, ZrO_2) and high density polyethylene particle on J774A.1 macrophages, Catelas et al.[36] reported a combined effect of particle size and concentration, referring to an overall "phagocytosable volume". Therefore, future *in vitro* studies may benefit from considering and comparing the effects of particle volumes per cell rather than strictly focusing on absolute particle size or number, particularly when comparing particles of a given composition.

Studies measuring the biological response to particles produced by MM implants have focused predominantly on the effects of micrometer-size CoCrMo particles, which are not the most clinically relevant. Some of these studies have reported significant decreases in cell viability[25] and significant increases in TNF-α release[21,22]. Results from other studies examining the biological effects of the more clinically relevant nanometer-size CoCrMo particles suggest that these particles have cytotoxic effects that appear to be high compared to those of the Cr_2O_3 particles shown in the present study. For example, Germain et al. found that, for particle volumes similar to the lowest ones used in the present study (50 μm^3 per cell), nanometer-size CoCrMo particles (mean size: 29.5 nm) induced a 43% decrease in macrophage viability after 1 day of incubation[20]. Similarly, Brown et al. reported that, for particle volumes comparable to the lowest ones used in the present study (50 μm^3 per cell), nanometer-size CoCrMo particles (mean size: 40 nm) decreased fibroblast viability by approximately 45% after 1 day, and increased significantly TNF-α release by human peripheral blood mononuclear cells *in vitro*[51]. The study of Kwon et al.[8], reporting the effects of nanometer-size chromium particles on macrophage viability, is perhaps the most comparable to the present study. Considering the propensity for nanometer-scale metal particles to oxidize instantaneously, the particles used by these authors were probably a form of chromium oxide[52]. Although Kwon et al. reported no significant effect of these particles on cell number, their results show, in agreement with the present study, a substantial decrease in cell number of up to approximately 30% after 1 day. Nevertheless, differences exist between the study of Kwon et al. and the present study, including cell lines (RAW 264.7 vs. J774A.1 murine macrophages) and the techniques used to measure cell viability. Furthermore, because Kwon et al. did not specify the number of particles incubated with the cells, a direct comparison of the results remains difficult.

Although a decrease in cell numbers, as well as an increase in late apoptosis (albeit very mild) and an increase in necrosis were observed in cells incubated with high concentrations of Cr_2O_3 particles, cell mortality remained low compared to that previously reported in cells incubated with lower concentrations of micrometer-size UHMWPE particles[27,53–55]. Indeed, previous *in vitro* studies showed that micrometer-size UHMWPE particles induced significant cell mortality[27,53–55] and pro-inflammatory cytokine release at lower particle concentrations[27,37,55,56]. This suggests that nanometer-size Cr_2O_3 particles have a lower cytotoxicity than micrometer-size UHMWPE particles. This difference may translate into different pathomechanisms in patient responses to wear particles from different types of implants. Interestingly, a recent study by Pal et al. revealed that nanometer-size UHMWPE wear particles induced significant dendritic cell activation, and consequently increased IL-6 and IL-1β secretion, but not TNF-α[57]. Nevertheless,

comparison of the effects of the nanometer-size UHMWPE wear particles described by Pal et al. with the effects of the nanometer-size Cr_2O_3 particles presented in the current study remains difficult because of differences in the cell models.

In conclusion, results showed overall low levels of cytotoxicity induced by Cr_2O_3 particles except at high concentrations, which induced significant decreases in total cell numbers and significant increases in necrosis. The 60 nm particles appeared to be even less toxic than the 700 nm particles. When combining size and concentration, results suggested an effect of particle volume. TNF-α, MCP-1, and MIP-1α cytokine release and gene expression remained low, probably because of the early cell mortality preventing cytokine/chemokine production. Overall, this study demonstrates that both nanometer-size particles of Cr_2O_3, a stable form of chromium oxide ceramic, have rather low cytotoxic effects on macrophages. Therefore, chromium oxide particles may not be the main culprit in initiating the inflammatory reaction in MM periprosthetic tissues. However, other parameters (such as potential intracellular damage) remain to be investigated.

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