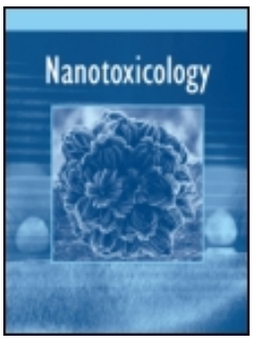

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The suitability of different cellular *in vitro* immunotoxicity and genotoxicity methods for the analysis of nanoparticle-induced events

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Abstract

Suitable assays and test strategies are needed to analyze potential genotoxic and immunotoxic health effects caused by nanoparticle exposure. The development and validation of such methods is challenging because nanoparticles may show unexpected behavior, like aggregation or interference with optical measurements, when routine *in vitro* assays are performed. In our interdisciplinary study, the effects of inorganic gold (4.5 nm) and iron oxide (7.3 nm) nanoparticles with a narrow size distribution were tested on human cells using different assay systems. The results show that cytotoxicity as well as immunotoxicity and genotoxicity induced by these two inorganic nanoparticles was low or absent when using a panel of cell-based tests in different laboratories. However, several technical issues had to be tackled that were specific for working with nanoparticles. The methods used, their suitability for nanotoxicity testing, and the technical problems encountered are carefully described and discussed in this paper.

Keywords: *Immunotoxicology, genotoxicology,* in vitro, *human cells*

Introduction

Nanotoxicology is an important field of study for the assessment of potential health effects of nanomaterials that are unintentionally generated (such as diesel exhaust particles) or are manufactured for specific purposes. The amounts and types of intentionally manufactured nanoparticles expand rapidly, leading to high occupational exposure, and exposure of the general public through product use and waste products (Tetley 2007). In addition, nanoparticles that are used for medical purposes will be directly administered to humans, in which case a direct contact between different tissues and the nanoparticles is intended. This increased exposure to the relatively unknown entities asks for methodologies to detect the potential effects of nanoparticles on human health. The need for the development of such methods has been described in several reviews and other publications (Oberdörster et al. 2005; Borm et al. 2006; Service 2008). However, designing assays that are suitable for nanotoxicity testing are hindered by a number of factors, one of which is the huge variation in different nanomaterials, with varying metrics and physicochemical properties. In the presented research the focus lays on nanotoxicity testing of purposely engineered, inorganic nanoparticles with a narrow size distribution.

In contrast to the assessment of chemical toxicity, where a limited number of factors such as chemical composition, solubility and chemical activity play an important role, nanotoxicology faces a large number of additional pitfalls.

The increasing interest in nanotechnology is due to the fact that properties of materials drastically change when they are in the nanometre range (< 100 nm).

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This holds true for example for the optical properties of metal nanoparticles (Kelly et al. 2003). These changes make nanoparticles attractive for different purposes in material sciences, cosmetics, medicine and many other fields. However, prediction of the immunotoxic or genotoxic effects of such particles is complicated by some of these altered characteristics. It is currently well accepted that the chemical composition is not the only parameter that plays a role in the analysis of such effects; particle size, surface, shape, coating, charge and stability are also of main importance (Wittmaack 2007). All these different parameters increase the number of samples that need to be tested, which also implies an increase in the costs (Choi et al. 2009). Moreover, the proteins and other components that are present in biological fluids, such as serum, mucus and lymph will influence the characteristics of the nanoparticles. Proteins and other components can coat the nanoparticles, thereby changing the surface a cell will 'see', and this coating could also either prevent or induce nanoparticle aggregation or agglomeration (Lynch et al. 2007; Dobrovolskaia et al. 2008; Faunce et al. 2008; Chen et al. 2009). Such interactions will therefore need consideration when performing *in vitro* and *in vivo* nanotoxicology studies.

There is a relatively high amount of contradictory data in the nanotoxicology literature (Gwinn and Vallyathan 2006). The nanotoxicology field is highly interdisciplinary, but most laboratories do not have the possibilities to perform sound toxicology experiments and at the same time perform careful nanoparticle analysis. Since the stability of colloidally dispersed particles largely depends on the dispersing media, particle analysis should ideally be performed at different stages of the experiment. Previous research has shown that factors such as the cell culture medium composition, and especially the serum content, are of extreme importance for the characteristics and stability of the particles (Chen et al. 2009). Moreover, the nanoparticle concentration in the stock solution also influences the formation of aggregates and agglomerates. Furthermore, treatment of the resuspended particles, for example by sonication or vortexing, will influence the aggregation state of the particles. In addition, the choice of solvent could also affect the stability of the particles thereby limiting or increasing the release of ions. Ideally, all these factors should be analyzed for each particle suspension, which is often not feasible. The need for such careful particle characterization and a recommended minimum approach has recently been published (Warheit 2008).

Currently, there are no methods that can be regarded as a gold standard for nanotoxicology studies. Initially, many cytotoxicity studies were performed using established assays, such as the MTT test or live/dead cell staining. These methods have turned out to be extremely cumbersome (especially live/dead cell counting) or their results altered because of the interference of the nanomaterials with the colour dyes used in such methods (Casey et al. 2007; Monteiro-Riviere et al. 2009). In addition, data from studies testing the effects of environmental pollutants, such as diesel exhaust particles or chemicals, showed that immunotoxic and genotoxic effects often occur at concentrations that are far below those inducing direct cytotoxicity (Don Porto Carero et al. 2001; Wichmann et al. 2005; Carfi' et al. 2007;

Table I. Cell viability methods used, including the cell type for which this assay was used, the maximum cell number that was used at the beginning of the assay (Max. cell number) and the affiliation of the group performing this specific assay according to the author list.

Method	Cell type	Max. cell number	Affiliation
CellTiterBlue (Promega)	A549 cells	5×10^4 cells/ml	1
	Jurkat cells	5×10^5 cells/ml	1
Cytokinesis Block Proliferation Index	Peripheral blood leukocytes	Full blood	2
Neutral Red Uptake Assay	BEAS-2B cells	12×10^4 cells/ml	3
Propidium iodide	Dendritic cells	1×10^6 cell/ml	3
MTT/XTT	CaCo-2 cells	5×10^5 cells/ml	4
	monocytes	2×10^6 cells/ml	4
Trypan Blue dye exclusion, visual inspection	CaCo-2 cells	5×10^5 cells/ml	4
	monocytes	2×10^6 cells/ml	4

Oostingh et al. 2008). In this manuscript, we will therefore focus on the immunological and genotoxic effects of nanoparticles with a narrow size distribution. Nevertheless, a range of different cytotoxicity tests were also included, as listed in Table I. This was done to determine on the one hand whether metallic nanoparticles had similar interfering effects on the optical assays as those observed for carbon nanomaterials and quantum dots, and on the other hand to exclude that possible immunological and genotoxic effects could be due to cell death. Cell viability was not affected by the nanoparticles used in this study, independent of cell type, nanoparticle type, and method of exposure.

The immune system is particularly exposed to potential effects of nanoparticles, since immune cells are abundant in the vicinity of the entry sites of these entities, such as skin, airways and gastrointestinal tract, and are specialized to recognize non-self materials. However, the selection of unambiguous endpoints is difficult when monitoring immune effects of these particles, since the immune system can be activated or suppressed and these effects can be general or limited to specific cells or locations. In addition, immune responses may be diverted (e.g., from inflammation to allergic responses) and effects may depend on an inactive immune system, or in contrast may only occur when an immune defence mechanism is switched on, e.g., when a person is ill. These diverse responses of the immune system make it difficult to develop the ideal assay for testing nanoparticle-induced immunotoxic effects.

Another aspect in the field of nanotoxicology, which is still far from being solved, is the possible effect at the DNA level. In fact, compared to the already acquired knowledge on the toxicological profiles of nanomaterials, genotoxicity data are still rare and for many materials altogether missing. This situation might be due to handling and test optimization problems, in other terms the lack of validated standard operating procedures (SOPs), appropriate for testing nanoparticle-induced effects. Another possible reason for the lack of data could be that the biological endpoints used for genetic toxicology screening, even if they are considered as biomarkers of effects and/or exposure, are far from being conclusive in terms of understanding the mechanisms behind the toxicological profiles. In fact, there is an ongoing debate in the scientific community whether nanoparticle-induced genotoxicity is a direct effect of the nanoparticle binding to the DNA, also known as a primary effect, or a secondary event, induced by activation of molecular pathways. These secondary events are mainly induced by the activation of oxidative events by the modulation of reactive oxygen/nitrogen species (ROS/RNS) generated during particle-elicited activation of intracellular molecular pathways (e.g., inflammation) (Schins and Knaapen 2007). With respect to the issues mentioned above, it might be assumed that the assays' SOPs, which have been optimized for chemicals only, might not provide reliable or relevant responses. Under these circumstances, using a panel of different tests might be the right strategy to obtain a complete picture of the potential effects of nanomaterials on human health.

Within the 6FP EU STREP project DIPNA (Development of an Integrated Platform for Nanoparticles Analysis to verify their possible toxicity and ecotoxicity) we have evaluated different immunological methods and genotoxicity tests. Most of these methods were already established and used for other purposes in the research groups involved, such as immunotoxicology or genetic toxicology screening of environmental pollutants, or basic immunological and genotoxicology research (Bossù et al. 2001; De Smedt et al. 2005; Verstraelen et al. 2005; Colognato et al. 2007b; Oostingh et al. 2008). Within the scope of the project, the different methods were analyzed for their usefulness in the field of nanotoxicology. The standardization of the protocols and methods within this project was carefully planned. The particles that were used in this study were synthesized in solution and were characterized at the different stages of the biological experiments (Figure 1 and Pfaller et al. 2009). This enabled the comparison between the results from the different laboratories within our consortium. Moreover, care was taken to warrant the use of non-aggregated particles with a narrow size distribution, a strategy which knowingly reduced the maximum mass concentration of particles in solution that could be used, but ensured the analysis of truly nanoparticle-induced effects. In addition, the cell culture medium used included serum components for all experiments, since the absence of serum is known to strongly affect the growth and behaviour of most cell types. Since nanoparticles will also come into contact with protein-rich bodily fluids before reaching most cells types (excluding the upper layer of the skin), the presence of serum components is probably more realistic compared to their exclusion. Considering the latter aspect, the micronucleus test was performed using whole blood samples instead of working with isolated lymphocytes, in order to maintain the physiologic environmental conditions as much as possible. Finally, the concentrations of nanoparticles used were exactly the same in all research groups. The above synchronization of protocols enabled comparison of the results between the different laboratories within this project.

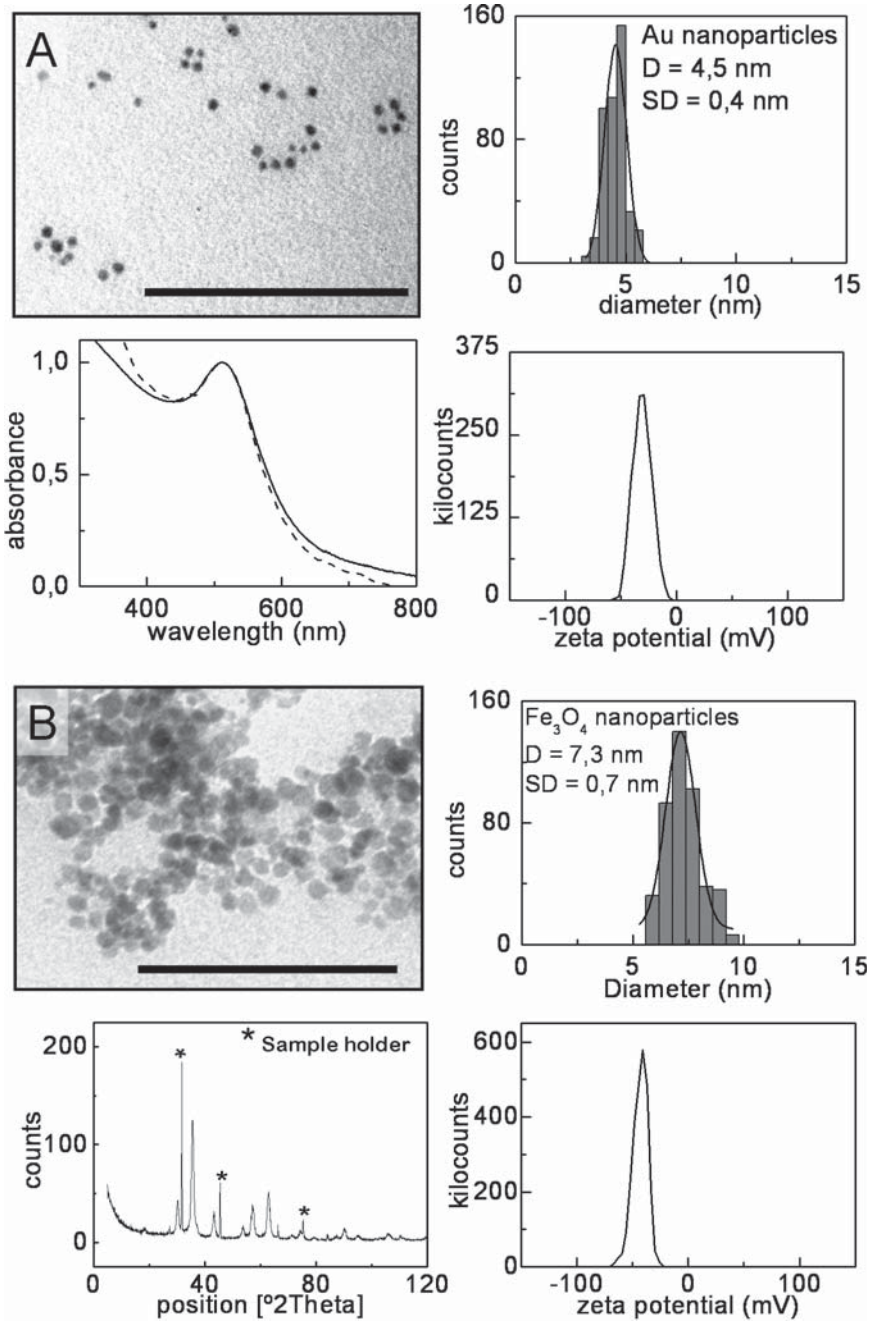

Figure 1. Detailed characterization of the nanoparticles used in the cell culture experiments. (A) Gold nanoparticles. Top two panels: TEM images and size distribution histogram. Mean diameter = 4.5 ± 0.4 nm; Bottom two panels from left to right: UV-visible spectrum of particles as-synthesized (solid line, peak of absorbance = 511 nm) and after 48 hours incubation in cell culture medium (dashed line, peak of absorbance = 512 nm); Zeta Potential: mean value = −33.5 mV. (B) Iron oxide nanoparticles. Top two panels: TEM images and size distribution histogram. Mean diameter = 7.3 ± 0.7 nm. Bottom two panels from left to right: X-Ray diffractogram of magnetic iron oxide NPs; Zeta Potential: mean value = −43.1 mV. The Fe_3O_4 nanoparticles were recovered from the cell culture media by centrifugation and once redispersed were stable in solution indicating that they did not aggregate during the experiment. Scale bars for TEM images are 100 nm. Parts of this Figure was reproduced from Pfaller et al. (2009) with permission from the publisher Taylor & Francis.

Methods

Particle preparation and characterization

Spherical nanoparticles were synthesized by wet chemistry methods starting with organo-metallic and metallic salt precursors either decomposed or reduced in the presence of stabilizers (Pfaller et al. 2009). For the current study, 4.5 nm gold and 7.3 nm iron oxide particles were selected. The particles were synthesized under conditions that enabled the collection of stable nanoparticles with a narrow size distribution, also called monodispersed particles (Figure 1). It is well accepted in the field of nanotechnology that particles with a size distribution where the standard deviation is below 10% can be considered as monodispersed (Puntes et al. 2002).

All reagents required for synthesis were purchased from Sigma-Aldrich and used as received. For the gold nanoparticles, 0.25 mM trisodium citrate was used as stabilizing agent and for the iron oxide nanoparticles 0.5 M deoxygenated tetramethylammonium hydroxide (TMAOH) was used as stabilizing agent. All glass material was sterilized and depyrogenated in an oven prior to use. Moreover, the particles were produced under conditions that reduced the levels of lipopolysaccharide (LPS) and other contaminants as much as possible. The exact procedure used for the synthesis of the nanoparticles and the methods used to characterize the particles are described in the Appendix (online version only).

The size distribution and size modification of the particles that were due to nanoparticle coating were both measured with transmission electron microscopy (TEM) (Figure 1) and dynamic light scattering (DLS). Despite the simplicity of DLS measurements when compared to TEM, the DLS method gave a broad range of results and lack of reproducibility, especially in the case of low refraction index materials. In fact, the DLS technique is not well suited for particles smaller than 10 nm, where the refraction index of the observed material is close to that of water. Therefore, even if DLS turned out to be useful for observing trends and comparisons (such as the comparison between particle sizes and coatings), the analysis of size distribution was more accurate and reproducible when using TEM.

An important issue that may often lead to confusion and lack of reproducibility of observed biological effects is the fact that cells are more susceptible to minute variations of the nanoparticles fine structure than conventional physicochemical characterization techniques. Thus, nanoparticles conjugated to different peptides may show identical characteristics when using TEM, X-Ray Diffraction (XRD), DLS, and Z-Potential, but very different biological response, as is the case for macrophages that were extremely sensitive to the type and degree of coating of gold nanoparticles (Bastús et al. 2009).

Handling of nanoparticles in cell culture

General procedure. The synthesized particles were used in cell culture at the highest concentration at which they remained stable, the stock concentrations were 6.05×10^{13} gold nanoparticles/ml and 6.6×10^{13} iron oxide nanoparticles/ml. No aggregation could be detected during the experiments (tested for 48 h in serum-containing cell culture medium), and therefore the doses were exact and comparable. The mass concentrations used in this study may seem low, but silver nanoparticles at these concentrations (0.1–10 μg/ml) were described to be toxic for viruses, prokaryotes and mammalian cells. In addition, these concentrations are not only relevant in toxicology, but also in medicine (Batarseh 2008).

The highest nanoparticle-concentrations used were calculated as depicted in Table II, i.e., in number concentration [particles/ml], molarity [nM], mass concentration [μg/ml], and surface area concentration [cm^2/ml] of the particles. The same maximum volumes of nanoparticle suspensions (9.1% (v/v) of the stock suspension) were used by all research groups, as well as a consistent use of 9.1% (v/v) solvent independent of the nanoparticle concentration used, thereby keeping the dilution factor of the nanoparticle suspension:cell culture medium constant (1:11) and ensuring that the final solvent

Table II. Characteristics of the nanoparticles, their solvents and the highest final concentrations as present in the cell culture medium.

Particle	Solvent	Diameter [nm]	Molarity [nmol particles/l]	Number conc. [particles / ml]	Mass conc. [μg / ml]	Surface Area conc. [cm^2/ml]
Gold	0.25 mM $Na_3C_6H_5O_7$ and 0.3 mM $NaBH_4$	4.5	9.1	5.5 x10^{12}	4.5	3.5
Iron oxide	5 mM TMAOH	7.3	10.0	6.0 x10^{12}	5.1	10

concentration was constant in all experiments. This amount of solvent did not induce significant changes in viability or growth of the cells (data not shown).

Assessing LPS contamination of nanoparticle batches. An important initial step when characterizing the biological effects of nanoparticles in cell culture systems is the assessment of the presence of contamination, for which LPS is generally used as a marker for bacterial contamination. Biological pyrogens, such as LPS, are potent activators of inflammatory responses in monocytes and other cell types expressing specific toll-like receptors. The unintended or unknown presence of LPS or other bacterial components can therefore be responsible for significant biological effects that may mistakenly be attributed to the nanoparticles. Even when working under sterile conditions, LPS derived from gram-negative bacteria can be present, due to its stickiness to many kinds of material. Thus, LPS can be easily introduced into nanoparticle batches through water and solvents, chemicals, raw materials, glassware and equipment used for synthesis (Vallhov et al. 2006; Hall et al. 2007). We have used the Limulus Amebocyte Lysate-based assay (LAL QCL-1000, Lonza Walkersville, MD, USA) to determine the levels of LPS in our samples and found that detectable amounts were present in most batches of nanoparticle suspensions and solvents. Evaluation of several batches of nanoparticles revealed a quite substantial batch-to-batch variability of the LPS contamination, which ranged from < 0.1–8.2 ng/ml for gold nanoparticles, and from 0.1–5.1 ng/ml for iron oxide nanoparticles. These levels of LPS are still relatively low and did not affect any of the biological read-out parameters tested, except for those assays in which monocytes were used. The exact amount of nanoparticles was therefore adapted for this specific cell type as described below.

Determination of intracellular reactive oxygen species (ROS) production in BEAS-2B cells. Among the possible mechanisms of nanoparticle toxicity, oxidative stress remains an important consideration. Some engineered nanoparticles are capable of inducing abiotic ROS production due to their intrinsic surface properties. Other nanoparticle properties that are responsible for interactions with sub-cellular organelles and biological systems could contribute to further, intracellular ROS production. In turn, ROS generation by particles can lead to protein, lipid, and DNA damage. Therefore, assessment of nanoparticle-induced ROS generation is important, although it only partially explains cellular oxidative stress (Li et al. 2008). Fluorogenic probes, such as 2′,7′-dichlorofluorescein diacetate (DCFH-DA), are considered reliable and efficient for quantifying overall oxidative stress in cells (Wang and Joseph 1999). The hydrophobic molecule DCFH-DA is non-fluorescent until it penetrates the cell where acetate groups are hydrolyzed by intracellular esterases and oxidation to its fluorescent product 2′,7′-dichlorofluorescein (DCF) occurs. In this study, the derivative 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA, Molecular Probes, Invitrogen, Paisley, UK) was used to assess intracellular generation of ROS. The additional chloromethyl group allows for longer retention within the cell due to covalent binding to intracellular thiol-containing components. Oxidation of the probe was detected by measurement of increased fluorescence with a flow cytometer, and was compared to measurements with a fluorescence microplate reader. In contrast to flow cytometry, the latter method allows to perform high-throughput analyzes using small test volumes for which equipment is available in most laboratories.

Since the airways are considered a major route of access for air-borne nanoparticles to the human body (Oberdörster et al. 2005) and the role of engineered nanoparticles in oxidative injury leading to lung diseases has been well documented (Li et al. 2008), the BEAS-2B cell line (ATCC, LGC Promochem, Teddington, UK) was selected as cell model. This cell line was originally derived from normal human bronchial epithelial cells obtained from autopsy of non-cancerous individuals. The exact method for the analysis of ROS production by BEAS-2B cells exposed to gold and iron oxide nanoparticles and the detection by flow cytometry or when using a fluorescence microplate reader has been described in the Appendix (online version only).

Results are expressed as mean fold-change of background-corrected values for exposed cells compared to cells treated with the respective solvent (mean fold-change of solvent = 1) ± standard deviation (SD). The Student's *t*-test on mean $\log_2$ fold-change values was used to assess statistically significant differences in intracellular ROS production between nanoparticle- and solvent-treated cells. *P*-values less than 0.05 were considered statistically significant.

Screening assay using stably transfected human reporter gene cell lines. Cytokines, such as interleukin (IL)-8, IL-6 and tumour necrosis factor (TNF)-α, play an important role in the communication between cells of the immune system, for example in inflammatory events. The pattern of secreted cytokines and chemokines and the expression of specific transcription

factors, such as NF-κB, can be used to monitor alterations in the activation status of the immune system. However, the conventional methods to analyze secreted cytokines, such as enzyme-linked immunosorbant assays and flow cytometry-based assays, are time-consuming and relatively expensive. The extremely large number of different nanoparticles asks for a cheaper, faster and more reliable method and for this purpose we tested the suitability of a panel of luciferase-based reporter cell lines that were established as part of two previously performed EU-projects (5FP MAAPHRI and 6FP NOMIRACLE). The data obtained with these cell lines can show potential immunomodulatory effects of the nanoparticles and whether these effects are of a specific nature, i.e., whether different cytokines and chemokines are affected differently, or if this response is of a more general nature. Using this reporter gene-based assay, the promoter can be analyzed in different cell types in a 96-well format at high-throughput rates.

The reporter gene-based screening method involves a panel of different cell lines, A549 (bronchial epithelial) or Jurkat (T cell lymphoma), that are stably transfected with a plasmid vector construct. This construct contains genes coding for ampicillin and neomycin resistance, allowing for selection of transfected bacteria and mammalian cells, respectively. The sequence for the enzyme luciferase is included as a reporter gene; this sequence is directly linked to a specific promoter region of a cytokine or the binding site for a transcription factor. The various cell lines used have different human cytokine promoters or transcription factor binding sites cloned in front of the luciferase gene. Activation of the promoter or binding site results in transcription of the luciferase gene. Following cell lysis, the released luciferase catalyses the oxidation of luciferin in the added substrate solution to oxyluciferin. The thereby produced light can be quantified using a luminometer. The exact description of the reporter cell lines was previously described (Oostingh et al. 2008) and a description of the method used can be found in the Appendix (online version only).

Innate immune response of human gut epithelial cells to nanoparticles. The human mucosal epithelial cell line CaCo-2 (colon carcinoma) is widely used as an *in vitro* model for studying intestinal epithelial cell function because CaCo-2 cells, if kept in culture for 10–14 days, acquire structural and functional characteristics of enterocytes, including minimal proliferation rate, formation of tight junctions and development of an epithelial layer with polarized villi (Sambuy et al. 2005).

Thus, the differentiated CaCo-2 model was used to examine the possible effects of nanoparticles on the defensive functions of the intestinal mucosal layer. In this study, differentiated CaCo-2 cells were exposed to nanoparticles in an acute fashion (a single dose for 24 h), then the expression levels of innate immunity related genes were analyzed as well as the cytokine secretion. The insensitivity of CaCo-2 cells to LPS allowed the use of the highest concentrations of nanoparticles. An exact methods description can be found in the Appendix (online version only).

Determination of dendritic cell maturation using flow cytometry. Dendritic cells (DC) as antigen-presenting cells play a pivotal role in adaptive immune responses, and therefore represent a suitable cell model to study immunotoxic effects. Dendritic cell activation/maturation has been widely studied by assessing cell surface expression of co-stimulatory molecules and other activation markers using flow cytometry. Besides chemicals (De Smedt et al. 2005), diesel exhaust particles and other airborne particulate matter have also been observed to potently stimulate DC maturation, mostly by exerting an adjuvant role in allergen-induced DC responses (Verstraelen et al. 2005; Porter et al. 2007; Inoue et al. 2009). Information on nanoparticle-induced effects is still scarce and limited to the observed promoting effect of carbon black nanoparticles on the maturation of mouse bone marrow-derived DC (De Haar et al. 2008; Koike et al. 2008).

In this study immature DC differentiated from human cord blood-derived $CD34^+$ progenitor cells were used as a highly relevant primary cell model showing *in vivo*-like DC characteristics. $CD34^+$-cell isolation and culture procedures have been described before (Schoeters et al. 2007), and a brief description of the method used for cell isolation as well as the analysis of expression levels of specific cell surface markers can be found in the Appendix (online version only).

Percentages of positive cells and mean fluorescence intensities (MFI) were determined and used to calculate stimulation indices (SI) as: (% positive cells × MFI) of treated cells, divided by (% positive cells × MFI) of solvent-treated cells. Results are expressed as mean SI (mean SI = 1 for solvent-treated cells) ± SD of four independent experiments using four different donor-derived CD34-DC cultures. The Student's *t*-test on mean $\log_2$ values was used to assess statistically significant differences in surface marker expression and percentage viability between nanoparticle- and solvent-treated cells. *P*-values less than 0.05 were considered statistically significant.

Innate immune responses of human monocytes to nanoparticles. Blood monocytes are primarily deputed to uptake and destruction of foreign particles/molecules. Since no direct cytotoxicity leading to cell death could be detected for the gold and iron oxide nanoparticles tested (visual inspection, trypan blue dye exclusion, MTT/XTT assay), the main objective was to determine whether the capacity of monocytes to mount appropriate innate defensive responses upon stimulation with danger signals could be influenced by nanoparticles. For this purpose, an *in vitro* model has been developed, based on the ability of human monocytes to up- or down-regulate a series of inflammatory vs. anti-inflammatory genes in response to prototypical stimuli.

Human monocytes were purified from buffy coats of healthy blood donors. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, then $CD14^+$ monocytes were isolated from PBMC by one-step positive selection with anti-CD14-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA). Only $CD14^+$ monocyte preparations with purity > 97% were used. Preliminary experiments were performed to standardize the monocyte culture conditions (number of cells in culture, vessel volume, vessel surface, composition of culture medium). The optimal culture conditions and the exact method used have been described in the Appendix (online version only).

Genetic toxicology methods. The most widespread assays used for DNA-damage screening are Single Cell Gel Electrophoresis (SCGE) or Comet assay for the evaluation of single and double strand breaks under alkaline conditions (Collins et al. 1996), and the Cytokinesis-Block MicroNucleus assay (CBMN assay) for aneuploidogenic and clastogenic events (Fenech 2007). The modified version of the Comet assay could be a suitable method to analyze nanoparticle-induced effects on oxidative DNA damage (Collins et al. 1996). With this assay, a clear view of the type of damage induced at the nucleotide level can be assessed. A possible technical problem that could be encountered when assessing nanoparticle effects is the high concentration of material that is normally tested. In fact, due to the presence of aggregates/agglomerates, the aggregates can be observed in the tail of the comet and this makes that the readout becomes unreliable (data not shown). The same problem occurs when applying the CBMN assay. For both assays the situation is more problematic when suspension cell lines are used. Fewer problems arise by applying the Comet and CBMN assays to adhering cell models, since several washing steps are included before the fixation procedure, where most of the residual nanoparticles are removed. For the two above-mentioned assays, SOPs are present and well established for testing chemical genotoxicity, but for testing nanoparticle-induced genotoxic effects it is clear that some modification should be introduced.

In this study, the CBMN assay has been tested for its suitability as a genetic nanotoxicology method using peripheral blood leukocytes. The CBMN assay was performed according to the procedure described by Colognato and colleagues (Colognato et al. 2008). An exact description of the method, as applied for the current study, can be found in the Appendix (online version only).

Two thousand binucleated cells (i.e., cells undergoing mitosis) were evaluated in a blinded fashion for the number of BiNucleated MicroNucleated (BNMN) cells containing one or more micronuclei, following the scoring criteria adopted by the Human Micronucleus Project (Bonassi et al. 2001). Moreover, 500 lymphocytes were scored to evaluate the percentage of binucleated cells. The Cytokinesis Block Proliferation Index (CBPI), as a cytotoxicity value, was calculated according to Surrallés et al. (1995).

Results

Results of nanoparticle-induced intracellular ROS production in BEAS-2B cells

After incubation of BEAS-2B cells to either 9.1% (v/v) solvent or nanoparticle suspension in complete growth medium for 24 h, no significant decrease of cell viability was determined as compared to untreated control cells using the neutral red uptake assay according to standard procedures.

Measurement of the fluorescence intensities by flow cytometry revealed no significant increased ROS generation in nanoparticle-exposed BEAS-2B cells as compared to the solvent-treated controls for any of the tested concentrations. As an example, results of gold nanoparticle suspensions are shown in Figure 2A. When compared to untreated control cells, the solvents of both nanoparticles also did not influence ROS levels (data not shown).

For comparison, ROS generation was also evaluated by measuring fluorescence intensities of nanoparticle-exposed BEAS-2B cells using a fluorometer. To this end, cells (or protein-coated wells in the case of cell-free controls) were pre-loaded with CM-H_2DCF-DA dye and only thereafter exposed to the gold and iron oxide nanoparticle suspensions for 0, 2, 4, 6 and 24 h. Cell cultures exposed to

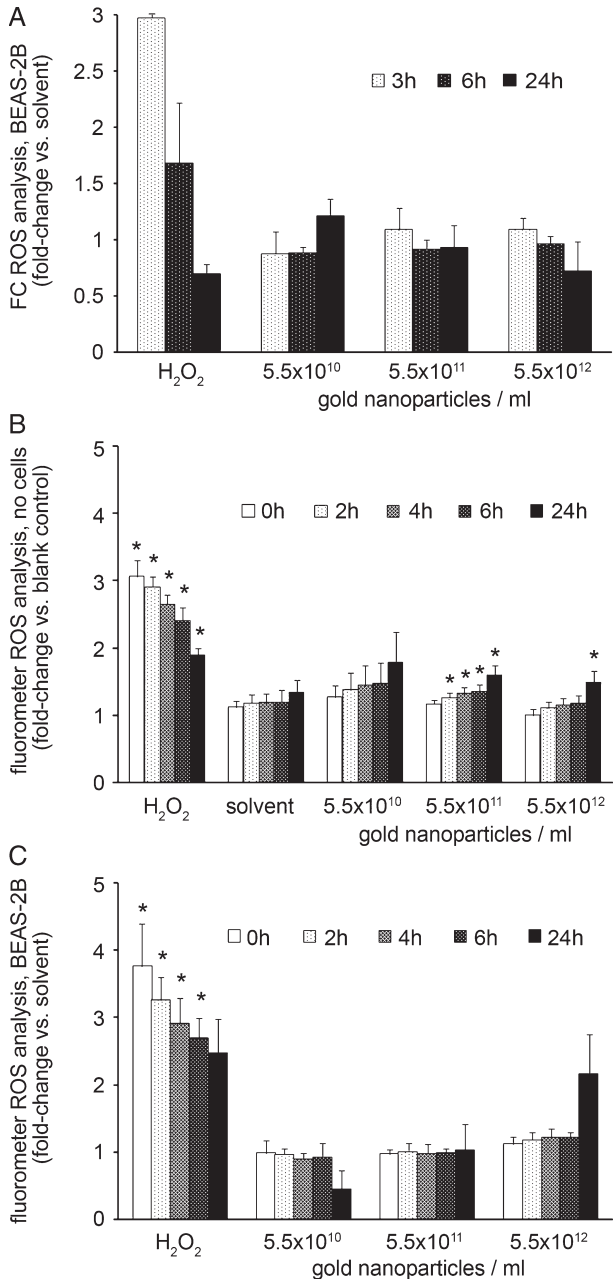

Figure 2. Effects of gold nanoparticles on ROS generation at several exposure times in human BEAS-2B bronchial epithelial cells assessed with CM-H_2DCF-DA for fluorescence analysis. (A) Flow cytometric (FC) analysis of exposed cells (duplicate measurements) presented as mean fold-changes ± SD as compared to the respective solvent controls (mean fold-change = 1) of 3–4 independent experiments. (B) Fluorometer analysis of background fluorescence signals of cell-free controls. Hydrogen peroxide (H_2O_2, 1 mM) was used as positive control. Data represent mean fold-changes ± SD as compared to blank cell-free controls (mean fold-change = 1) of triplicate measurements from one experiment. (C) Results from fluorometer analysis of exposed BEAS-2B cells showing mean fold-changes ± SD as compared to the respective solvent controls (mean fold-change = 1) of triplicate measures from one experiment. Hydrogen peroxide (1 mM) was used as positive control. * indicates $p < 0.05$ compared to the respective control values.

hydrogen peroxide (1 mM) were used as positive control. Background fluorescence signals of cell-free controls of gold and iron oxide nanoparticle suspensions, the solvent of the gold particles and the positive control were observed to be increased above blank controls containing cell culture medium only. This effect was progressive with time and was statistically significant for hydrogen peroxide and several of the nanoparticle concentrations at the different incubation times. In Figure 2B this is shown

for gold nanoparticles as an example, whereas results for iron oxide nanoparticles were similar, but less pronounced.

Baseline fluorescence intensities were measured immediately after exposure and showed significantly increased ROS generation (mean fold-change ± SD: 3.8 ± 0.6; $p = 0.02$) in BEAS-2B cells that were exposed to H_2O_2 as compared to unexposed, control cells (Figure 2C). Although fluorescence intensities gradually decreased with prolonged incubation times, significantly increased levels were maintained up to 6 h.

In cells that were incubated with gold nanoparticles at the highest concentration (9.1% v/v = 5.5×10^{12} NP/ml), intracellular ROS levels were increased after 24 h up to a mean fold-change of 2.2 (±0.6), but did not reach statistical significance compared to the corresponding solvent control (Figure 2C). The solvent of the gold nanoparticles did not show any oxidative effect compared to untreated cells (data not shown). Iron oxide particles and the corresponding solvents did not induce altered oxidative activity up to 24 h incubation (data not shown).

Suitability of fluorescent intracellular ROS detection assays for assessment of nanoparticle-induced oxidative stress

Oxidative stress involves intracellular production of ROS and downstream responses to the ROS, which are dictated by cell type-dependent antioxidant defence mechanisms. ROS generation has been observed in response to large fibres, ambient particles and a number of nanoparticles (Hussain et al. 2005; Park et al. 2008; Herzog et al. 2009b). Nanoparticle-induced ROS can be explained by the chemical composition and catalytic activity on the one hand, and the size and mobility of the particles leading to uptake into the cell on the other hand (Limbach et al. 2007). A close link has been established between nanoparticle-mediated oxidative damage, and immune and genotoxic effects in human cells. Assessment of oxidative stress as one possible explanation for observed biological effects of nanoparticles is therefore considered an essential component in a toxicity screening strategy (Li et al. 2008).

In the present study a DCFH-DA-based assay was evaluated for analysis of intracellular ROS production by *in vitro* cultured lung epithelial cells exposed to the engineered iron oxide and gold nanoparticles suspensions. Two different protocols using either fluorescent spectroscopy or flow cytometry were compared. For spectroscopical measurements cell-free controls containing the different inducers (nanoparticle suspensions, solvents or positive control) were included to determine the background signals for subtraction. These were found to be significantly enhanced for both nanoparticles at differing concentrations and for hydrogen peroxide compared to blank cell-free controls up to 24 h. This suggests that hydrogen peroxide and the nanoparticles interacted with the DCFH dye that adhered to the protein coating on the bottom of the 96-well plates, thus enabling further enhancement of the fluorescent signal generated by spontaneous hydrolysis, and atmospheric and/or light-induced oxidation of the dye. Indeed, due to specific physicochemical characteristics, such as size and chemical composition, nanoparticles may interfere with fluorescence measurements. In particular the presence of nearby metallic nanoparticles is known to enhance spectral properties of fluorophores (Aslan et al. 2005). Fluorescent spectroscopy measurements also revealed increased ROS levels after exposure of the cells to the highest concentrated solution of gold nanoparticles in a time-dependent way, whereas this effect was not observed using flow cytometry. This difference in observation between the two methods may be caused by a different quantification of the fluorescence signal, which represents a population of cells in the case of spectroscopy, whereas point signals on individual cells are counted in the case of flow cytometry. Given the fact that DCFH is not completely trapped intracellularly, but can leak out, and nanoparticles are still present during measurement, background signals are thus expected to be increasing time-dependently using spectrophotometry. In contrast, using a flow cytometry-based method fluorescence detection is possible at a single cell level and the DCFH-DA dye is added at the end of the incubation period after which most nanoparticles are washed from the cells, giving rise to truly intracellularly generated fluorescence signals. On the other hand, quantification of point signals by flow cytometry is subjected to more variation, as can also be concluded from our data, and at the same time is less well suited for high-throughput screening formats. It can therefore be concluded that both spectroscopical and flow cytometric methods have their specific benefits and limitations, but if suitable control measurements are included, they are both useful for evaluation of nanoparticle-induced ROS generation. Furthermore, when spectral interference with fluorescence is to be expected, as is the case when metallic nanoparticles are used, it is recommendable to use additional non-fluorescence-based techniques to confirm the observed effects.

Results of nanoparticle-induced immunotoxicity as determined by the reporter gene assay

The stably transfected A549 and Jurkat cell lines were used to investigate the influence of two specific

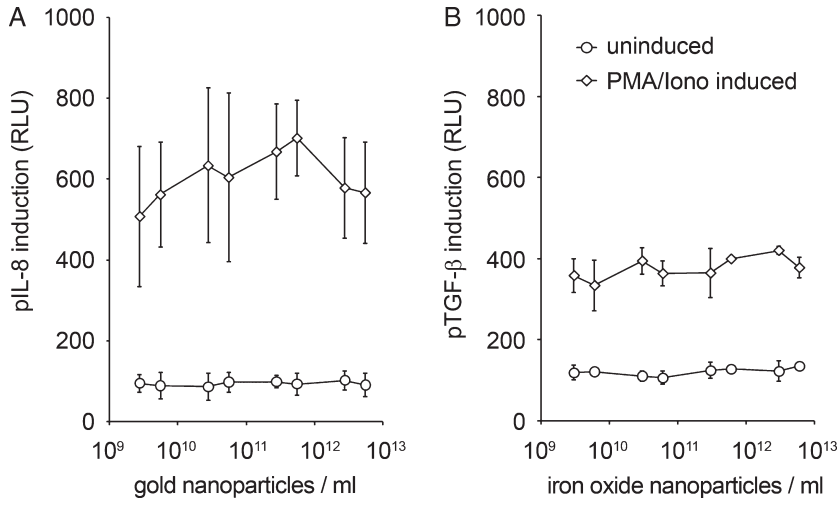

Figure 3. Effects of nanoparticles on stably transfected human reporter cell lines. Effects of gold nanoparticles on the induction of the IL-8 promoter (pIL-8) gene construct in Jurkat cells (A), and effects of iron oxide nanoparticles on the induction of the TGF-β promoter (pTGF-β) gene construct in Jurkat cells (B) are shown as representative examples of the results obtained with the different cell lines after 24 hours of exposure. For both panels, the lower line shows the level of luminescence related to the induction of the cytokine promoter in Jurkat cells. The upper line refers to Jurkat cells which were stimulated with PMA/Iono. The values are shown as mean ± SD of the relative luminescence (untreated and unstimulated cells = 100%). The solvent alone did not affect the cytokine promoter induction.

nanoparticle suspensions on cytokine regulation. The induction of the IL-8, IFN-γ and TGF-β promoters or the NF-κB binding sequence in Jurkat cells, and of the IL-6, IL-8, TNF-α promoters and the NF-κB binding sequence in A549 was tested. The effects of the different nanoparticle suspensions, including the ones described in this manuscript, on the different stably transfected A549 cell lines has previously been published (Pfaller et al. 2009). The effects on the cytokine promoter induction were low or absent, and the same accounts for the effects on the NF-κB binding sequence. The results obtained with the different stably transfected Jurkat cell lines, which have not been previously described, showed similar results to those obtained with the A549 cell lines. Up- or down-regulation of the activity of the promoter or binding sequences was very low and did not reach statistical significance, as tested by the Student's *t*-test, for any of the Jurkat cell lines tested.

In Figure 3, a representative example of the results obtained with IL-8 (A) or TGF-β (B) promoter-transfected Jurkat cells incubated with gold or iron oxide nanoparticles, respectively, in the presence and absence of PMA/Iono is depicted. The stimulation with PMA/Iono results in an activation of the cytokine promoter simulating an ongoing immune reaction, as is the case in inflammation or several immune diseases. The results showed that the nanoparticle-induced effects on this cell line were very small, independent of the addition of a cellular stimulus.

Suitability of the stably transfected reporter cell lines for detection of nanoparticle- induced immunomodulatory effects

Due to the need for screening methods to evaluate possible nanoparticle-caused immunomodulatory effects, the use of reporter gene assays is also under discussion. An appropriate cell-based screening method should in principle reduce the number of *in vitro* and more importantly *in vivo* experiments. The presented method has now been extensively tested for the screening of immunomodulatory effects of chemicals (Oostingh et al. 2008) and (nano-) materials (Herzog et al. 2009a; Pfaller et al. 2009), and there are some clear advantages and disadvantages to this method. First of all, the human immune system is very complex, and the data obtained with a cell-based assay that determines the effects on proximal promoters will not describe all possible impacts of nanoparticles on the immune system, but will only take a defined part into consideration. Secondly, even when the production of luciferase in the reporter gene assay is related to cytokine promoter activation, it is still not direct proof for the production and secretion of the cytokine itself. Nevertheless, we have formerly published results obtained with this screening method (Oostingh et al. 2008; Herzog et al. 2009a; Pfaller et al. 2009), and in these studies we could show that the results obtained with the reporter gene assay could largely be confirmed by direct analysis of secreted cytokines, using the enzyme linked immunosorbant assay (ELISA). The reporter gene

assay can thus be used as an indication for the occurrence of immunomodulatory events. Another positive aspect of this assay is that two different cell types, both playing key roles in immune defence, and a number of different cytokines and chemokines involved in different cell signalling pathways can be tested in parallel. This method is thus more robust than many other methods that are currently used and in this respect forms a good alternative for first line screening of immunomodulatory effects. Positive results from these screening studies will have to be followed up with additional methods to gain a deeper insight in the ongoing events, in which case primary cells and tissues, as well as animal models will have to be included. Moreover, this quick and relatively cheap screening method can be performed with routine equipment available in many cell culture laboratories and could therefore be used at many specific locations of interest. When it is taken into account that the results that were obtained using this method are as sensitive as those obtained when performing enzyme linked immunosorbent assays (ELISA) or Multiplex bead-analysis on the same cell types, it becomes obvious that the presented reporter gene assay could be a useful screening method.

Effects of nanoparticles on the innate immune response of human gut epithelial cells

The experiments were performed by exposing differentiated CaCo-2 cells to a single dose of either gold or iron oxide nanoparticles (9.1% v/v) for 24 h. The corresponding dilution of nanoparticle solvent was used as negative control. Cells stimulated with rhuIL-1β (10 ng/ml) were used as positive control. At the end of the incubation period, cytokine production and gene expression were evaluated. No direct cytotoxicity leading to CaCo-2 cell death could be detected for gold or iron oxide nanoparticles or for their solvents, by visual inspection (very useful for long-term cultures with continuous cell monolayers as in this case) and MTT/XTT assay. Preliminary results suggest that nanoparticles do not have significant effects on the expression of the genes examined. An example is shown in Figure 4 for expression of two genes of key importance in inflammation, i.e., IL-1β and its cleaving enzyme caspase-1. While stimulation with IL-1β strongly up-regulates IL-1β and caspase-1 gene expression, nanoparticles do not have a significant effect when compared to medium and/or solvent treated cells (Figure 4). It is important to underline the fact that solvent alone indeed had some effect in gene-upregulation, stressing the importance of including the chemical solutions used for nanoparticle suspension when testing their interaction with biological systems, in order to avoid misinterpretation of results.

When examining the levels of cytokine secretion in cell supernatants, differentiated, untreated CaCo-2 cells were found to be able to secrete IL-1Ra, but not IL-1β, IL-18, or IL-18BP. The secretion of IL-1Ra was not affected by the presence of nanoparticles, while secretion of other cytokines was not induced by nanoparticles (data not shown).

Thus, results obtained in the *in vitro* human gut epithelial cell model suggest that addition of gold or iron oxide nanoparticles to cells does not significantly

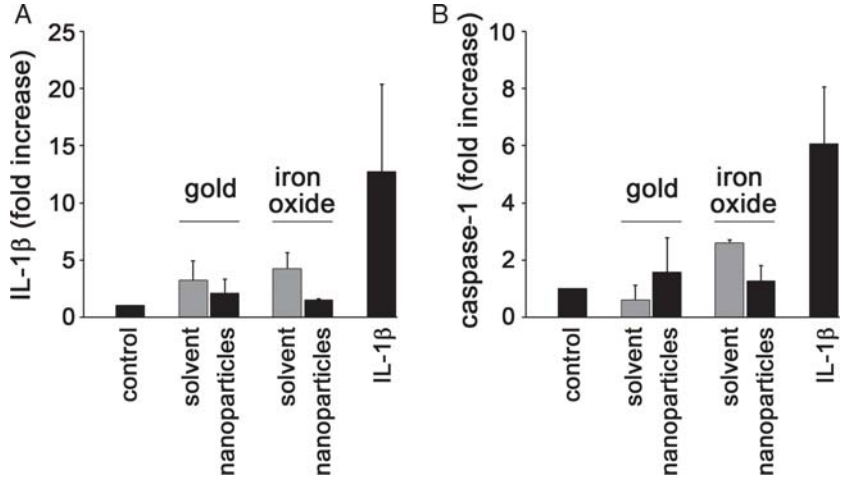

Figure 4. Effect of nanoparticles in innate activation of human gut epithelial cells. Differentiated human CaCo-2 cells were exposed to nanoparticles (9.1% v/v) in culture for 24 hours. Gene expression for IL-18 (A) and caspase-1 (B) was measured by real-time PCR. Medium alone represented the negative control, while IL-1β (10 ng/ml) was used as positive control. Real-time PCR results are expressed as fold-increase vs. the medium control. Data are the mean ± SEM of results from three independent experiments.

affect the basal or stimulated expression of innate immunity-related genes and cytokines.

Suitability of the human gut epithelial cell assay for the analysis of nanoparticle-induced innate immune response

The intestinal mucosa plays an important role in the protection against microorganisms and toxic compounds, with mucosal epithelial cells being directly involved in defensive immune reactions by producing defence mediators (chemokines, cytokines, and other inflammatory molecules). Mucosal epithelial cells in the gut represent one of the major routes of entrance into the human body for nanoparticles (e.g., upon ingestion). As is the case for professional defence cells such as monocytes, mucosal epithelial cells are also able to react to the presence of foreign particles by mounting an innate immune response that may become uncontrolled and pathological if the foreign bodies are not eliminated. The presence of inorganic, non-biodegradable and non-biocompatible particles (although not in the nano-size) has been detected in gut pathologies, such as fibrosis and Crohn's disease (Powell et al. 1996). It is therefore of particular relevance to evaluate the possible role of nanoparticles in amplifying or co-stimulating innate immunity defensive responses at the gut level.

The *in vitro* human gut epithelial cell model has a series of advantages that can make it particularly suitable for its application to the toxicological screening of nanoparticles. It represents a reliable model of the human gut mucosal epithelial layer that can be tested at the same time for innate/inflammatory response (as in this case) and for barrier functions, i.e., to examine whether nanoparticles can go through the barrier and how (permeability of junctions, transcytosis). Since the mucosal layer does not only include enterocytes, but also other cells that are important for antigen/particle uptake, a more comprehensive study has been initiated that includes evaluation of nanoparticle effects on M cells (specialized epithelial cells responsible of uptake of molecules/particles from the gut lumen and their transcytosis to the sub-epithelial space), dendritic

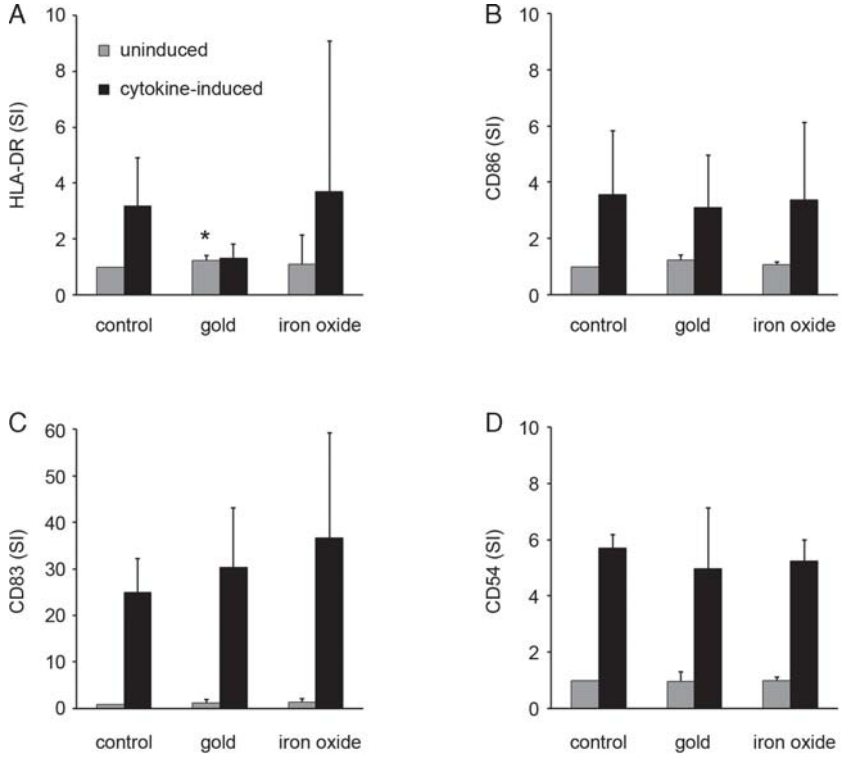

Figure 5. Expression of HLA-DR (A), CD86 (B), CD83 (C) and CD54 (D) in human dendritic cells exposed to gold and iron oxide nanoparticles for 24 hours. Immature CD34-DC were left untreated (control) or exposed to 9.1% (v/v) nanoparticle suspension in the absence (uninduced) or presence (cytokine-induced) of a mixture of IL-1β and TNF-α (5 ng/ml), which was used as positive control. Stimulation indices (SI) as compared to the respective solvent controls (SI = 1) are shown. Data represent the mean SI ± SD of four independent experiments using different donor-derived CD34-DC cultures. * indicates $p < 0.05$ compared with the corresponding solvent control, either uninduced or cytokine-induced.

cells (that protrude their dendrites between epithelial cells up to the gut lumen and take contact with foreign particles), and monocytes/macrophages (that encounter foreign particles in the sub-epithelial space). Preliminary evidence suggests that neither M cells (data not shown), nor monocytes/macrophages (see below) are affected in their innate immune response by gold or iron oxide nanoparticles. However, a co-culture model that includes the diverse cellular elements may be particularly suited to assess the different aspects of the human gut mucosa reactivity to nanoparticles.

Effect of nanoparticles on the expression of cell surface molecules in CD34-DC

After treatment with IL-1β and TNF-α for 24 hours, cell viability was not significantly reduced compared to untreated control CD34-DC using propidium iodide staining (data not shown). The cytokine mixture potently induced maturation of the cells, as was observed by significant stimulation of expression of all four surface markers (Figure 5; mean SI ± SD of HLA-DR: 3.2 ± 1.70, CD86: 3.6 ± 2.3, CD83: 25.0 ± 7.2, CD54: 5.7 ± 0.5).

The gold and iron oxide nanoparticle suspensions did not influence cell viability after 24 h exposure when compared to their solvents. The solvents also did not cause a significant reduction of cell viability as compared to untreated control cultures (data not shown). The gold nanoparticle suspension induced a statistically significant increase in the expression of HLA-DR, but the observed stimulatory effect was small (mean SI ± SD: 1.3 ± 0.2, $p = 0.047$) (Figure 5). Compared to their solvent, treatment of the cells with iron oxide nanoparticles had no effect on any of the studied markers.

To assess the potential amplifying effect of the studied gold and iron oxide nanoparticles on cytokine-induced maturation, CD34-DC were co-treated with IL-1β and TNF-α, and the nanoparticle suspensions. This co-treatment caused significant stimulation of CD86, CD83 and CD54 expression, which was comparable to that obtained with cytokines alone (Figure 5). On the other hand, upon co-treatment with cytokines and nanoparticles the expression of HLA-DR was not statistically different from either its expression in the absence of cytokines (solvent control) or with cytokines alone, due to the high variability of the results (Figure 5).

Suitability of the dendritic cell maturation assay for the analysis of nanoparticle-induced immune activation

Expression of DC maturation/activation markers has been studied extensively by flow cytometry as a possible biomarker for sensitization in response to chemical exposure. For this purpose HLA-DR, CD83, CD86 and CD54 are among the most frequently tested surface markers, and increased CD86 expression is the most promising biomarker to date (De Smedt et al. 2005; Dos Santos et al. 2009). In the present study, we have investigated the potential of two engineered inorganic nanoparticles to induce maturation of CD34-DC by measuring expression of HLA-DR, CD83, CD86 and CD54, but we did not observe any significant effects. When the cells were co-stimulated with a known maturing cytokine mixture, consisting of IL-1β and TNF-α, the addition of nanoparticle suspensions gave no additional enhancement or reduction of the significant cytokine-induced DC maturation response. These results for gold and iron oxide nanoparticles do not agree with the observations of direct inducing capacity and amplifying activity of carbon black nanoparticles (14–56 nm) on the maturation of mouse bone marrow-derived DC that were reported in two recent studies (De Haar et al. 2008; Koike et al. 2008). Discrepancies with our findings may result from differences in applied mass concentrations, which were lower in our experiments (4.5 μg/ml and 5.1 μg/ml for iron oxide and gold nanoparticles, respectively, vs. > 10 μg/ml for carbon black). Other reasons might be the different agglomeration/aggregation states of the particles (particles with a narrow size distribution were used in our study, whereas in Koike et al. (2008) carbon black particles were autoclaved at 250°C, a process known to increase aggregation), different sizes and chemical composition of the particles (4.5 nm gold and 7.3 nm and iron oxide, vs. 14–56 nm carbon black), or different source and differentiation protocol of DC (human CD34-DC vs. mouse bone marrow-derived DC). Nevertheless, all these studies with primary cell cultures have demonstrated the usefulness of flow cytometric analysis of DC maturation for *in vitro* screening of nanoparticle immunotoxicity. DC are recognized to be professional antigen-presenting cells and no equivalent, alternative techniques for assessment of their phenotypic maturation/activation status are available. For instance, molecular techniques such as RT-PCR do not provide evidence for functional protein expression. As already mentioned, flow cytometry offers the additional advantage of measuring fluorescence at the individual cell level after removal of residual nanoparticles, thus minimizing possible spectral interference (see previously). Disadvantages of the method include a lack of information on T cell activation as a crucial factor in the DC maturation/activation process, and a lack of interaction of DC with other cells, which has been demonstrated to be the determining factor in

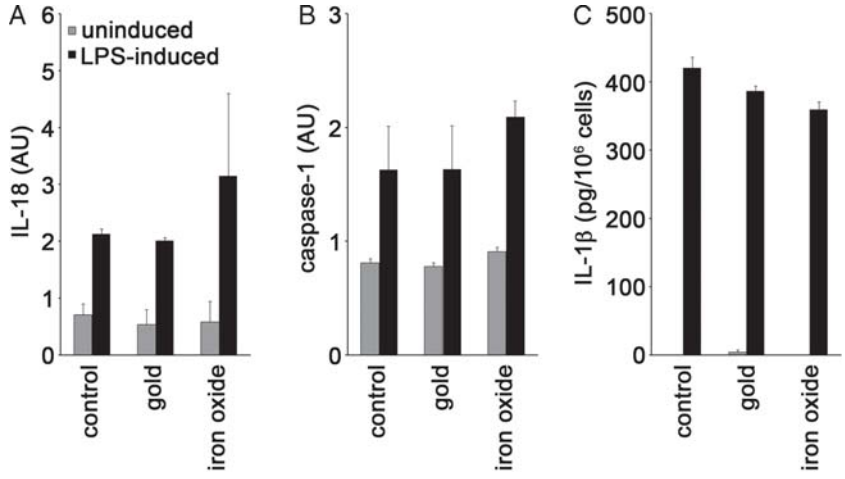

Figure 6. Effect of nanoparticles on innate immune activation of human blood monocytes. Human $CD14^+$ blood monocytes were cultured for 4 or 24 hours in medium alone or containing LPS (50 ng/ml). Nanoparticles (gold, iron oxide) and their solvents were added at the beginning of the culture. Because of LPS contamination, both nanoparticle batches were used at 4.55%, and their solvent at the corresponding 1:22 dilution. (A) Expression of IL-18 mRNA after 4 h of culture. (B) Expression of caspase-1 after 4 h of culture. Real-time PCR results are expressed in arbitrary units (AU). Data are the mean ± SD of results obtained from three donors. (C) Production of IL-1β in the 24 h-supernatants of the same experiments. Levels of released IL-1β were analyzed by ELISA and expressed as pg/10^6 monocytes.

phenotypic and functional enhancement of DC responses by diesel exhaust particles (Bleck et al. 2006). Furthermore, we have used human CD34-DC as a primary source of dendritic cells in this study. The use of primary dendritic cells suffers from a number of drawbacks, such as dependency on blood donors and related ethical issues, donor-to-donor variation in the background immune status, extensive cell culture protocol, variable cell yield, and a limited shelf life. However, among the available *in vitro* DC models they represent mostly the *in vivo* DC physiology (Dos Santos et al. 2009).

Effects of nanoparticles on the innate immune response of human monocytes

Due to the extreme reactivity of monocytes to LPS, nanoparticle suspensions were all tested for LPS contamination. Only few of the preparations could be used in monocyte cultures at high concentrations, but never at the highest concentration of 9.1% (v/v) used in other cellular systems (see Table II). Thus, the highest concentration tested was 4.55% (v/v), which corresponded to a final amount of LPS in culture < 5 pg/ml. Solvents were used accordingly at 4.55% (v/v) instead of 9.1%. Monocytes (4×10^6 cells in 2 ml) from three different donors were exposed to nanoparticles for 4 and 24 h and simultaneously challenged with LPS (inflammatory stimulus) or IL-4 (anti-inflammatory stimulus). Preliminary results show that nanoparticles do not have any significant effect on gene regulation under these conditions. Among genes tested, results reported hereafter specifically regard expression of IL-18, an inflammatory cytokine that is a potent inducer of IFN-γ, and caspase-1, the enzyme responsible for the maturation and activation of IL-18 itself and of the major inflammatory cytokine IL-1β. As an example, data regarding IL-18 and caspase-1 expression in monocytes challenged with LPS for 4 h are reported in Figure 6 (panels A and B). Basal expression of both genes is significantly up-regulated by LPS, while the presence of nanoparticles does not change either the basal expression or the LPS-stimulated up-regulation. Nanoparticle solvents, used as controls at the same dilution as for nanoparticle suspensions, likewise had no significant effect either on basal gene expression or on stimulus-induced expression (data not shown). The production of cytokines was also examined in the monocyte supernatants after 24 h of exposure to nanoparticles. No significant effects were detected. As an example, production of the highly inflammatory cytokine IL-1β, undetectable in unstimulated monocytes, is potently induced by LPS (Figure 6C). Nanoparticles had no significant effect on either LPS-induced IL-1β production or on spontaneous production. Experiments performed with lower nanoparticle concentrations likewise did not show any effect of nanoparticles on monocytes gene expression and cytokine secretion (data not shown).

Although preliminary, as these results need to be confirmed with a larger number of donors, the presented data suggests that, under rigorous experimental conditions, the addition of these nanoparticles to

human monocytes in culture do not have any significant effect either on direct monocyte activation or in modulating the innate immune reactivity of these cells to microbial agents.

Suitability of the monocytes cell assay for the analysis of nanoparticle-induced innate immune responses

The major role of monocytes/macrophages is to recognize and eliminate foreign material. They display remarkable plasticity and readily react to environmental changes by initiating different activation programmes. Classical monocyte/macrophage activation in response to microbial products (e.g., LPS) or interferon-γ (IFN-γ) gives rise to potent inflammatory effector cells (M1), which mediate the destruction of microorganisms and tumour cells and produce inflammatory cytokines and chemokines. On the other hand, alternatively activated M2 macrophages (e.g., in response to IL-4, IL-10, TGF-β) can tune inflammatory reactions, down-regulate Th1 responses, stimulate Th2 responses, scavenge debris, and promote angiogenesis, tissue remodelling and repair (Martinez et al. 2008).

Nanoparticles are foreign agents, and it is therefore expected that monocytes can be directly involved in the uptake of nanoparticles, their degradation and clearance from the bloodstream and from tissues. Nanoparticles in biological fluids and tissues are usually covered with biological molecules (e.g., proteins) which can facilitate their interaction with monocytes/macrophages, either through surface receptors or by non-receptor-mediated pinocytosis or lipid-raft-mediated uptake. After interaction with, or uptake of nanoparticles, it is possible that monocytes display alterations in their ability to respond to other activating stimuli. Given the central role of monocytes in the innate defence mechanisms of the organism, it is therefore of particular importance to study the 'immunotoxicity' of nanoparticles. In this case, rather than direct toxicity and subsequent cell death, the aim is to examine the capacity of nanoparticles to alter the reactivity of monocytes to danger signals (such as bacterial components) and to mount an appropriate defensive response. Alteration of defensive responses can result either in hypo-responsiveness (inadequate capacity of eliminating dangerous microorganisms, increased susceptibility to diseases), or in exaggerated reaction (uncontrolled reactivity, chronic inflammation).

The *in vitro* model of human monocyte activation that has been set up for detecting nanoparticle effects had to be carefully standardized in order to avoid false negative and false positive results. False negative results could be due to the lack of nanoparticle-cell interaction; thus in this system a high number of monocytes (4×10^6) has been seeded on a large culture surface (9.6 cm^2), allowed to adhere and form a dense cell monolayer, and covered with a thin film of culture medium containing nanoparticles. This was chosen to provide a sufficient volume of fluid and nutrients to allow cell survival for 24 h without drying out, but thin enough to favour the maximal interaction between cells at the bottom and nanoparticles in suspension. False positive results could be due to unwanted stimulation of monocytes, which are very reactive cells, by chemicals or contaminants present in the nanoparticle preparations. This has been evaluated by examining the effect of solvents on monocytes in which the nanoparticles were suspended, and by measuring the endotoxin (LPS) contamination of the various batches of nanoparticle suspensions. Indeed, despite the particular care taken during the synthesis, several nanoparticle and solvent batches contained LPS contamination high enough for triggering a significant monocyte reaction (which is already substantial with LPS concentrations as low as 10 pg/ml; data not shown). Thus, the extreme sensitivity of the human monocyte assay could make it difficult to reach the strict requirements needed for accurate testing of nanomaterial-induced effects. Nevertheless the assay is both relevant (targeting the human cells firstly involved in reaction to foreign materials) and sensitive (cells can react to very minute amounts of foreign molecules). The donor-to-donor variability, typical of experiments on human primary cells, is not a major issue here, as relative differences are usually maintained (e.g., early increase of IL-18 expression in LPS-stimulated cells vs. untreated monocytes) even if absolute values may differ.

Results of nanoparticle-induced genotoxicity as determined by the CBMN assay

The genotoxic effects of gold and iron oxide nanoparticles were determined for two different healthy blood donors. The indices of genotoxicity and cytotoxicity, defined as the frequency of binucleated micronucleated leukocytes (BNMN ‰) and the CBPI respectively, were assessed for both donors (Figure 7). The results showed that for the gold nanoparticles no significant concentration-dependent effects for both endpoints could be observed for the two donors tested. In contrast, the results obtained with the iron oxide nanoparticles showed that, even though these particles did not induce any cytotoxicity (panel B), some effects were observed with respect to

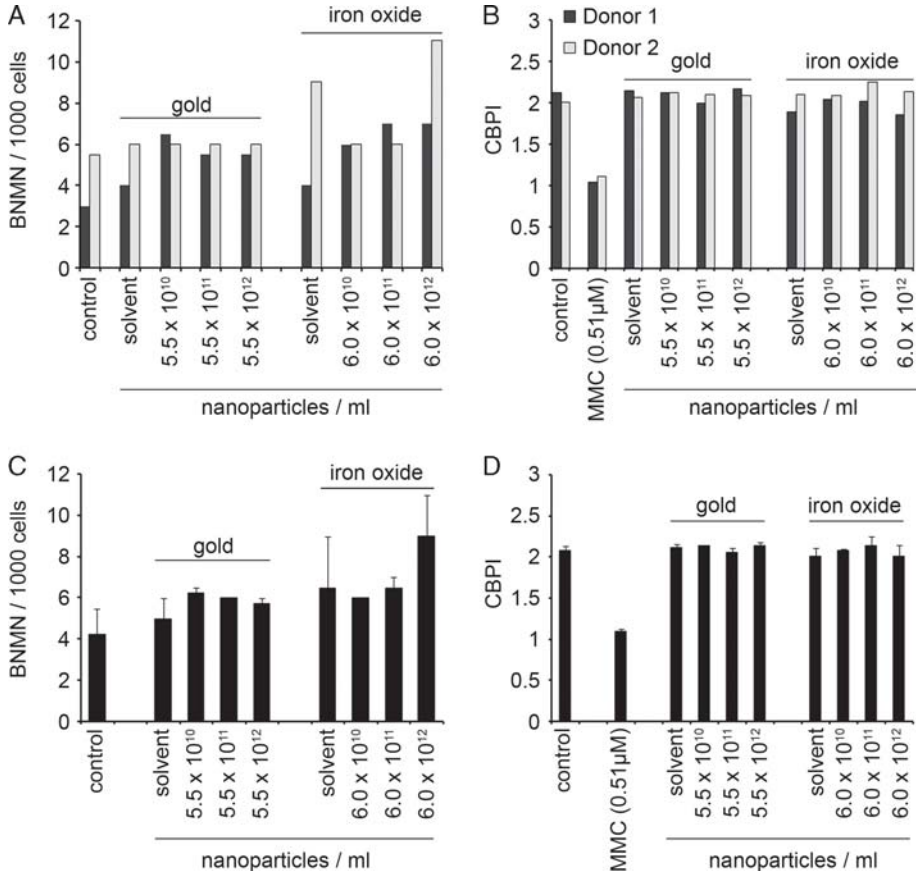

Figure 7. Genotoxic effects of gold and iron oxide nanoparticles on human peripheral leukocytes. Cells of two healthy donors were treated for 48 hours with the nanoparticles in the presence of phytohaemagglutinin. Panel (A) shows the genotoxicity effect evaluated by analyzing the frequency of micronucleated binucleated cell (BNMN ‰) for both nanoparticles. Panel (B) shows the effects of the same treatment with nanoparticles on the cytotoxicity index (CBPI). Results of the two donors were averaged and expressed as mean ± SD (C and D). Addition of 0.51 µM mitomycin C (MMC) resulted in a BNMN frequency of > 100 for each donor tested.

the genotoxicity. These results were not consistent between the two donors tested, implying either donor-specific effects or assay variability for this specific kind of particles. The cells from donor 1 showed a slight increase in genotoxicity when incubated with iron oxide nanoparticles compared to the control, but this effect was not nanoparticle concentration-dependent. The cells from donor 2 showed an increase with the solvent, as well as with the highest particles concentration, whereas no effect was observed for the other two particle concentrations tested. The mean values of the two donors with the standard deviations showed that, when the data are taken together, there is no significant difference between treated and control cells (Figure 7, panel C and D). The slight increase observed for the highest concentration of iron oxide particles was in this case not significant compared to the solvent-treated cells. In summary, no effects of these two nanoparticle suspensions on the cytotoxicity could be observed and only small effects on the genotoxicity were observed when using iron oxide nanoparticles, but not when using gold particles. These effects did not reach statistical significance.

Suitability of the CBMN assay for the analysis of nanoparticle-induced genotoxicity

Some examples of genotoxic effects of different nanomaterials are already available (Colognato et al. 2007a, 2008; Lindberg et al. 2009), but these data refer to materials with an elevated poly-dispersive character (ions leaching, aggregation and agglomeration effects, changes of surface charge in media). In those studies, real nanoparticle-induced genotoxicity is difficult to verify and confounding factors are likely to play a pivotal role. The current study has the main advantage that the nanoparticle suspensions used are well characterized and the particles have a narrow size distribution. The reproducibility and specificity was very low in previous studies, whereas the evaluation of

genotoxic effects under the experimental procedures used in this study showed that the results obtained present a good reproducibility, at least when observing the trends obtained from the two donors analyzed. The data presented did not show any significant dose-dependent increase of the genotoxicity endpoint considered. Nevertheless, it should be kept in mind that the experiments were based on only two donors, that the treatment performed was an acute procedure, and that the concentrations tested were relatively low. The assay is labour-intensive, which makes it less suitable as a general screening method for the analysis of nanoparticle-induced genotoxic effects. However, the results are exact and small alterations caused by genotoxicity can be readily observed. This study also emphasizes one of the problems encountered when primary cells are used – the donor-to-donor variation – which already becomes obvious when analyzing the controls (untreated cells) from the donors in the current study. Such a variation does represent the expected deviation in responses in the population, but causes problems with respect to standardization of assays and results.

Discussion

The methods and data presented in this manuscript have been a result of an interdisciplinary study in which one of the main aims was to test already established methods for their suitability in nanotoxicology. Overall, the cytotoxic, immunotoxic and genotoxic results from this study indicated that the nanoparticle-induced effects were small or absent. First of all, these results have to be placed in the light of the particles used. For this study, small (below 10 nm), inorganic nanoparticles were applied to the different *in vitro* cell culture-based systems at concentrations that ensured the particles to be dispersed in solution. The particles were produced by wet synthesis and carefully characterized after synthesis, but also when diluted in cell culture medium and after incubation in medium for prolonged periods of time. The particles were stable during the experiments and no aggregates or agglomerates were formed in the presence of serum-containing cell culture medium as was previously shown (Pfaller et al. 2009). However, the occurrence of aggregation or agglomeration on the cell membrane or inside the cell cannot be excluded.

The use of particles with a narrow size distribution smaller than 10 nm also directly implied that the final particle mass was much lower compared to other studies where a similar type of particles was used (Hussain et al. 2005; Lewinski et al. 2008). The reasoning behind the use of synthesized nanoparticles with narrow size distributions was that this is an appropriate approach to determine real nanoparticle-induced toxic effects and not the toxicity of nano-derived materials that are no longer in the nanometre scale, or mixtures of both types of particles. The application of undefined aggregates and agglomerates induces extra uncertainty factors and introduces an additional complication to interpreting the obtained data. However, in the search for a positive nanoparticle control, many different commercially available nanopowders were tested for their cytotoxicity, immunotoxicity and genotoxicity during this study. Unfortunately, effects were only observed at high particle concentrations in which case the particles were all visibly aggregated within minutes to hours depending on the dispersion method used (e.g., sonication). The particles tested, their state after suspension, and the methods used are depicted in Table I of the Appendix (online version only).

Nanoparticles are known to be optically active due to their high absorption and scattering effects. Therefore, the presence of the nanoparticles may influence the readings by over- or under-estimation in biological assays based on optical measurements. This was firstly reported for carbon nanotubes (Wörle-Knirsch et al. 2006), but this principle can theoretically be applied to all non-transparent materials. Moreover, the optical response can also depend on the degree of aggregation of the nanoparticles, the scattering of 10 nm gold nanoparticles might be negligible, but aggregated gold nanoparticles on a substrate or on the cells might not be negligible (Jain et al. 2007). Precipitation on a substrate or on cells could produce a red colour when analyzed by confocal microscopy (Pujals et al. 2009). The gold and iron oxide nanoparticles used in this study were selected because of their broad use in nanomedicine and industrial applications, respectively. They did not interfere with any of the absorbance-based methods tested at the concentrations used. When using fluorescent spectroscopy, spectral interference of the metallic nanoparticles was observed. These effects may result from known interactions of the excited-state fluorophores with free electrons in the metals. The use of metallic nanostructures on planar surfaces or as colloidal suspensions to favourably modify the spectral properties of fluorophores has been widely used in medical diagnostics and biotechnology (Aslan et al. 2005). Removing the nanoparticles from the solution before performing optical or fluorescence-based tests might therefore be the appropriate procedure for biological assays, but this might not always be possible.

One of the problems that we faced, and which became very apparent when testing immunomodulatory effects using monocytes, was bacterial contamination of the nanoparticle suspensions. Potential problems associated with lipopolysaccharide (LPS) as one factor of contamination of nanoparticles has been highlighted in very few previously performed studies (Vallhov et al. 2006; Hall et al. 2007; Schulze et al. 2008). We tested the levels of LPS in the samples used in this study and even though these levels were low, because great care was taken to avoid contamination, they were still detectable and did affect the assay using monocytes. This batch-to-batch variability demands that analysis of contamination is performed in every single sample that is used for cell-based *in vitro* assays or *in vivo* methods, and data should be presented in the light of this analysis. It should be emphasized that sterilization of nanoparticles is not sufficient to prevent problems associated with contamination with LPS and other bacterial compounds, since these compounds are not destroyed by sterilization procedures and continue to be immunostimulatory even in the absence of living bacteria.

The suitability of the tested assays can be evaluated in two different aspects. First of all, the analysis of potential toxic effects of nanoparticles in general asks for rapid and reliable screening methods. For this purpose, nanoparticle-induced cytotoxicity testing, combined with a screening method, such as the reporter gene method or ROS analysis using one or more defined cell lines, might be the appropriate choice. Other methods, which make use of primary cells or which are time- and material-consuming, might be less appropriate for this purpose. Methods using primary cells are more difficult to standardize and are affected by donor-to-donor variability, as was observed in our study for the CBMN assay used to detect genotoxicity of nanoparticles. The same can also be inferred from the relatively large standard deviations of the data obtained with $CD34^+$ progenitor-derived dendritic cells. Secondly, methods that make use of primary cells or that determine the effects of specific pathways within a certain cell system will provide relevant and important information about the mechanism(s) behind nanotoxic effects that most likely will not be unravelled when using cell line-based screening methods. Moreover, we have now shown in several studies that primary cells do respond with higher sensitivity compared to their immortalized counterparts, and the responses of such cells could therefore be more relevant to the responses found *in vivo* (Pfaller et al. 2009).

Providing standardized protocols for particle treatment in biological assays might be too restrictive at present, since this treatment largely depends on the scientific question asked (Dawson et al. 2009). Therefore, clear description of the source, treatment and dispersion protocols used for the preparation of nanoparticles is needed to compare independently performed experiments. This could help reconciling some of the differences in the biological responses that can be found in the literature. The same holds true for the *in vitro* toxicological tests, exact protocols with respect to the source of cells, the cell number, cell culture medium, and incubation times are all factors of extreme importance for validation of results and to enable comparison between studies. *In vivo* studies also need a careful description, for which animal species, strains, age, sex, methods of applying nanoparticles and vehicles used, will be of main importance. Notably, for both *in vitro* and *in vivo* toxicology methods, there is a need for the inclusion of the appropriate controls to test possible effects of solvents, adherence of the analyzed proteins to particles, effects caused by ions released by the particles, potential contamination of samples, optical interference of the particles with the method used, and many other factors. When all these factors are taken into consideration, a panel of methods could be introduced that allow the testing of nanotoxic effects at different levels and provide us with a coherent picture of potential nanotoxic effects.

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