Innate defence functions of macrophages can be biased by nano-sized ceramic and metallic particles

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ABSTRACT. Nano-sized particles of ceramic and metallic materials are generated by high-tech industrial activities, and can be generated from worn-out replacement and prosthetic implants. The interaction with the human body of such nanoparticles has been investigated, with a particular emphasis on innate defence mechanisms. Human macrophages (PMA-differentiated myelomonocytic U-937 cells) were exposed *in vitro* to non-toxic concentrations of TiO_2, SiO_2, ZrO_2, or Co nanoparticles, and their inflammatory response (expression of TLR receptors and co-receptors, and cytokine production) was examined. Expression of TLR receptors was generally unaffected by exposure to the different nanoparticles, except for some notable cases. Exposure to nanoparticles of ZrO_2 (and to a lesser extent TiO_2), upregulated expression of viral TLR receptors TLR3 and TLR7. Expression of TLR10 was also increased by TiO_2 and ZrO_2 nanoparticles. On the other hand, TLR9 expression was decreased by SiO_2 nano-particles, and expression of the co-receptor CD14 was inhibited by Co nanoparticles. Basal and LPS-induced production of cytokines IL-1β, TNF-α, and IL-1Ra was examined in macrophages exposed to nanoparticles. SiO_2 nanoparticles strongly biased naïve macrophages towards inflammation (M1 polarisation), by selectively inducing production of inflammatory cytokines IL-1β and TNF-α. SiO_2 nanoparticles also significantly amplified the inflammatory phenotype of LPS-polarised M1 macrophages. Other ceramic nanoparticles had little influence on cytokine production, either in resting macrophages, or in LPS-activated cells. Generally, Co nanoparticles had an overall pro-inflammatory effect on naïve macrophages, by reducing anti-inflammatory IL-1Ra and inducing inflammatory TNF-α. However, Co nanoparticles reduced production of IL-1β and IL-1Ra, but not TNF-α, in LPS-polarised M1 macrophages. Thus, exposure to different nanoparticles can modulate, in different ways, the defence/inflammatory capacities of macrophages. A thorough analysis of these biasing effects may shed light on the mechanisms of pathogensis of several diseases based on dysregulation of the immune response (allergies, autoimmunity, tumours).

Keywords: nanoparticles, inflammation, macrophages, TLR receptors, cytokines, innate immunity

INTRODUCTION

The fast-growing development of nanotechnologies in many scientific, technological, and industrial application areas is raising new questions about possible risks to human health and environmental safety. Nano-sized particles can also be released from medical implants (such as bone and tooth replacements) upon prolonged mechanical stress. The reduced size of nanoparticles allows their entrance to and diffusion within the body, and their interaction with different types of cells. The diffusion capacity of nanoparticles is being extensively studied for drug delivery exploitation [1-3], but on the other hand the possibility of interference with cellular functions remains relatively unexplored.

The body is primarily protected from foreign invasion by the innate immune system, which is geared towards discriminating between self and alien/abnormal molecular patterns and mounting the first set of inflammatory and defence responses. Macrophages are among the first innate immune cells recruited to a site of invasion, and to come into contact with the foreign agents. Macrophages are versatile, plastic cells, which respond to environmental signals with diverse functional programmes. Classical macrophage activation in response to microbial products (e.g., bacterial lipopolysaccharides, LPS) and interferon-γ (IFN-γ) gives rise to potent effector cells (M1), which kill microorganisms and tumour cells and produce inflammatory cytokines and chemokines (including IL-1 and TNF-α). On the other hand, alternative macrophage activation (e.g., with IL-4) induces M2 macrophages, which tune inflammatory responses and adaptive Th1 immunity, scavenge debris, and promote angiogenesis, tissue remodelling and repair [4-6]. The microenvironment thus influences macrophage activation and their subsequent functions.

Mechanical damage due to particulate agents of different chemical composition usually leads to necrotic cell damage and to a potent innate and inflammatory reaction with activation of M1 macrophages. This will eventually lead to elimination of the foreign agent, development of specific immune response, and tissue repair by M2 macrophages. In other cases, as in silicosis, silica particles induce fibrosis and macrophage apoptosis, leading to chronic pathology as particles cannot be removed by scavenging cells [7].

A very important aspect in the initiation of macrophage activation is the presence of receptors of the TLR/IL-1R family. TLR/IL-1R are an important class of receptors involved in the initiation of the inflammatory response and of innate immunity reactions, characterised by a common signalling pathway. TLR/IL-1R include the receptor chains for the inflammatory/immune cytokines IL-1 [8] and IL-18 [9], and the TLR subfamily. TLR (Toll-like receptors) are germ-line encoded receptors that can be activated by different microbial components (LPS, lipopeptide, dsRNA, GU-rich ssRNA, flagellin, CpG-rich DNA, etc.) and endogenous stress-related proteins (heat shock proteins, fibrinogen), thereby initiating inflammatory and innate immune responses [10, 11]. TLR are mainly expressed by myeloid cells (macrophages), although their presence has been described in several other cells and organs [10, 11].

In this study, the interaction of macrophages with nanoparticles was investigated. Nanoparticles of ceramic (silica, SiO_2; titania, TiO_2; zirconia, ZrO_2), and metallic materials (cobalt, Co) have been assessed for their ability to modulate the vital and functional parameters of human macrophages (the U-937 myelomonocytic cell line differentiated in culture with PMA). After evaluating the possible cytotoxic effect of nanoparticles, their capacity to affect macrophage defence functions was examined in terms of modulation of expression of TLR, and in functional assays of M1 macrophage polarisation (production of the inflammatory cytokines IL-1β and TNF-α) *versus* M2 polarisation (production of the anti-inflammatory mediator IL-1Ra).

METHODS

Nanoparticles

Ceramic, metallic, and polymeric nanoparticles were examined for experimental use. Ceramic materials were: silica (silicon oxide, SiO_2), titania (titanium oxide, TiO_2, widely used use as a coating in dental and orthopedic implants), zirconia (zirconium oxide, ZrO_2). Of the metals, cobalt and nickel were considered, as both are known to be allergenic. Nanoparticles of nickel did not resuspend homogeneously in aqueous medium, and formed micro-sized particulate agglomerates, and were therefore discarded. Polymeric nanoparticles of polyvinylchloride (PVC) were not assayed for technical reasons (floating particles did not interact with adherent cells in culture).

Nanoparticles of SiO_2, TiO_2, and ZrO_2 were prepared by TAL Materials, Inc. (Ann Arbor, MI, USA) by flame spray pyrolysis, i.e. by combusting metallo-organic alcohol solutions with oxygen at 1200-2000 °C, followed by rapid quenching, to produce unagglomerated, single particle nanopowders. The average nanoparticle size is 15 nm for SiO_2 (range 4-40 nm), and 70 nm for TiO_2 (range 20-160 nm). The size of ZrO_2 nanoparticles ranged between 5-30 nm. Cobalt nanoparticles (Cobalt 100 g - FLUKA cod. 60784 – Lot & filling code: 334014/1 21901) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and had a size varying between 50 and 200 nm. Chemical characterisation of Co nanoparticles revealed the presence of iron, in addition to cobalt and oxygen. Before use in culture, powders were further depyrogenated at 180 °C for 4 h. Working batches of nanoparticles were prepared in sterile PBS and stored in depyrogenated glass vials. All nanoparticle suspensions were vigorously shaken (vortexed for 30 sec at max speed) before each transfer for dilution or dispensing. Routine checking by microscopy was peformed on nanoparticle suspensions to assess the homogeneity of nanoparticle distribution in dilution procedures.

Cells

The human myelomonocytic cell line U-937 (histiocytic lymphoma; ATCC n. CRL-1593.2, obtained from the Bank of Cell Lines of the National Institute for Cancer Research, Genova, Italy) was used. Cells were grown to subconfluency in RPMI-1640 medium supplemented with 50 μg/mL gentamycin sulfate, 2 mM L-glutamine, 25 mM HEPES buffer, and 2 g/L $NaHCO_3$ (all from Euroclone Life Sciences Division, Pero, Italy) (hereafter referred to as culture medium), with the addition of 10% FBS (Euroclone). To induce macrophage differentiation, subconfluent cultures were exposed to 10 nM PMA (Sigma Chemical Co.) for 72 h at 37 °C. Exposure to PMA induced strong adherence, cytoskeletal rearrangements and the appearance of macrophage-like morphology. All media and supplements were endotoxin-free. Cultures were routinely tested for mycoplasma contamination (Mycoplasma detection kit, Roche Diagnostics, Milano, Italy).

Cell proliferation and survival

For vitality and proliferation experiments, cells were plated at $5x10^4$ cells/well of Cluster^{96} plates (Costar, Cambridge, MA, USA) in 100 μL culture medium + 1% AB serum (Sigma Chemical Co.), and incubated for 24 h. Increasing doses of nanoparticles were then added in 100 μL of fresh medium for increasing time periods. Proliferation (number of metabolically active cells) was evaluated as formazan dye formation at 492 nm, with the XTT assay (Roche Diagnostics). To estimate the number of necrotic cells, 4 μg/mL trypan blue dye (Merck, Darmstadt, Germany) were added to each well. The percentage of dye-uptaking cells (dead cells with permeable membrane, i.e., necrotic cells) was calculated by counting at least 100 cells/well under an inverted microscope. All counts were performed within three min from addition of the dye. Based on the results of cell proliferation/survival assays, the maximal non-toxic concentrations of nanoparticles were selected for subsequent experiments (50 μg/10^6 cells/24 h for Co, 400 μg/10^6 cells/24 h for other nanoparticles) *(figure 1)*.

Semi-quantitative RT-PCR for TLR expression

Cells were cultured for 24 h in medium + 1% AB serum, in the presence or absence of nanoparticles (Co nanopar-

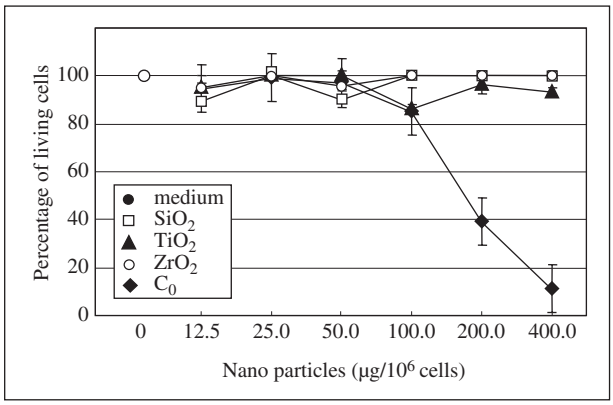

Figure 1
Toxicity of different nanoparticles on human macrophages (PMA-differentiated U-937 cells) in culture. Data reported were obtained with the XTT assay, which measures metabolically active cells, after 24 h of exposure to nanoparticles. Qualitatively overlapping data were obtained at different time points (12 h, 48 h, 72 h, 96 h), using the trypan blue dye exclusion assay.

ticles: 50 μg/10^6 cells; SiO_2, TiO_2, and ZrO_2 nanoparticles: 400 μg/10^6 cells). Messenger RNA (mRNA) was extracted from cells using MicroPoly(A)Pure™ (Ambion, Inc., Austin, TX, USA), resuspended in 30 μL RNase-free water, and quantified by measuring absorbance at 260 nm. Aliquots of 2 μg mRNA were reverse-transcribed using Ready-To-Go™ You-Prime First-Strand Beads (Amersham, Little Chalfont, UK) in a final volume of 33 μL. Semi-quantitative RT-PCR was performed on cDNA aliquots of 3.3 μL by using a Multiplex PCR Kit (Qiagen GmbH, Hilden, Germany), and specific primer pairs for TLR (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10) and co-receptor molecules (CD14, MD-2). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. The list of primers is reported in *table 1*. After an initial denaturation step, amplification was carried out for 20, 23, 26, 29, 32, 35, 38, 41, 44, 47 cycles under the following conditions: 94 °C for 30 sec, 57 °C for 90 sec, 72 °C for 30 sec. Densitometric values of amplification products of the genes under investigation were calculated by image analysis with the programme MIP (Medical Image Processing) (developed by Marco Paterni, Institute of Clinical Physiology, CNR). Values were plotted and compared with those obtained for GAPDH, corrected for the amplification efficiency, and expressed in arbitrary units, taking the GADPH value as 100. For analysis of TLR10, whose amplification product migrated very close to that of the housekeeping gene, a different pair of primers was designed for GAPDH in order to generate a shorter amplification product (see GAPDH (2) in *table 1*). Expression of GAPDH was identical to both pairs of primers.

Cytokine production by macrophages

PMA-differentiated cells ($2x10^5$ cells/well of Cluster^{24} plates; Costar) were exposed to nanoparticles (50 μg/10^6 cells for Co nanoparticles; 400 μg/10^6 cells for SiO_2, TiO_2, and ZrO_2 nanoparticles) in 1.0 mL medium + 1% AB serum for 24 h in the presence or absence of 100 ng/mL lipopolysaccharide (LPS, from E. coli 055:B5; Sigma Chemical Co.). The presence of the cytokines IL-1β, TNF-α, and IL-1Ra was evaluated in culture supernatants by specific ELISA (R&D Systems, Minneapolis, MN, USA). Results were expressed as mean ± SD of two to three separate determinations. Statistical significance was assessed by Student's t test.

RESULTS

Exposure of human macrophages (PMA-differentiated U-937 cells) to increasing doses of nanoparticles for different times did not cause cell death or inhibition of cell proliferation, with the exception of Co nano-particles. Two different assays were used to assess cell vitality/proliferation. The XTT assay measures the number of metabolically active cells (thus reflecting cell survival and proliferation). The trypan blue dye exclusion assay determines the presence of necrotic cells. Ceramic nanoparticles had no toxic effect at doses as high as 400 μg /10^6 cells for up to 96 hours *(figure 1)*, whereas Co nanoparticles showed significant toxicity at doses exceeding 100 μg/10^6 cells/24 h. Results obtained with the two assays were qualitatively and quantitatively very similar, thus only the results of the XTT assay are shown. For each type

Table 1
Primers for semi-quantitative RT-PCR analysis of TLR expression

Gene	Forward	Reverse	Fragment length	Accession N.
TLR1	CAGTGTCTGGTACACGCATGGT	TTTCAAAAACCGTGTCTGTTAAGAGA	104 bp	U88540
TLR2	TCACCTACATTAGCAACAGTG	GTAGATCTGAAGCATCAATCTC	365 bp	U88878
TLR3	GGATCCAAAAACTCTGTACATTA	TCCTAGAAGAGATGTAATTGTG	169 bp	NM_003265
TLR4	CCTAAGGAAACCTGATTAACAC	GATATTAGCTTATAGGCAAGAC	148 bp	U88880
TLR5	GTTCAAGGACCATCCCCAGGGCAC	TCCTGGAGATCCTCAGGCCACCTC	437 bp	AB060695
TLR6	AGAACTCACCAGAGGTCCAACC	GAAGGCATATCCTTCGTCATGAG	482 bp	NM_006068
TLR7	GATAACAATGTCACAGCCGTCC	GTTCCTGGAGTTTGTTGATGTTC	321 bp	AF240467
TLR8	GTGCCACCCAAACTGCCAAGCTCC	GATCCAGCACCTTCAGATGAGGC	309 bp	AF245703
TLR9	GGACCTCTGGTACTGCTTCCA	AAGCTCGTTGTACACCCAGTCT	153 bp	AF245704
TLR10	TCTGTCATACAATAAATTGTCTG	GGATTTCTTCCCGCATTTA	286 bp	AF296673
CD14	TGGATCTTAGGCAAAGCCCC	TTCCTGGTCCCTGGAACT	143 bp	M86511
MD-2	CAATCATGTTACCATTTCTGTTT	TTCTAATTTGAATTAGGTTGGTG	487 bp	AB018549
GAPDH (1)	GGTGAAGGTCGGAGTCAACGG	GAGGGATCTCGCTCCTGGAAGA	239 bp	NM_002046
GAPDH (2)	AACGTGTCAGTGGTGGACCTG	AGTGGGTGTCGCTGTTGAAGT	160 bp	NM_002046

of nanoparticle, the highest, non-toxic dose assayed was subsequently used for functional experiments. This was 400 μg/10^6 cells/24 h for SiO_2, TiO_2, and ZrO_2, and 50 μg/10^6 cells/24 h for Co.

Expression of TLR has been evaluated in human macrophages exposed to nanoparticles by semi-quantitative RT-PCR. Data in *figure 2* show that in resting conditions (empty columns), human macrophages express all the TLR chains except TLR3. Modulation of TLR gene expression was examined following exposure for 24 h to nanoparticles of SiO_2, TiO_2 , or ZrO_2 (400 μg/10^6 cells), or Co (50 μg/10^6 cells). It was seen that nanoparticles have different effects on the expression of different TLR genes. Of the ceramic materials (grey columns), SiO_2 nanoparticles (light gray) had little effect on TLR expression, except for the decreased expression of TLR9 (receptor for bacterial/viral DNA). SiO_2 nanoparticles also appeared to induce a very low expression of the viral receptor TLR3; however, the biological relevance of this finding needs to be confirmed. Nanoparticles of ZrO_2 (dark gray), showed a selective capacity for inducing/increasing expression of viral receptors TLR3 and TLR7, as well as that of TLR10, but did not significantly affect expression of TLR8 (another putative viral receptor). Likewise, TiO_2 nanoparticles (medium gray) increased expression of TLR7 and TLR10, but not TLR3, and they also significantly enhanced expression of the TLR4 co-receptor MD-2. Co nanoparticles (black columns) showed no significant effect on the expression of most TLR, however they significantly decreased expression of the TLR4 co-receptor CD14.

The production of the inflammatory and defence mediators IL-1β and TNF-α, and of the anti-inflammatory cytokine IL-1Ra, was evaluated in human macrophages cultured for 24 h in the presence or absence of nanoparticles. As shown in *table 2*, the low level of basal IL-1β production was not affected by a 24 h treatment with TiO_2 or Co nanoparticles, whereas it was significantly enhanced by SiO_2 nanoparticles (130-fold increase). Nanoparticles of ZrO_2 had a very limited stimulating effect on IL-1β production (two-fold increase). By examining production of TNF-α, both SiO_2 and Co nanoparticles were seen to induce significant production of the inflammatory mediator (20-30-fold increase), whereas the other nanoparticles were inactive. On the other hand, the spontaneous production of the anti-inflammatory mediator IL-1Ra was enhanced only three-four fold by ceramic nanoparticles, while significantly inhibited by nanoparticles of Co (>70%).

The ability of nanoparticles to modulate cytokine production in LPS-activated (M1 polarised) macrophages was then assessed. When exposed to an optimal concentration of LPS (100 ng/mL), macrophages produce enhanced amounts of pro-inflammatory cytokines (9-30-fold) and a smaller increase of anti-inflammatory IL-1Ra (3.5-fold) *(table 2)*. LPS-activated macrophages, in the presence of SiO_2 nanoparticles, produced amounts of IL-1β and TNF-α that were significantly higher than the sum of those

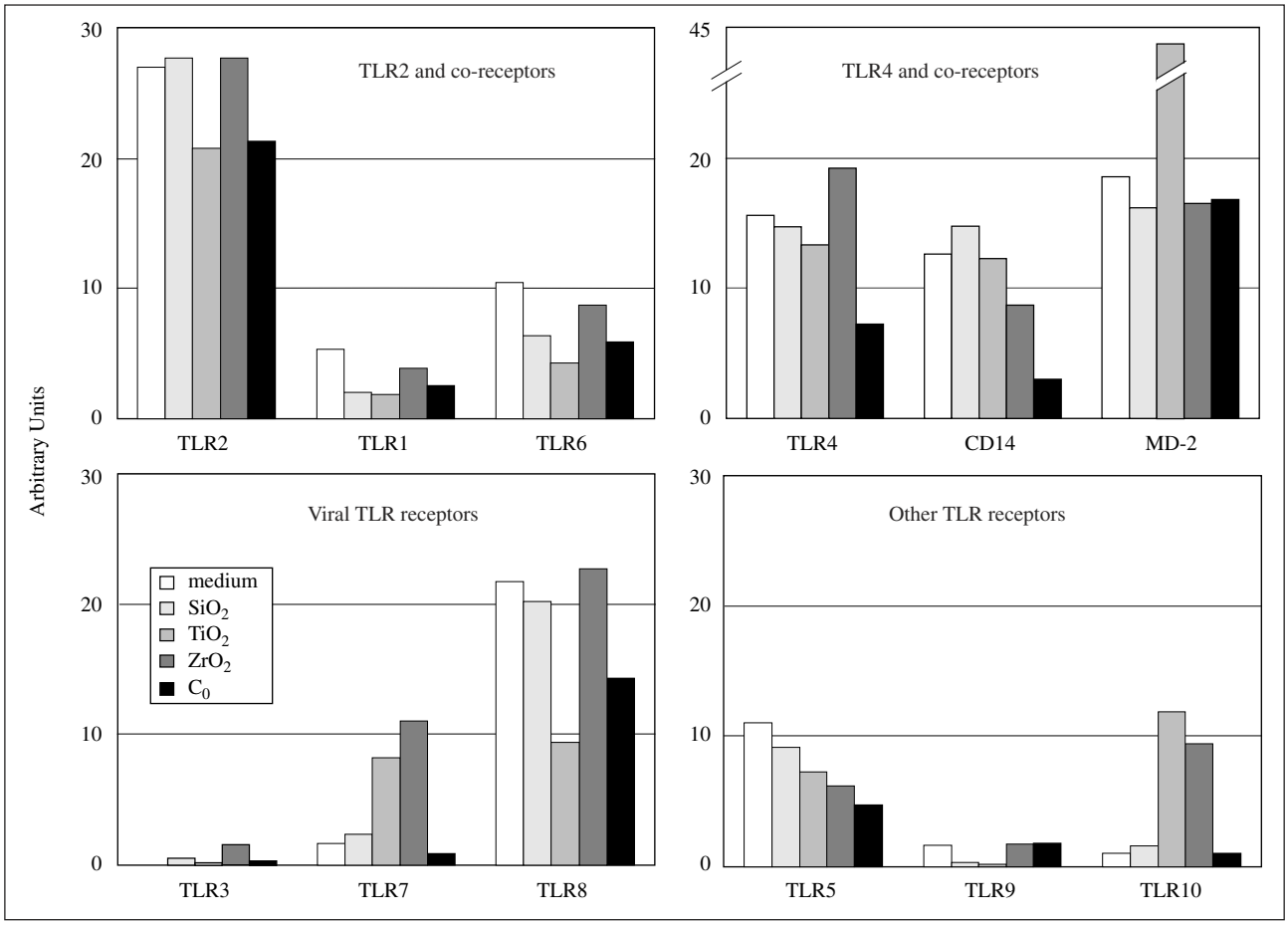

Figure 2

Expression of TLR in human macrophages exposed to nanoparticles in culture. Cells were incubated with nanoparticles for 24 h before mRNA extraction and semi-quantitative RT-PCR analysis. The housekeeping gene GAPDH was used as reference. Results are representative of at least two independent experiments.

Table 2
Production of cytokines by human macrophages

Cytokine	*Treatment of macrophages*	*Cytokine production (pg/10^6 cells/24 h; mean ± SD) by macrophages exposed to nanoparticles*				
		none	*SiO_2*	*TiO_2*	*ZrO_2*	*Co*
IL-1β	medium	7.0 ± 2.8	896.1 ± 38.0*	9.1 ± 0.5	17.1 ± 3.7*	12.6 ± 6.5
	LPS	60.8 ± 11.6*	2,253.7 ± 79.1***	45.8 ± 4.6*	214.7 ± 21.3***	21.6 ± 5.9**
TNF-α	medium	0.9 ± 0.1	20.5 ± 0.9*	1.5 ± 1.1	1.9 ± 0.8	32.0 ± 3.1*
	LPS	27.1 ± 2.4*	190.3 ± 0.1***	19.8 ± 0.4*	26.0 ± 0.1*	56.9 ± 1.1***
IL-1Ra	medium	3,850.8 ± 359.7	14,093.5 ± 687.0*	12,192.9 ± 29.4*	11,388.8 ± 1,242.8*	1,052.6 ± 385.9*
	LPS	13,282.3 ± 1,030.8*	13,011.8 ± 555.8	6,827.5 ± 307.0***	7,894.9 ± 914.0*	754.3 ± 5.8**

* significant difference ($p<0.05$) from respective medium control. ** significant difference ($p<0.05$) from LPS alone. *** significant difference ($p<0.05$) from LPS alone and nanoparticles alone.

induced by exposure to either stimulus alone, indicating synergistic induction. On the other hand, no variation in the production of IL-1Ra was observed in M1 macrophages upon exposure to SiO_2 nanoparticles. TiO_2 and ZrO_2 nanoparticles had a partial, pro-inflammatory effect on M1-polarised macrophages, as both slightly decreased production of IL-1Ra, and ZrO_2 nanoparticles also increased LPS-induced IL-1β. Finally, metallic Co nanoparticles had an additive effect on TNF-α production by LPS-activated macrophages, while they inhibited induction of IL-1β and IL-1Ra.

DISCUSSION

The different types of nanoparticles represent a new species of non-biodegradable environmental pollutants that are generated in substantial amounts by modern nanotechnological activities. Nano-sized particles can be released from everyday items (e.g., TiO_2 is used as whitener for paper, paint, food products, toothpaste), can be found as environmental contaminants of industrial locations and factories, and can be released into the body by worn prosthetic implants (e.g., dental bridges, bone replacement implants). In war zones, a variety of nanoparticles can be generated by blasting of high technology weapons and these can contaminate agricultural and zootechnical products, soil and water. In some of these situations (e.g. war zones, industrial settings), the extent of nanoparticulate contamination can be very high, although the lack of reliable data on the type and level of nanoparticulate contamination in human environments does not allow a precise assessment of exposure risks. Whereas the inflammatory effect of micro-sized particles is well-documented (readily activating the scavenging activity of macrophages and eliciting potent inflammatory responses) and dependent on their size (which limits their diffusion), the possible effects of nanoparticles on cell function is largely unknown. The reduced size of nanoparticles is expected to result in failure to trigger a normal inflammatory response, leading to the hypothesis that these may be "inactive" as far as physiological function of cells are concerned. However, they are, in fact, potentially far more active than larger particles, due to the very large surface area (up to 1000 square meters/gram), which enormously increase their potential reactivity upon cell contact. There is evidence that nanoparticles can escape classical scavenging defence mechanisms and, after adhering to the cell surface, can be internalised through a non-phagocytic mechanism, and have been detected in the cell cytoplasm and in the nucleus [1-3, 12].

In order to evaluate the possible influence of nanoparticles on host ability to cope with external insults, their effect on the function of professional, innate defence cells has been examined, using as the cellular model, the human histiocytic lymphoma line U-937, differentiated to macrophages with PMA. Macrophages (professional scavengers and inflammatory cells) are among the first immune cells to come into contact with foreign particles and microorganisms, to initiate the innate defence response. Macrophages recognise foreign particles through TLR, a family of evolutionarily conserved pattern recognition receptors that can sense and specifically recognise pathogenic microorganisms and endogenous stress factors, thereby initiating immune responses [13-16]. In humans, ten different TLR proteins have been identified so far, of which nine (TLR1-9) have been characterised functionally [17-19]. Inflammatory stimuli, such as bacterial components (e.g., LPS which triggers TLR4) or inflammatory cytokines (IFN-γ), can bias macrophages to become potent inflammatory effector cells (M1-polarised macrophages), able to produce inflammatory mediators and sustain the inflammatory and immune reaction eventually leading to elimination of the foreign body.

The possibility that non-toxic doses of ceramic (TiO_2 SiO_2, and ZrO_2) and metallic nanoparticles (cobalt) could modulate the innate defensive function of macrophages was examined. This was evaluated by two parameters: the expression of innate immunity receptors (TLR1-10 and the TLR4 co-receptors CD14 and MD-2), and M1 polarisation (evaluated as the production of inflammatory cytokines IL-1β and TNF-α, in comparison with the anti-inflammatory cytokine IL-1Ra). In these assays, nanoparticles were used at the highest, non-toxic concentrations measured, in order to simulate exposure in heavily contaminated environments. However, since no concrete data are available on nanoparticle contamination in different environments, or on the possible effects of accumulation, the dose selected for the *in vitro* experiments are to be considered only as an indication.

Results obtained show that nano-particles have significant effects on the expression of some TLR molecules, suggesting that they could affect cell reactivity to infections by altering the expression of innate receptors. Particularly interesting is the finding that nanoparticles (both ceramic and metallic) can enhance expression of TLR chains important for viral-dependent stimulation. In fact, in normal conditions macrophages do not express TLR3, the receptor

activated by dsRNA [17], but this can be significantly induced upon exposure to ZrO_2 nanoparticles. Another receptor important in virus recognition is TLR7, which binds viral ssRNA and triggers IFN-α/β production through an IRF-3-dependent signalling pathway [19, 20]. Expression of TLR7 is potently enhanced by TiO_2 and ZrO_2. A similar enhancement by TiO_2 an ZrO_2 nanoparticles was also observed for expression of TLR10, a receptor of unknown function expressed at low levels in resting macrophages. Thus, it could be inferred that macrophages exposed to nanoparticles of different chemical natures (in particular zirconia) are hyperreactive to viral infections, which may therefore cause excessive inflammation and pathological tissue damage. On the other hand, a decrease in the expression of TLR9, the receptor that recognises CpG motifs within bacterial and viral DNA, was observed in macrophages exposed to silica and titania nanoparticles. Since CpG ligands of TLR9 are being developed as novel effective vaccine adjuvants, because of their ability to stimulate innate immunity [21, 22], it will be important to know that vaccine efficacy might be hampered by nanoparticulate SiO_2 and TiO_2 contamination in the organism.

No significant variation was noticed in macrophages exposed to nanoparticles as regards expression of TLR2 and its co-receptors TLR1 and TLR6, of TLR5, TLR8, and TLR4. However, it is interesting to note that nanoparticles of Co and of TiO_2 could modulate expression of TLR4 co-receptors. In particular, macrophages exposed to Co nanoparticles have decreased expression of CD14 (a feature typical of M2-biased macrophages), which should possibly reduce their overall responsiveness to LPS. On the other hand, TiO_2 nanoparticles significantly increased expression of MD-2, supposedly enhancing MD-2-dependent LPS responsiveness.

The capacity nanoparticles to modify the production of inflammatory defence cytokines by resting or LPS-activated (M1) macrophages was examined. IL-1β is a pivotal cytokine in the initiation of the inflammatory/defence response, responsible of pathological tissue destruction in chronic inflammatory and autoimmune diseases [8]. TNF-α is a multifunctional cytokine with a central role in the effector functions of activated macrophages and other defence cells, and is also greatly involved in pathological inflammatory conditions [23]. Conversely, the receptor antagonist of IL-1, IL-1Ra, is a cytokine which down-regulates inflammation by inhibiting IL-1 activity [24]. All cytokines are produced upon macrophage activation, with a preferential induction of inflammatory (IL-1β, TNF-α) *versus* anti-inflammatory (IL-1Ra) cytokines in M1 *versus* M2 macrophages [4-6].

In resting cells, SiO_2 nanoparticles show a potent capacity to activate macrophages to produce inflammatory cytokines. The effect was particularly evident for IL-1β production, which was increased by over 100-fold in macrophages exposed to silica nanoparticles as compared to untreated cells. Conversely, other types of nanoparticles had little or no direct capacity to induce inflammatory cytokines, apart from Co nanoparticles, which were selectively able to induce TNF-α, but not IL-1β production. By examining production of anti-inflammatory IL-1Ra, it was noted that the basal level of cytokine production was increased three-four-fold by ceramic nanoparticles, while it was inhibited by Co nanoparticles (>70%). These observations suggest that the presence of different types of nanoparticles can bias resting macrophages towards different effector functions by altering the balance between pro-inflammatory and anti-inflammatory mediators. SiO_2 nanoparticles are dramatically pro-inflammatory and act as M1-polarising stimulus, similarly to LPS. In fact, they significantly enhance the production of IL-1β (130-fold) and TNF-α (20-fold), while being much less potent in increasing IL-1Ra production (four-fold). Also, Co nanoparticles promote macrophage inflammatory functions, although with a different profile. In parallel with increasing TNF-α production, Co nanoparticles increase IL-1β activity through a reduction of its antagonist IL-1Ra. Conversely, ceramic nanoparticles of titania and zirconia have an overall anti-inflammatory effect, as they do not significantly affect production of inflammatory mediators, while increasing that of IL-1Ra.

The capacity of nanoparticles to alter the balance between pro-inflammatory cytokines and anti-inflammatory mediators was extended to macrophages already activated for inflammatory functions, with bacterial LPS (M1-polarised effector macrophages). In response to LPS, macrophages produce increased amounts of inflammatory cytokines (in this study, LPS induced a 10-fold increase of IL-1β and a 30-fold increase of TNF-α), as compared to anti-inflammatory mediators (3.5-fold increase of IL-1Ra), thus shifting the pro-inflammatory/anti-inflammatory balance towards inflammation. In these already biased macrophages, all types of nanoparticles studied further shifted the cytokine balance towards inflammation, thus amplifying inflammatory M1-effector functions. In particular, SiO_2 nanoparticles greatly amplified IL-1β and TNF-α production in M1-polarised macrophages, while having no effect on the production of IL-1Ra. The other ceramic nanoparticles also have an inflammation-promoting capacity, although more limited, as ZrO_2 enhanced IL-1β production, and both TiO_2 and ZrO_2 partially decreased IL-1Ra synthesis. On the other hand, macophages exposed to Co nanoparticles showed a partial unresponsiveness to LPS (possibly dependent on down-regulation of the co-receptor CD14), as both LPS-stimulated IL-1β and IL-1Ra production were reduced. On induction of TNF-, the effect of Co nanoparticles was additive to that of LPS suggesting that, at variance with IL-1β and IL-1Ra, induction of TNF-α is partially independent of CD14 at the LPS concentration used [25].

Thus, despite the lack of macroscopic, mechanical inflammatory effects, nanoparticles of metallic or ceramic materials can profoundly modulate the innate/inflammatory response of macrophages. The effects of nanoparticles on gene expression and cellular functions may depend on some parameters typical of the materials. In contrast to ceramic materials, cobalt nanoparticles release ions from the surface, which can interact with ionic components of cells. The surface charge of cobalt nanoparticles can also attract polar molecules, forming stable and/or insoluble compounds, thus altering cellular equilibrium. Silica nanoparticles differ from more inert titania and zirconia nanoparticles as regards solubility. It is known that silica in an aqueous environment turns into a silica hydrated gel [26, 27], which can attract calcium and phosphorus ions and promote their precipitation as insoluble calcium phosphate, which may in turn affect macrophage activation. Titania and the more inert zirconia nanoparticles can elicit significant cellular responses, once they are internalised in

the cytoplasm (A.M. Gatti, unpublished) [12]. Modulation of innate defence receptors in cells exposed to nanoparticles runs the risk of inadequate defences against certain infections (as in the case of TLR9 inhibition by nanoparticles of silica and titania, which may result in impaired resistance to some viruses and bacteria), or the risk of chronic inflammation (as in the case of zirconia nanoparticles, which induce the expression of TLR3 and increase that of TLR7, thus amplifying virus-triggered inflammation). A further risk of inflammatory pathological dysregulation could lie in the ability of nanoparticles, in particular those of SiO_2, to bias naïve macrophages towards the inflammatory M1 functional phenotype, and to amplify effector functions of M1-polarised macrophages. On the other hand, titania and zirconia nanoparticles do not induce an inflammatory functional phenotype in naïve macrophages, and have only a limited effect in enhancing the inflammatory effector functions of M1-polarised macrophages. This is in agreement with previous data showing strong inflammation and apoptosis caused by silica particles *in vivo*, as opposed to the weak and transient effect caused by TiO_2 particles [28, 29].

The notion that nanoparticles of allegedly inert materials can affect cellular functions is of major importance. Indeed, the nanotechnological industry develops and produces nanoparticles of widely different chemical composition, while the risk to human health associated with exposure is unknown. Nanoparticles are also released into the environment by high temperature processes (incineration, cement plants, high technology weapons' blasting, etc.), where they persist and can access the living organisms through inhalation, or ingestion of contaminated food and water. Of particular relevance is the possibility that the functional anomalies of the innate immune system caused by nanoparticles may be among the causes of the dysregulated amplification of the immune response and concomitant chronic inflammation characteristic of autoimmune diseases [30]. On the other hand, down-modulation of inflammatory innate receptors and cytokines (as observed with Co nanoparticles, which partially reverse the M1 polarisation of macrophages by inhibiting CD14 expression) may hamper immune surveillance and allow tumour development [4, 31].

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