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Reaction of human macrophages on protein corona covered TiO_2 nanoparticles

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Abstract

The cytokine secretion of primary cells of human macrophages during the interaction of TiO_2 nanoparticles (with an average primary size of 100-120 nm) is investigated down to concentration levels suggested to be relevant for *in vivo* conditions. We find that high TiO_2 concentrations induce the cytokines Interleukin IL-1β, IL-6, and IL-10 secretion, while at low concentrations only IL-6 secretion is observed. To obtain further evidence on *in vivo* conditions we investigated the development and structure of the protein corona of the nanoparticles. We demonstrated that the surface of TiO_2 particles attract preferably secondary modified proteins which then induce cytokine secretion of macrophages. Our results indicate that concentration of corona covered TiO_2 particles below 1 μg/ml induce IL-6 secretion which is reported to be responsible for the development of autoimmune diseases as well as for the secretion of acute phase proteins.

From the Clinical Editor: This study investigates the effects of protein corona covered titanium dioxide nanoparticles on human macrophages, concluding that concentration of such particles below 1 μg/ml induces IL-6 secretion, which may be responsible for the development of autoimmune diseases as well as for the secretion of acute phase proteins. This finding has important implications on future applications of such nanoparticles.
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Key words: Titanium dioxide; Nanoparticles; Human macrophage; Protein corona; Immunology; Nanotoxicology; Interleukin-6

Titanium dioxide (TiO_2) nanoparticles have been widely used as an functional additive in liquid and solid surface coatings due to their high optical refractive index and photocatalytic activity.[1,2] In nanomedicine, their use as an antimicrobial agent has attracted high attention, and their light scattering and absorption properties are responsible for their cosmetic application in sunscreens.[3-6]

The high surface-to-volume ratio of nanoparticles grants them the ability to bind molecules to their surface when exposed to a biological environment. In bio-systems this biological layer around the nanoparticle is called "protein corona". The protein corona influences the biodistribution and, thus, the potential effects of the nanoparticles in the organism.[7-9]

The studies available so far regarding the contact of TiO_2 nanoparticles with human cells present multiple differing and controversial results. Lautenschlager et al. reviewed that in the dermis of the human skin TiO_2 nanoparticles are not inducing

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any damages.[10] In contrast, the particles are reported to induce cell death in human neural cells and fibroblasts and induce oxidative stress in human umbilical vein endothelial cells (HUVEC).[11,12] It was also described that TiO_2 nanoparticles induce inflammation in a human acute monocytic leukemia cell line.[13] In addition to these reports different types of nanoparticles are described to induce chronic autoimmune diseases like rheumatoid arthritis.[14] The putative mechanism underlying the development of such disorders is described for single wall carbon nanotubes (SWCNT) which induce a change in the citrullination of proteins.[15] This change in the secondary modification results in a misleading recognition of proteins by the immune system.

In this work, the interactions of primary cultures of human macrophages isolated from peripheral blood with TiO_2 nanoparticles were systemically investigated with a special regard to the role of the protein corona. As part of the innate immune answer they are involved in the first recognition of foreign materials. After characterization of the nanoparticles by zeta-potential measurements and scanning electron microscopy (SEM) the composition of the protein corona was determined by mass spectroscopy. The secretion of the proinflammatory granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFNγ), interleukin (IL)-1β, IL-2, IL-5, and IL-6 as well as the anti-inflammatory cytokines IL-4, and IL-10 was detected after incubation of cells with different concentrations of TiO_2. These cytokines where chosen as examples for proteins that are secreted by inflammatory M1 and anti-inflammatory M2 macrophages thus covering a significant part of macrophage function related to inflammatory processes.

Our results provide clear evidence that secondary modified proteins accumulated on TiO_2 nanoparticles induce in macrophages the release of IL-6 which is responsible for the development of chronic diseases. This cytokine is even released in the presence of low concentrations of nanoparticles.

Methods

Materials

The commercially available TiO_2 nanoparticles were obtained from Sigma Aldrich (Steinheim, Germany). McCoy's 5A medium and the ELISA kits were obtained from Life Technologies GmbH (Darmstadt, Germany). Fetal bovine serum, L-glutamine and nonessential amino acids were purchased from Biochrom GmbH (Berlin, Germany). Buffy coats from healthy donors were obtained from the German Red Cross. Trypsin (sequencing grade) was purchased from Roche Diagnostics, Mannheim, Germany.

Macrophages

Human macrophages were isolated from overall 8 buffy coats from peripheral blood of healthy volunteers obtained from the Red Cross by Ficoll gradient centrifugation followed by density gradient centrifugation on Percoll and the subsequent isolation of CD14 positive cells by magnetic associated cell sorting (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of at least 95% of positive cells was determined by flow cytometry in an Agilent 2100 Bioanalyzer (Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

Cell culture

After isolation, cells were cultured in McCoy's 5A medium supplemented with 15% fetal bovine serum, 1% L-glutamine, and 2% nonessential amino acids. The tests were performed immediately after the isolation procedure at 37 °C and 7% CO_2.

Nanoparticle preparation

Nanoparticles were suspended in water for embryo transfer (Sigma Aldrich GmbH, Steinheim, Germany) and cell culture medium. Ultrasonication at 20% of amplitude was performed for 2 minutes (Ultrasonic Processor UP400S, Hielscher Ultrasonics GmbH, Teltow, Germany). The stock suspension (1 mg/mL) was serially diluted to the final concentrations in ultra pure water.

Scanning electron microscopy

The morphology and diameter of the nanoparticles were determined by scanning electron microscopy (LEO1530VP Gemini, Carl Zeiss NTS GmbH, Oberkochen, Germany). Different concentrations of nanoparticle suspensions were prepared. A drop was placed on a silicon wafer, and allowed to dry before submitted to analysis. The detector used was SE1 at a working distance of 3 mm. The images were analyzed using the ImageJ software (version 1.46 r, Java 1.6.0_65, Wayne Rasband, National Institutes of Health, USA).

Zeta potential analysis

The zeta potentials of nanoparticles were measured in a Malvern Zetasizer Nano ZS instrument (Malvern Instruments GmbH, Herrenberg, Germany). Samples in different concentrations, dispersed in water and in McCoy's 5A medium supplemented with 15% fetal bovine serum, 1% L-glutamine, and 2% nonessential amino acids were prepared and loaded into a special cell for zeta potential analysis.

SDS-PAGE electrophoresis and Western blotting

Nanoparticles were incubated with McCoy's 5A medium supplemented with 15% fetal bovine serum, 1% L-glutamine, and 2% nonessential amino acids. Hereafter, the samples were centrifuged (15,000 *g* for 30 min) to remove the medium. After repeating this procedure three times, the proteins attached to the nanoparticles were desorbed by adding a SDS-sample buffer [0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, glycerol, β-mercaptoethanol, 0.5% (w/v) Bromophenol blue] to re-suspend the pellet and submitting the mixture at 95 °C for 5 minutes. Afterwards, nanoparticles were separated by centrifugation repeated three times and the supernatants were collected and analyzed by sodium-docecyl-sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The prepared medium was used as control. Samples were loaded onto the gel and a constant voltage of 130 V was applied for 45 min to separate proteins according to their molecular weight. After SDS-PAGE was performed, proteins were blotted on a polyvinylidene difluoride membrane. The membrane was stained in Coomassie staining solution (0.25% (w/v) Coomassie Brilliant

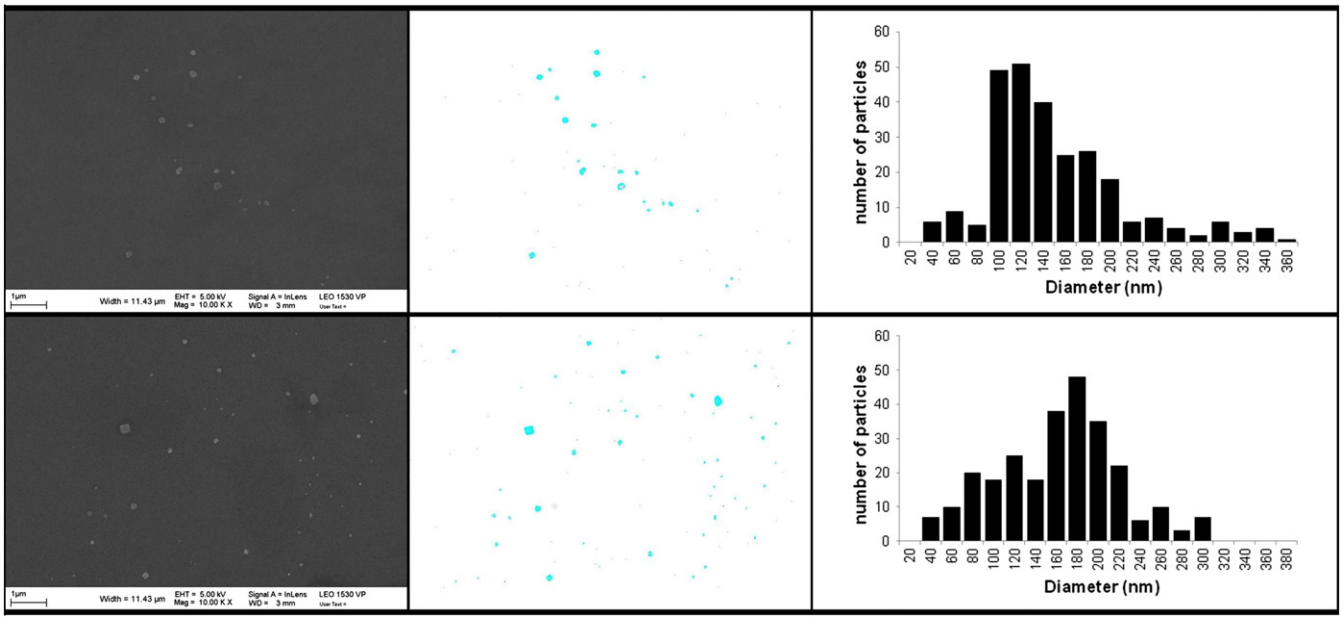

Figure 1. Diameter distributions of TiO_2 nanoparticles before (upper) and after (lower) incubation in cell culture medium. SEM pictures of nanoparticles (left); pictures obtained by ImageJ software (middle) and diameter distribution graphs (right).

Blue R-250 in methanol:acetic acid/water at 4:1:5 (v:v:v), and followed by destaining in methanol:acetic acid:water at 4:1:5 (v:v:v).

Identification of proteins by de-novo sequencing of peptides using nanoESI Q-TOF mass spectrometry

Protein bands were subjected to in-gel digestion according to Shevchenko et al.[16]. Briefly, bands were excised from the gel, cut into small cubes and destained with 100 mM ammonium bicarbonate/acetonitrile (ACN) (1:1, v/v). The supernatant was discarded and gel pieces were washed with pure ACN until gel pieces became opaque and shrank. After removal of ACN trypsin solution (25 ng/μL in100 mM ammonium bicarbonate) was added and incubated on ice bath for 30 min in order to saturate the gel pieces with protease. Then, additional protease solution was added and incubation continued on ice bath for another 90 min. The gel pieces were covered with ammonium bicarbonate buffer and incubated overnight at 37 °C. The peptides were extracted by adding extraction buffer [1:2, 5% formic acid (FA)/ACN v/v] to each tube and incubated for 15 min at 37 °C in a shaker. The supernatant was collected into a fresh tube, dried *in vacuo* and reconstituted in aqueous 0.1% trifluoroacetic acid (TFA) for desalting using C_{18} Ziptips pipette tips (Millipore, Schwalbach, Germany). Briefly, tips were equilibrated five times (10 μL each) with ACN and five times with 0.1 % TFA. Peptides were loaded onto the tips by 10 aspirating/dispensing cycles followed by washing 5 times with 10 μL 0.1% TFA and 5% methanol solution. Subsequently, peptides were eluted 10 times with 10 μL 50% ACN solution containing 0.1% TFA and finally dried *in vacuo*. Nanoelectrospray mass spectrometry (nanoESI Q-TOF MS) experiments were carried out using a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) in the positive ion mode [ESI(+)] as described recently.[17] Briefly, a Z-spray atmospheric pressure ionization (API) source was used, with the source temperature set to 80 °C and a desolvation gas (N_2) flow rate of 75 L/hour. Homemade nanospray capillaries were used, with the capillary tip set to a potential of 1.1 kV and a cone voltage of 40 V. For low-energy collision-induced dissociation (CID) experiments, the peptide precursor ions were selected in the quadrupole analyzer and fragmented in the collision cell using a collision gas (Ar) pressure of 3.0×10^{-5} mbar and collision energies of 20-40 eV (E_{lab}). Proteolytic peptide ions were fragmented by CID and their amino acid sequences were deduced by evaluating the fragment ion series (mainly b- and y-type ions for amide bond cleavages) observed in the corresponding CID spectra manually. Since the amino acids isoleucine (I) and leucine (L) are isobaric they cannot be discriminated by means of low energy CID experiments. Similarly, the masses of glutamine and lysine differ only by 36 mDa and are not unambiguously distinguishable with the Q-TOF instrument used. Thus, in general, amino acids exhibiting an increment mass of 128 Da are assigned to Q or K if proven by tryptic cleavage. Proteins were identified by searching the derived peptide sequence stretches against non-redundant databases from the National Center for Biotechnology Information (NCBI) and Swiss-Prot using NCBI BLAST2 (http://web.expasy.org/blast/). Proteins were only taken into consideration when consecutive sequence stretches of at least 10 amino acid residues were matching.

Enzyme-linked immunosorbent assay (ELISA)

Macrophages were incubated together with the TiO_2 nanoparticles, in the respective concentrations in McCoy's 5A medium supplemented with 15% fetal bovine serum, 1% L-glutamine, and 2% nonessential amino acids for different incubation times. Supernatant of centrifuged nanoparticles suspensions (15,000 *g*

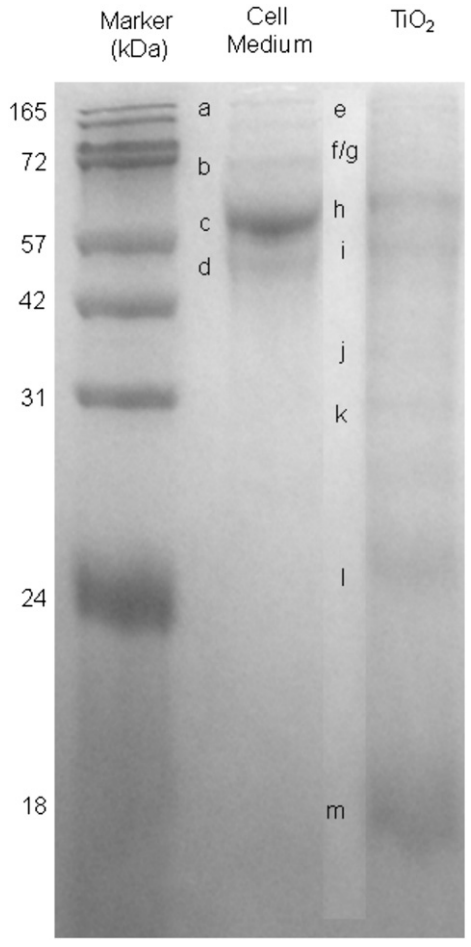

Figure 2. SDS-PAGE gel of the protein corona of TiO_2 nanoparticles after incubation of the particles in serum containing cell culture medium.

for 30 min.) were also analyzed. After different incubation times, the medium was centrifuged and ELISA IL-1β, IL-6, and IL-10 assays were performed following the manufacturer's guidelines.

Luminex® assay

Luminex® assay (Life Technologies, Darmstadt, Germany) was performed for quantification of cytokines in the supernatant of the samples according to the manufacturer's protocol.

Fluorescent protein corona

After overnight incubation of nanoparticles in 10% albumin from bovine serum FITC conjugated (Life Technologies GmbH, Darmstadt, Germany) at 37 °C, the nanoparticles were washed three times, diluted and added to the cell culture. After 3 and 24 hours incubation the wells were washed with PBS and 0.5 mL of PFA 4% was added to each well followed by incubation at 37 °C for 15 minutes. The wells were washed twice and observed under the microscope. The nanoparticles used as a control were incubated for 24 hours under the same conditions in the dark, followed by observation under the microscope.

Table 1
Identified proteins obtained by BLAST search against NCBI/UniProt databases using experimentally obtained peptide sequence stretches.

Band	Accession number	Identified protein (*bos taurus*)	MW (kDa)
a	Q7SIH1	Alpha-2-macroglobulin[a]	167.6
b	Q29443	Serotransferrin[a]	77.8
c	B0JYQ0	ALB protein	69.3
d	P12763	Alpha-2-HS-glycoprotein precursor (Asialofetuin)[c]	38.4
e	Q7SIH1	Alpha-2-macroglobulin[a]	167.6
f	Q3SX14	Gelsolin (Actin-depolymerizing factor)[b]	80.7
g	Q29443	Serotransferrin[a]	77.8
h	B0JYQ0	ALB protein	69.3
i	P12763	Alpha-2-HS-glycoprotein precursor (Asialofetuin)[a,b,c]	38.4
j	P34955	Alpha-1-antiproteinase precursor (Alpha-1-antitrypsin)[a]	46.1
k	P15497	Apolipoprotein A-I precursor (Apo-AI)[c]	30.3
l	Q2KIS7	Tetranectin	22.1
m	P02070	Hemoglobin subunit beta	15.9

[a] *N*-glycosylation.
[b] *O*-glycosylation.
[c] Phosphorylation.

Statistical analysis

Data were expressed as mean ± standard deviation. The statistical hypothesis *t*-test was used with a confidence level of 95%.

Results

The nanoparticles were characterized after dispersion in water and in cell culture medium. The diameters were measured by scanning electron microscopy. Using the free software ImageJ, the diameter distributions were determined (Figure 1). The software performs a digital count and a graph that relates the diameters to the number of particles was developed. The most modal diameter was in buffer 120 nm and in cell culture medium 180 nm. The size distribution was presented in Figure 1.

Nanoparticles were also characterized according to their surface charge expressed as zeta potential. The measured zeta potential of titanium nanoparticles in water was −42.3 ± 0.7 mV. The relevant absolute value of the zeta potential, the magnitude, decreased after incubation in cell culture medium (−40.5 ± 0.3 mV). In order to analyze the formation of the protein corona around nanoparticle surfaces, they were incubated in cell culture medium. Proteins present in the corona of titanium dioxide nanoparticles were separated by gel electrophoresis. Figure 2 shows a Coomassie-stained SDS-PAGE of proteins obtained from cell culture medium prior to treatment with TiO_2 nanoparticles and after formation of the corona. While separation of proteins from the cell culture medium only gives rise to four distinct faint bands in the molecular weight range between 50 and 150 kDa, more species were detected from the TiO_2 nanoparticle sample (Figure 2, lanes 2 and 3). SDS-PAGE of the corona gave rise to a smeared band covering the whole molecular weight range investigated. However, several zones of the gel exhibited the presence of faint bands pointing to a specific enrichment of proteins by the nanoparticles to some extent.

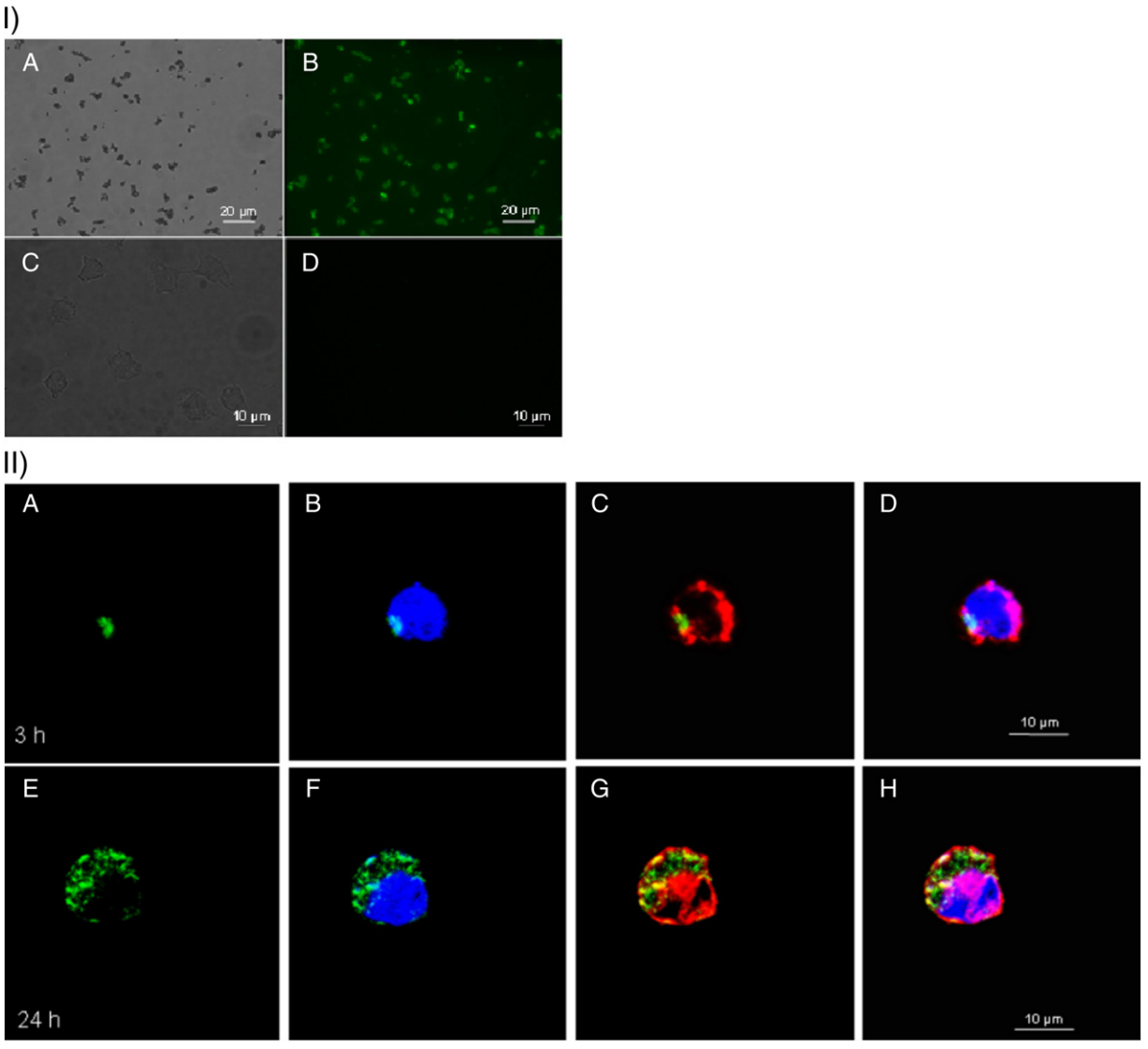

Figure 3. TiO_2 nanoparticle protein corona interaction with human macrophages. I. TiO_2 nanoparticles 24 hours after incubation with fluorescent labeled albumin visualized by light **(A)** and fluorescence **(B)** microscopy. Autofluorescence of cells visualized by light **(C)** and fluorescence **(D)** microscopy. II. TiO_2 nanoparticles, covered with a fluorescent albumin corona incubated with cells visualized by cell organelle staining. 3 hours **(A–D)** and 24 hours **(E–H)** after interaction (green A, E: fluorescent stained nanoparticles; blue B, F: DAPI staining of the nucleus; red C, H: DiD staining of cellular membranes; overlay D, H).

Proteins were identified by proteolytic digestion using trypsin as protease and resulting peptides were submitted to nanoESI Q-TOF MS analysis. *De-novo* sequencing of selected precursor ions using low energy collision-induced dissociation (CID) revealed confined sequence stretches which were searched against the database (Table S1).

As a representative example, Figure S1 displays a CID spectrum of doubly charged peptide precursor, ions at *m/z* 769.38 obtained from a tryptic digest of band h exhibiting the most intense staining (Figure 2). The complete b- and y-type fragment ion series clearly demonstrated the peptide sequence 421LGEYGFQNELIVR433 originating from ALB protein (Accession number B0JYQ0), i.e., bovine serum albumin. A synopsis of the proteins identified is given in Table 1.

To follow up the interaction of the protein corona with the cells we used as model system titanium dioxide nanoparticles incubated in a solution containing fluorescent labeled albumin to allow the formation of a bright corona detectable by fluorescence microscopy. The coated nanoparticles were added to the cell culture and pictures were made after 3 and 24 hours, using the same exposure time (Figure 3). After 3 hours the interaction of the cellular membrane with particles was detected. After 24 hours the nanoparticles were taken up together with their protein corona.

The effect of the attachment of the nanoparticles to the cellular surface after 3 hours was shown by incubation of the human macrophages with TiO_2 concentrations of 10, 50, and 100 µg/mL, respectively. We tested the secretion of the inflammatory cytokines Granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFNγ), IL-1β, IL-2, IL-5, and IL-6, and the anti-inflammatory cytokines Il-4 and IL-10. Only IL-1β, IL-6 and IL-10 are secreted under these

Table 2
Cytokine secretion by human macrophages after incubation with TiO_2 nanoparticles.

	Concentration in cell culture medium (pg/mL)		
	TiO_2 10 μg/mL	TiO_2 50 μg/mL	TiO_2 100 μg/mL
inflammatory			
GM-CSF	-[a]	-[a]	-[a]
IFNγ	-[a]	-[a]	-[a]
IL-1β	61.822 ± 2.579	94.349 ± 6.794	114.642 ± 5.530
IL-2	-[a]	-[a]	-[a]
IL-5	-[a]	-[a]	-[a]
IL-6	659.167 ± 6.207	859.412 ± 21.449	1026.173 ± 113.871
Anti-inflammatory			
IL-4			
IL-10	6.916 ± 0.784	11.971 ± 0.469	24.956 ± 6.952

[a] Calculated concentrations less than the lowest detectable standard; empty-not detectable.

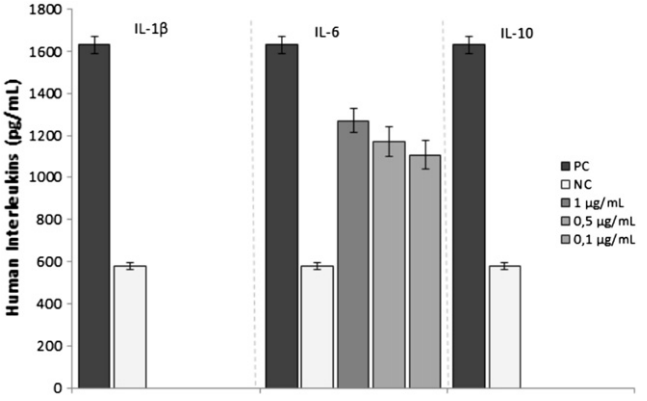

Figure 4. Cytokine secretion of macrophages in response to incubation with TiO_2 nanoparticles. Positive control (PC; cells incubated with LPS), negative control (NC; cells in medium), IL-1β, IL-6, and IL-10 in cell culture medium secreted by macrophages after incubation with different concentrations of nanoparticles.

conditions with the concentrations IL-6 >IL-1β > IL-10 (Table 2).

Additionally, after 3 hours-incubation of nanoparticles with cells at lower concentrations of 0.1, 0.5, and 1 μg/mL the secretion of IL-1β, IL-6, and IL-10 was determined. IL-6 was the only interleukin detected in the cell culture medium from the cytokines investigated in our experiments (Figure 4). We show 1 out of 4 similar experiments. The complete dataset is depicted in Table S2. The grey shaded cells are the basis of the graph. All the samples presented statistically significant differences from the control by Student's *t*-test ($P < 0.05$).

Discussion

The nanoparticles used were characterized with regard to their diameters and surface charges. The diameters of the TiO_2 nanoparticles were determined by scanning electron microscopy. The particles showed mainly a size distribution between 100 and 200 nm with a maximum at 120 nm. When suspended in cell culture medium, the particles presented a slight increase in diameter and a decrease in the absolute value of the zeta potential. The main number of particles showed here a diameter of 180 nm with a range between 80 and 240 nm. The change of the surface charge is an indication of protein adsorption.[18]

The characterization of nanoparticles is an important matter helping to understand the profile of their protein corona. Lundqvist et al. showed that, for a fixed material type, the diameter and the surface characteristics of the nanoparticles can change the nature of the proteins in the corona.[19] Later on, it was shown that the protein corona mediated the interaction of nanoparticles with cells.[20,21] The binding sites of the proteins can interact in a short time with receptors of the cell membrane and activate them. Accordingly, mapping of the protein corona of TiO_2 nanoparticles was performed by using SDS-PAGE and mass spectrometry. Interestingly, most proteins identified in the corona of TiO_2 nanoparticles are reported to comprise post-translational modifications such as *N*- and *O*-glycosylations as well as phosphorylations. The latter finding is corroborated by previous reports on selective enrichment of phosphorylated as well as glycosylated proteins from complex mixtures using titanium dioxide as a stationary phase in solid phase extraction and chromatography.[22] Beside post-translationally modified proteins, the protein corona of TiO_2 nanoparticles harbors albumin as one of the major constituents. The preferred association of albumin to titanium dioxide surfaces as well as its capacity to bind various substrates is well known and has been reported previously.[23]

The affinity of serum albumin to the nanoparticles was used to demonstrate the interaction of the TiO_2 nanoparticles with human macrophages. Bovine serum albumin conjugated to a fluorescent tag was used as a model protein for microscopic observations of the corona of nanoparticles to evaluate the interaction of the TiO_2 nanoparticles. After attachment nanoparticles are able to enter the cells by various mechanisms of endocytosis. Phagocytosis, a type of endocytosis special for macrophages, forms phagosomes that fuse with lysosomes to phagolysosome. Enzymes present inside the lysosomes are responsible for the digestion of biological substances.[24] After an incubation time of 24 hours an uptake of the nanoparticles including their corona was observed. It might be suggested that during further incubation the corona will be degraded and, after the death of the cell, the particles are exposed to the surrounding tissue, starting with a new protein layer.

However, before cellular uptake the protein corona of nanoparticles interacts with cell membranes. Here, the corona interacts with surface receptors, suggesting activation of signaling proteins followed by inducing cytokine secretion in the cells (Figure 4). The cytokines chosen for this article represent the activity of macrophages of the M1 and M2 type.

We observed that within the selected cytokine portfolio only IL-1β, IL-6, and IL-10 were secreted after incubation at particle concentrations higher than 10 μg/mL (Table 2). In order to assess the induction of cytokines after cultivation in the presence of low TiO_2 nanoparticle concentrations (<1 μg/mL), the particles were incubated with the cells for 3 hours and IL-1β, IL-6, and IL-10 were quantified in the supernatant by ELISA. This was the method chosen due to its highly accepted sensitivity and good correlation with multiplex assays.[25]

As a result, low TiO_2 nanoparticle concentration did not induce anti-inflammatory markers such as IL-10 which can suppress immune and inflammatory responses. Also the strong inflammation activator IL-1β was not detected in the supernatant under these conditions.

As a surprise we observed for human macrophages of the peripheral blood in our experiment only an IL-6 release even at TiO_2 concentrations of 0.1, 0.5, and 1 μg/mL as compared to controls. The obtained data show the typical variations of primary immune cells, especially in their reactivity to LPS. As the binding structures for nanoparticles and LPS and as a consequence the intracellular signaling differs, the quantitative comparison of the cytokine secretion should been seen critically. Basically it just shows the different activability of the leukocyte preparations. Nevertheless, and most important is that the IL-6 secretion is in all cases significantly higher than negative controls. For the interleukins investigated in this work, this was not demonstrated before.

IL-6, a cytokine crucial for the acute phase response, dictates together with its soluble receptor, sIL-6Rα, the change from acute to chronic inflammation by altering the nature of leukocyte infiltration, as Cem Gabay reported. [26] Thus IL-6 might play a crucial role in the development of chronic diseases in presence of protein corona covered TiO_2 nanoparticles. An overexpression if IL-6 is also reported for different type of autoimmune diseases as well as for some cancers. Tocilizumab, a monoclonal antibody against IL-6 is meanwhile established in treatment of rheumatoid arthritis.[27]

In conclusion, secondary modified proteins are accumulated on the surface of TiO_2 nanoparticles as a protein corona which is supposed to be degraded by the cells after uptake. It can be speculated that this accumulation results in a change of the structure of the proteins or to unusual combination of protein structures on the particle surface which triggers the cells of the immune system to release IL-6. Since the proinflammatory IL-6 is a signaling molecule not only for inflammation and cancer but also mediates autoimmune response the secretion of IL-6 might induce chronic diseases and cancer even during cell exposure at low TiO_2 nanoparticle concentration. The activation of cells of the immune system by an accumulation of secondary modified proteins on nanoparticles is, to our knowledge, not yet described and comprise an additional mechanism to the citrullination of proteins which is reported to induce autoimmune diseases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.10.001.

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