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The Importance of an Endotoxin-Free Environment during the Production of Nanoparticles Used in Medical Applications

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ABSTRACT

We investigated the effect of spherical gold nanoparticles on immature dendritic cells (DCs). Conventionally produced nanoparticles had a maturating effect on the DCs—a result of lipopolysaccharide (LPS) contamination. By modification of the production process, low-LPS particles were obtained, which had practically no effect on phenotypic maturation or cytokine production of the DCs. Our findings emphasize the importance of high purity in the production of nanoparticles, since possible contaminants may interfere with the assessment of biological/medical effects. They also highlight that nanoparticles can function as carriers of immune modulating contaminants.

Nanomaterials are of growing importance not only for industrial applications but also in bioscience and medicine. Near-term applications include drug delivery,[1] improved contrast agents for imaging,[2] fluorescence biomarkers,[3] and chip-based nanolabs capable of monitoring and controlling individual cells.[4] In addition, current vaccine development includes the use of nanoparticles to optimize antigen delivery to antigen-presenting cells.[5]

When new materials are introduced into the medical field, possible hazardous or immunomodulatory effects need to be explored. Since the physiochemical properties of nanomaterials are different from those of their bulk counterparts,[6] their interaction with biological systems is expected to be different. The effects may vary between different kinds of nanoparticles, depending, for instance, on chemical composition, size, and shape.[7,8] We started to explore the possible immune modulating effects of gold nanoparticles on human antigen-presenting dendritic cells. The choice fell on gold nanoparticles because gold is inert,[9] gold nanoparticles are relatively easy to synthesize, and gold surfaces readily bind a wide range of biomolecules such as DNA[10] and proteins,[11] a property which is desired for quantitative analyses.[12] Furthermore, gold nanoparticles have photothermal properties that may be of use for localized heating or drug release, increasing their potential for therapeutic applications.[13]

To investigate possible immunostimulatory or immunomodulatory effects of gold particles, we decided to use dendritic cells (DCs), described as "the sentinels of the immune system", since they are the most efficient type of antigen-presenting cells, having an exceptional capacity to initiate both primary and secondary immune responses in vivo. By expressing different levels of cytokines and costimulatory molecules such as CD80, CD83, and CD86 and peptides loaded onto major histocompatibility complex (MHC) class I and II, they can induce different types of immune responses.[14,15] Inflammatory stimuli induce DC maturation, leading to increased expression of the molecules mentioned above, as well as production of cytokines such as interferon (IFN)-α, interleukin (IL)-10 and IL-12, depending on the type of stimulus and the DC subset.[16] Maturation of DCs reduces their ability to take up antigen, while increasing their potential for antigen presentation, hence also increasing their capacity to stimulate T cells. DCs have therefore become very attractive targets in immunotherapy.[17]

In a first set of experiments we sought to determine whether gold nanoparticles can be used as inert carriers in therapeutic approaches or whether they affect DC maturation. We produced 7 nm spherical gold particles with sodium

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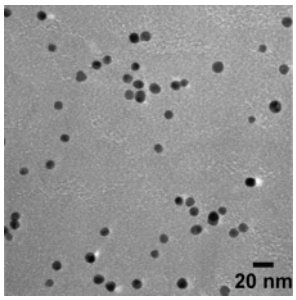

Figure 1. Transmission electron microscopy image of a representative gold nanoparticle formulation. Gold nanoparticles were produced with sodium borohydride as the reducing agent and were coated with HSA, suspended in PBS, and placed on copper grids. (TEM images were recorded at a voltage of 200 kV with a JEOL JEM-2000EX.)

borohydride as a reducing agent[18] (Figure 1) which we added to human MDDCs with a typical immature phenotype.[19] MDDCs were generated as described previously with IL-4 and GM-CSF for 6 days.[20] Flow cytometric analysis of MDDCs cocultured with the gold nanoparticles for 24 h resulted in an increased expression of the costimulatory molecule CD86 and antigen presenting MHC class II (Figure 2), as well as of CD80 and CD83 (data not shown). This was shown both as an increase of percentage of positive cells and as an increased surface expression on individual cells, as indicated by the mean fluorescence intensity (MFI). An up-regulation of CD40 and MHC class I was only observed by increased levels of MFI (data not shown). Collectively, this implied maturation of MDDCs. Similar results were observed after 6 h (data not shown). The study included three different concentrations of gold nanoparticles. At the lowest concentration, 0.5 μg of Au/mL, the effect on the cells was smaller than that at the two higher concentrations, 5 and 50 μg Au/mL, which in turn had essentially equivalent effects, implying saturation (Figure 2). Human serum albumin (HSA), which was added to gold nanoparticle suspensions to prevent aggregation of the particles in phosphate-buffered saline (PBS), had no observable effect on the MDDCs (data not shown) and was therefore used in further experiments.

Another way to study the effect of nanoparticles on DCs is to investigate the production of cytokines by myeloid DCs (mDCs) and plasmacytoid DCs (pDCs).[16] Thus, peripheral blood mononuclear cells (PBMCs), containing, among others, these two cell types, were incubated with particles, and cytokine production was examined with ELISpot analysis. We found that the gold nanoparticles induced production of IL-12, known to be produced by mDCs, but not of INF-α, which is produced by pDCs (Figure 3C). These findings indicated that the gold nanoparticles induced maturation of mDCs. Intriguingly, addition of even more gold particles resulted in lower production of IL-12 (data not shown) suggesting that high concentrations of the particles may inhibit the expression of IL-12.

However, effects detected in cell or animal experiments may be due to other effects, e.g., contamination of the materials under study. The strong maturation of the MDDCs and the notable contrast between the expression of IL-12 and IFN-α seen after exposing DCs to gold nanoparticles led us to suspect that the gold nanoparticles were contaminated with endotoxin. Endotoxins are found in the outer membrane of Gram-negative bacteria and are members of a class of phospholipids called lipopolysaccharides (LPS).[21] LPS is naturally present everywhere in our surroundings and can therefore be introduced to the system through the water used as the solvent, chemicals, raw materials, or equipment used in the preparation of the nanoparticles. LPS is known to induce a variety of cells to produce cytokines and chemokines, which in turn affect DC movement and maturation.[14] LPS induces IL-12 production by mDCs but not pDCs since the latter cells lack the toll-like receptor 4 (TLR4).[22] pDCs, which have TLR9, respond instead to viral products such as those containing unmethylated deoxycytidyl-deoxyguanosine (CpG) motifs, resulting in production of IFN-α.[22] A common pathogenic effect of LPS in vivo is fever, but exaggerated responses to LPS may lead to the life-threatening condition known as endotoxic shock.[21]

To investigate whether the nanogold colloids were contaminated with LPS, analyses were performed with the Limulus Amebocyte Lysate (LAL) endochrome assay. The results revealed concentrations of 12−51 ng of LPS/mL in the four different batches prepared in an uncontrolled environment, corresponding to final concentrations of 0.5−11 ng of LPS/mL in the cell cultures incubated with the highest concentration of gold nanoparticles, 50 μg of Au/mL. LPS concentrations of 1 ng/mL are known to be sufficient to stimulate MDDCs to produce IL-12p70,[23] but concentrations as low as 0.01 ng/mL have been reported to stimulate human PBMCs, which include DCs.[24] This suggests that the LPS concentrations found in the gold colloids were sufficient to induce maturation of DCs.

To explore the possibility that the up-regulation of maturation-related molecules was indeed induced by the LPS and not by the nanoparticles themselves, we performed experiments where the LPS-blocking agent polymyxin B was added. The increased expression of costimulatory molecules seen earlier with increased dose of nanoparticles was abolished by addition of polymyxin B (Figure 3A). Similarly, addition of polymyxin B to the particles abolished the induction of IL-12 (Figure 3C). These results support our hypothesis that LPS was responsible for the maturation of the DCs, rather than the nanopaticles themselves. Control experiments showed that polymyxin B by itself did not have a maturating effect on the phenotype of MDDCs (partly shown in Figure 3B) or cytokine production (Figure 3D).

Furthermore, to verify that polymyxin B did not have a direct inhibitory effect on DC maturation but only inhibited LPS-induced maturation, CD40 ligand (L) produced by L cells (a fibroblast cell line transfected with CD40L) was used as a complementary maturation control to LPS. Flow cytometry showed that the maturation effect of CD40L was unaffected by polymyxin B (Figure 3B), thus suggesting that polymyxin B inhibited MDDC activation by binding to LPS rather than by any cellular interaction. The mean fluorescence intensity (MFI) values and percentages of cells expressing CD80, CD83, CD86, and MHC class I and II were similar

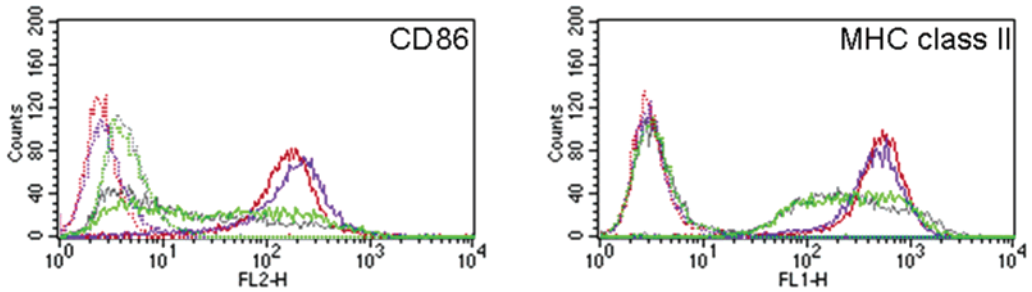

Figure 2. Initial experiments with gold nanoparticles cocultured with immature MDDCs. Expression of the costimulatory molecules CD86 and HLA-DR was analyzed by flow cytometry after 24 h. MDDCs at a concentration of 4×10^5 cells/mL were incubated with 50 μg of Au/mL (red line), 5 μg of Au/mL (blue line), or 0.5 of Au/mL (green line) for 24 h. The gray line represents MDDCs cultured in medium alone, and dashed lines represent isotype controls. Results shown are representative of four independent experiments using MDDCs generated from different healthy blood donors. Ten thousand cells were analyzed per sample.

in nontreated cells and cells treated simultaneously with polymyxin B and the LPS concentration seen in the gold colloids. This implies that polymyxin B is able to block the effects of LPS, at least in the amounts present in the gold colloids used in cell experiments (partly shown in Figure 3B).

Further studies were done to optimize production of nanoparticles to minimize LPS contamination. Efficient removal of LPS is difficult to achieve due to its extreme heterogeneity, both in terms of composition and structure, and also due to size differences caused by aggregation.[21] In addition, LPS is thermostable and fairly insensitive to pH changes. Applying high temperatures, such as 250 °C for 45 min, or high concentrations of acids and bases would, according to the Food and Drug Administration (FDA, www.fda.gov), be an efficient method to remove LPS, but such treatment may also affect the properties of nanoparticles, e.g., causing aggregation. An alternative way to remove LPS is by running the samples through polymyxin B columns. However, this may lead to loss of nanoparticles, especially if the LPS binds to the surface of the particles. Moreover, it is not clear whether LPS can be removed from the surfaces of the nanoparticles by repeated washing. A safer approach is to keep the entire production process contaminant-free. Therefore, we modified the synthesis procedures to avoid nanoparticle contamination. This meant synthesizing nanoparticles in fume hoods, replacing distilled water with ultrapure water (<0.25 ng of LPS/mL), and the consistent use of gloves. In addition, both the surroundings and the glassware were cleaned with PyroCLEAN before being used in the synthesis experiments. In this way, LPS concentrations were lowered 16−425 times to 0.12−0.75 ng/mL, resulting in a final endotoxin concentration of 3−48 pg/mL, for the cells treated with 50 μg of gold nanoparticles/mL. These levels are within the critical limits mentioned before[23,24] and are also low enough to be administered to humans according to FDA guidelines (www.fda.gov).

When MDDCs were exposed to gold nanoparticles with a low endotoxin content (13 pg/mL), there was only a minor up-regulation of most surface molecules detected after 6 h (Figure 3A), with a similar pattern after 24 h (data not shown). Corresponding results were seen for the expression of IL-12 and IFN-α, where a few or no cytokine producing cells could be detected (Figure 3D). We can therefore conclude that the gold nanoparticles with low LPS contamination induced hardly any maturation of the MDDCs.

Since a major function of DCs is to deliver signals for T-cell stimulation, the functionality of MDDCs incubated with gold nanoparticles was assessed by investigating the impact of nanoparticles on lymphocyte activation by MDDCs. In an autologue setting, MDDCs preincubated with low-LPS nanoparticles did not induce a proliferative response in CD14-depleted PBMCs, while high-LPS nanoparticles did (Figure 4A), suggesting that high-LPS nanoparticles can trigger an unwanted immune response. Furthermore, in an allogeneic setting, high-LPS nanoparticles potentiated the allogeneic reaction, whereas low-LPS nanoparticles did not (Figure 4B). These results suggest that low-LPS nanoparticles are not interfering with MDDC-T cell interactions.

Our results support the suitability of gold nanoparticles for use in biomedical applications. The findings also emphasize the importance of combining knowledge within materials science with that of biomedicine when generating new advanced materials, to avoid misinterpretation of results. At present, nanoscience focuses mainly on the search for new materials and techniques, and safety and biomolecular aspects are often postponed to future studies. Contaminants, such as LPS, as found in this study, may lead to erroneous conclusions that cause developers to abandon what might be a promising material. It is also of importance to keep in mind that LPS is a very real problem, not only in the field of nanomedicine but also in the general medical research field, as seen in the literature.[24−26] Moreover, this study highlights the exquisite sensitivity of DCs to endotoxin contaminants, which also suggests the usefulness of IL-12 ELISpot as a complement to the LAL test assay.

It is worth mentioning that LPS also may have advantageous synergetic effects, depending on the desired cellular/medical effects. One study shows that exposure to LPS has a crucial role in the development of tolerance to allergens, thus having potential to lower the prevalence of allergy.[27] In addition, Blander and Medzhitov recently showed that the efficiency of antigen presentation of a specific antigen complex by DCs is dependent on the coexistence of TLR ligands within the same complex.[28] These data suggest that the use of adjuvants such as LPS can in some cases be desirable. However, if they are to be used, they need to be applied in a controlled way.

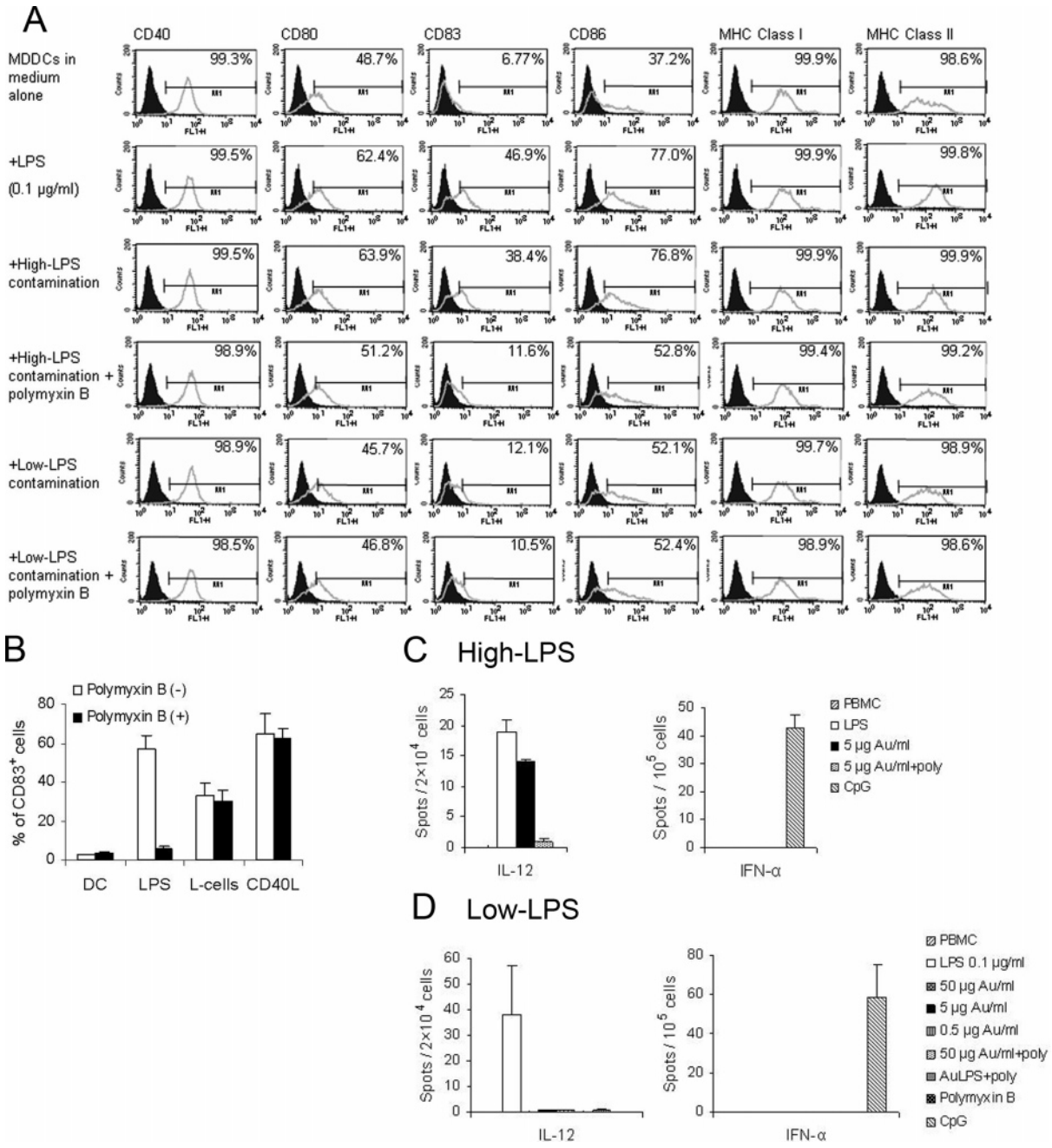

Figure 3. Maturation of MDDCs is mediated by LPS in the gold nanoparticle preparations. (A) Expression of the costimulatory molecules CD40, CD80, CD83, and CD86 and of MHC class I and II, comparing MDDCs (4×10^5 cells/mL) cultured in medium alone, with cells treated with high-LPS contaminated gold nanoparticles (0.5 ng of LPS/mL when incubating cells with 50 μg of Au/mL) with or without polymyxin B (10 μM) and with low-LPS contaminated gold nanoparticles (48 pg of LPS/mL when incubating cells with 50 μg of Au/mL) with or without polymyxin B (10 μM) for 6 h. For each sample 10000 cells were counted and analyzed. The percentage of positive cells is shown in each image. Filled bars represent isotype control. LPS (0.1 μg/mL) was used as a positive control. The results shown are from one experiment of two experiments yielding similar results. (B) Percentage of $CD83^+$ cells of nontreated MDDCs (DC), cells incubated with 0.1 μg of LPS/mL (LPS), with L cells or with L cells producing CD40L (CD40L), with (+) or without polymyxin B (−) for 24 h. The results are shown as mean ± standard error of the mean of three independent experiments using cells from different donors. (C, D) ELISpot analysis of the production of IL-12 and INF-α by PBMCs (2×10^4 or 10^5 cells/well, respectively) after 20 and 40 h of incubation, respectively, with (C) high-LPS gold nanoparticles (0.24 ± 0.02 ng of LPS/mL when incubating cells with 5 μg of Au/mL) and (D) low-LPS nanoparticles (corresponding to 11 ± 3, 1.1 ± 0.3, 0.11 ± 0.03 pg of LPS/mL for the different gold concentrations, respectively) in the absence or presence of polymyxin B (poly). LPS and CpG were included as positive controls. Results represent the mean ± standard error of the mean from three independent experiments using cells from different donors.

Our data further suggest that exposure to contaminated nanoparticles during the production and handling of nanomaterials might have an immunomodulatory effect. Nanoparticles are easily spread in the environment and can reach the lungs by inhalation, where they will encounter DCs,[29] and may move further into the systemic circulation.[30] Therefore, to minimize the risks, the contamination of nanomaterial used in nonbiomedical applications should also be avoided.

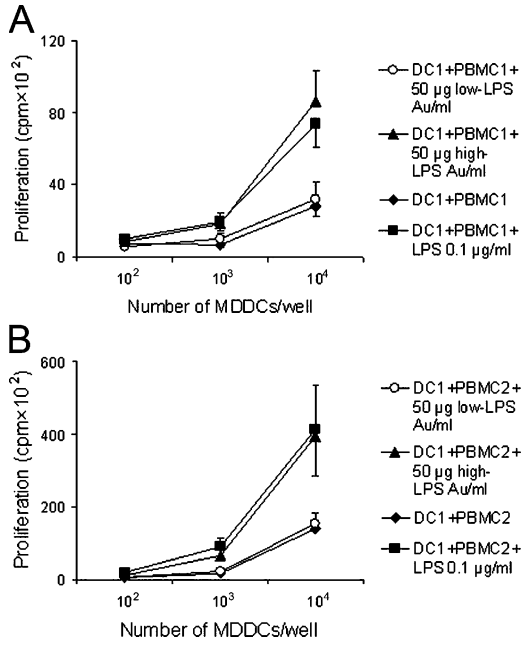

Figure 4. MDDCs preincubated with pure gold nanoparticles do not affect proliferation in allogeneic or autologous CD-14 depleted PBMCs, while LPS-contaminated particles do. MDDCs were cultured in only medium, or preincubated with high (2.4 ± 0.2 ng of LPS/mL) or low (16 ± 1.7 pg of LPS/mL) contaminated gold nanoparticles at a density of 4×10^5 cells/mL for 24 h. LPS (0.1 μg/mL) was used as a positive control. The MDDCs (1×10^2, 1×10^3, and 1×10^4 cells per well) were thereafter cocultured with either autologous (PBMC1; A) or allogeneic (PBMC2; B) CD14-depleted PBMCs (1×10^5 cells per well) in triplicates for 4 days, and then ^{3}[H]-thymidine was added for another 18 h. The results are shown as mean $\pm$ standard error of the mean of three independent experiments. The phytohaemagglutinin (PHA)-induced proliferation in the CD14-depleted PBMCs was $60 \times 10^3 \pm 4 \times 10^3$ cpm ($n = 3$), and background proliferation in the CD14-depleted PBMCs was $12 \times 10^2 \pm 3 \times 10^2$ cpm ($n = 3$).

In conclusion, the present study implies that (i) LPS contamination of gold nanoparticle preparations can stimulate DCs leading to an increased expression of costimulatory and MHC class I and II molecules and production of cytokines, (ii) awareness and control of contaminants in the surroundings is important, especially during the production of particles intended for use in medical applications, and (iii) gold nanoparticles produced in a clean environment have a low impact on maturation and functionality of DCs, thus making them suitable as inert carriers for biomedical applications.

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Supporting Information Available: Methods and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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