

Local Effects on Airway Inflammation and Systemic Uptake of 5 nm PEGylated and Citrated Gold Nanoparticles in Asthmatic Mice

Albert J. Omlor, Duc D. Le, Janine Schlicker, Matthias Hannig, Raphael Ewen, Sebastian Heck, Christian Herr, Annette Kraegeloh, Christina Hein, Ralf Kautenburger, Guido Kickelbick, Robert Bals, Juliane Nguyen, and Q. Thai Dinh*

Nanotechnology is showing promise in many medical applications such as drug delivery and hyperthermia. Nanoparticles administered to the respiratory tract cause local reactions and cross the blood–air barrier, thereby providing a means for easy systemic administration but also a potential source of toxicity. Little is known about how these effects are influenced by preexisting airway diseases such as asthma. Here, BALB/c mice are treated according to the ovalbumin (OVA) asthma protocol to promote allergic airway inflammation. Dispersions of polyethylene-glycol-coated (PEGylated) and citrate/tannic-acid-coated (citrated) 5 nm gold nanoparticles are applied intranasally to asthma and control groups, and (i) airway resistance and (ii) local tissue effects are measured as primary endpoints. Further, nanoparticle uptake into extrapulmonary organs is quantified by inductively coupled plasma mass spectrometry. The asthmatic precondition increases nanoparticle uptake. Moreover, systemic uptake is higher for PEGylated gold nanoparticles compared to citrated nanoparticles. Nanoparticles inhibit both inflammatory infiltrates and airway hyperreactivity, especially citrated gold nanoparticles. Although the antiinflammatory effects of gold nanoparticles might be of therapeutic benefit, systemic uptake and consequent adverse effects must be considered when designing and testing nanoparticle-based asthma therapies.

A. J. Omlor, D. D. Le, J. Schlicker, R. Ewen, S. Heck, Prof. Q. T. Dinh
Department of Experimental Pneumology and Allergology
Saarland University Faculty of Medicine
Kirrberger Str. 100, D-66421 Homburg/Saar, Germany
E-mail: thai.dinh@uks.eu

Prof. M. Hannig
Clinic of Operative Dentistry
Periodontology and Preventive Dentistry
Saarland University
Kirrberger Str. 100, D-66421 Homburg/Saar, Germany
Dr. C. Herr, Prof. R. Bals, Prof. Q. T. Dinh
Department of Internal Medicine V
Pneumology, Allergology and Respiratory Critical Care Medicine
Saarland University Faculty of Medicine
Kirrberger Str. 100, D-66421 Homburg/Saar, Germany

DOI: 10.1002/sml.201603070

Dr. A. Kraegeloh
INM–Leibniz Institute for New Materials
Campus D2 2, D-66123 Saarbrücken, Germany
Dr. C. Hein, Dr. R. Kautenburger, Prof. G. Kickelbick
Institute of Inorganic Solid State Chemistry
Saarland University
Campus Dudweiler, Am Markt Zeile 3-5
D-66125 Saarbrücken, Germany
Prof. J. Nguyen
Department of Pharmaceutical Sciences
School of Pharmacy and Pharmaceutical Sciences
303 Kapoor Hall, SUNY Buffalo, NY 14214, USA



1. Introduction

Nanotechnology is a promising development in medical applications and therapeutics. This is particularly true in oncology, where nanoparticle-based carrier systems can be used to design targeted antitumor therapies with fewer side effects.^[1] Second-generation nanocarriers such as BIND-014 and Aurimmune (Cyt-6091) are already being tested in clinical trials of patients with several different cancer types.^[2–4] In addition to the targeted transport of antitumor drugs, nanoparticles are also being used for hyperthermia-based tumor treatment, with a commercial product called AuroShell (Nanospectra Biosciences, Inc.) currently being tested in a phase I clinical trial.^[5,6]

Due to their ready availability and unique physicochemical properties, gold nanoparticles were used in early nanotechnology applications and remain a contemporary research topic, with both Aurimmune (Cyt-6091) and AuroShell based on gold nanoparticles. In addition, the National Institute of Standards and Technology (NIST) has suggested the use of gold nanoparticles as a model system for nanotoxicological research.^[7]

To date, several studies have examined the local and systemic effects of administration of gold nanoparticles to the respiratory tract. The majority of these studies either focus on pulmonary toxicity and inflammation^[8–11] or examine translocation to extrapulmonary organs.^[12–16] However, little is known about how these effects are influenced by chronic respiratory diseases such as asthma.

Understanding nanoparticle–host interactions is essential for the accurate evaluation of nanotechnology safety and also sheds light on how allergic airway conditions are affected by inhaled pollutants. With this in mind, this study examines both the local effects of gold nanoparticles on the airways and their systemic uptake to extrapulmonary organs in mice with an allergic airway precondition. Most therapeutically used nanoparticle drugs have a polyethylene glycol (PEG) coating, and PEGylation has been shown to have a strong influence on nanoparticle uptake, stability, and cell interactions.^[2,17,18] Therefore, PEG-coated (PEGylated) gold nanoparticles were the focus of this study. However, to ensure comparability with previous toxicological studies,^[13,19–21] we also studied citrated gold nanoparticles.

2. Results and Discussion

With possible future therapeutic uses and toxicological understanding in mind, this study focused on the local and systemic impact of gold nanoparticles when applied via the respiratory tract. Few studies have investigated the effects of gold nanoparticles in the context of allergic airway inflammation. This is the first study to examine the influence of allergic airway inflammation on the systemic uptake of different gold nanoparticles.

2.1. OVA-Induced Allergic Airway Inflammation

The OVA-induced airway inflammation model is a well-established method to simulate allergic airway inflammation

in BALB/c-mice.^[22–25] All groups in our model are labeled by three syllables, the first two defining the asthma status (Figure S1, Supporting Information). The phosphate-buffered saline (PBS)/PBS groups without intraperitoneal (ip) sensitization or inhalative challenge to ovalbumin and the OVA/PBS groups with ip OVA sensitization but only PBS challenge served as control groups, and these mice showed no symptoms of allergic airway inflammation. Mice in the OVA/OVA groups received both ip sensitization to OVA and an OVA challenge. As a consequence, they developed an asthma-like airway reaction, which we simply call “asthma,” and these mice demonstrated: (i) a large increase in inflammatory cells in hematoxylin and eosin (H&E)-stained lung sections; and (ii) mucus overproduction in periodic acid-Schiff (PAS)-stained sections compared to other groups. The nonasthma groups (PBS/PBS and OVA/PBS) contained fewer immune cells and less mucus in the respective lung sections (Figure 1).

The morphological observations were consistent with bronchoalveolar lavage (BAL) cell-counting results (Figure 2). All three asthma groups (OVA/OVA/pbs, OVA/OVA/cit, and OVA/OVA/peg) contained significantly more ($p < 0.01$) total immune cells, macrophages, and eosinophils (eosinophils were greatly increased in the asthma groups and almost zero in the control groups). Neutrophils were more numerous in all three asthmatic groups than their respective nonasthmatic groups, although this was not significant in all cases. Lymphocyte numbers, however, were variably different between groups: the OVA/OVA/peg group was significantly greater than the respective nonasthma groups, the OVA/OVA/pbs group was greater but not significant, while the OVA/OVA/cit group was similar. These results demonstrate that the OVA model is functional and creates an eosinophilic inflammatory environment with macrophages. Moreover, total IgE and OVA specific IgE measurements showed the high sensitization status of OVA-treated mice. Both IgE levels were significantly higher for the sensitized groups (OVA/PBS and OVA/OVA), than for the nonsensitized groups (PBS/PBS).

A main objective of this study was to evaluate the local effects of nanoparticles on the airways in the context of asthma, so the effect of intranasal nanoparticle treatment was compared between asthma and nonasthma groups. The intranasal route of administration was chosen because it is less invasive but similarly effective as intratracheal application.^[26] The asthma groups treated with pbs, citrated (citrate/tannic-acid-coated) gold nanoparticles, and PEGylated gold nanoparticles were all significantly different. The pbs-treated group (OVA/OVA/pbs) had the most immune cells of all the asthma groups ($182\,000 \pm 6250$ cells mL^{-1}), while the group treated with citrated gold nanoparticles (OVA/OVA/cit) had the least ($91\,300 \pm 4890$ cells mL^{-1}). The PEGylated (OVA/OVA/peg) group ranged in between ($136\,000 \pm 13\,700$ cells mL^{-1}). The difference between citrated nanoparticles (cit) and pbs is highly significant at the 0.001 level, the difference between PEGylated and pbs is significant at the 0.05 level, and the difference between PEGylated and citrated is significant at the 0.01 level. In contrast, there were no significant differences in total cell counts between the three different treatments

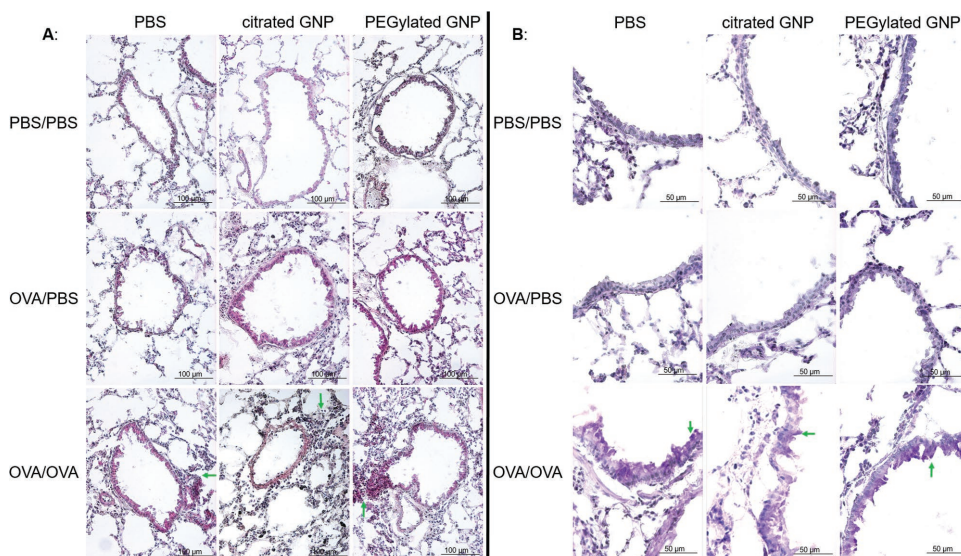


Figure 1. A) Representative photomicrographs of H&E-stained lung sections. Lung sections from OVA-sensitized and challenged mice (OVA/OVA) showed a large increase in inflammatory cell numbers (arrows). Mice only treated with PBS (PBS/PBS) and mice sensitized to OVA but only challenged with PBS (OVA/PBS) did not contain aggregated inflammatory cells. This behavior was seen for the groups that did not receive any nanoparticles (pbs), for the groups that received citrated nanoparticles (cit), and the groups that received PEGylated nanoparticles (peg). B) Representative photomicrographs of PAS-stained lung sections. Lung sections from OVA-sensitized and challenged mice (OVA/OVA) showed high mucus secretion (arrows) compared to mice only treated with PBS (PBS/PBS) or mice sensitized to OVA but only challenged with PBS (OVA/PBS). This behavior was seen for the groups that did not receive any nanoparticles (pbs), for the groups that received citrated nanoparticles (cit), and the groups that received PEGylated nanoparticles (peg).

for the nonasthma groups, which were all in the range of 50 000 cells mL⁻¹.

When responses of individual immune cell types to nanoparticles were examined, macrophages showed a similar pattern as the total cell count number: 146 000 ± 10 100 cells mL⁻¹ for OVA/OVA/pbs, 63 700 ± 8720 cells mL⁻¹ for OVA/OVA/cit, and 99 700 ± 7289 cells mL⁻¹ for OVA/OVA/peg. This was unsurprising, because the total cell count was dominated by macrophages, which made up more than 70%. Similarly, for eosinophils, the highest count was observed in the OVA/OVA/pbs group (24 100 ± 3420 cells mL⁻¹), the OVA/OVA/cit group had the lowest count (4070 ± 1050 cells mL⁻¹), and the OVA/OVA/peg group was in between (15 800 ± 5930 cells mL⁻¹).

These cell count data suggest that gold nanoparticles have an antiinflammatory effect in mice with OVA-induced airway inflammation since the total cell, macrophage, and eosinophil counts were reduced in the nanoparticle groups. This antiinflammatory effect was smaller for PEGylated than citrated nanoparticles. This might be explained by the coating shielding the gold from interacting with cells, but might also be related to different agglomeration behavior and phagocytosis. Of note, there were no significant cell reductions as a result of nanoparticle treatment in the nonasthmatic animals. This strongly suggests that gold nanoparticles do not affect the inflammatory response in healthy animals. The antiinflammatory mechanism of gold nanoparticles is poorly understood, but one putative mechanism is direct binding of gold nanoparticles to proinflammatory cytokines such as interleukin-1β and, therefore, their inactivation.^[19]

The above patterns were not found for lymphocytes and neutrophils, which showed a tendency for higher cell counts

in nanoparticle-treated groups. The highest number of neutrophils was found in animals treated with citrated gold nanoparticles. Two recent studies using the OVA asthma model with TiO₂ nanoparticles (primary particle size 22 nm) and graphene oxide (particle thickness 0.61 nm, other dimensions between 20 nm and 4 μm) showed similar results and a shift from a Th2-dominated reaction to a Th1 pattern with fewer eosinophils but more neutrophils as a result of nanoparticle treatment.^[27,28] Also in other contexts, immunomodulation with Th1 shifts has been observed for nanoparticles of very different chemistry and size, such as sulfonate- and phosphate-functionalized polystyrene nanoparticles (245 and 227 nm), iron oxide nanoparticles (100 nm), aqueous fullerene C₆₀ nanoparticles (100 nm), and selenium nanoparticles (60 nm).^[29–32] It is not clear whether the immunomodulation depends on specific nanoparticle properties, such as a negative surface charge as the only common feature of the above-mentioned particles, or whether this is a more unspecific phenomenon. In our experiment, the nanoparticles were applied at two different time points. The first dosing occurred at the end of the OVA sensitization, while the second dosing was done shortly after the second OVA challenge. Therefore, the observed immune shift could be due to a nanoparticle influence on either sensitization, challenge or both. It is also possible that the increased numbers of neutrophils and lymphocytes in the nanoparticle-treated groups are a direct irritative effect of the gold nanoparticles, especially the citrated, rather than an immunomodulative effect. This however would not explain why the number of macrophages and eosinophils is reduced as well. In addition to immune cell counts in the BAL, the local effect of nanoparticles on asthma mice was also evaluated by airway resistance measurements (**Figure 3**).

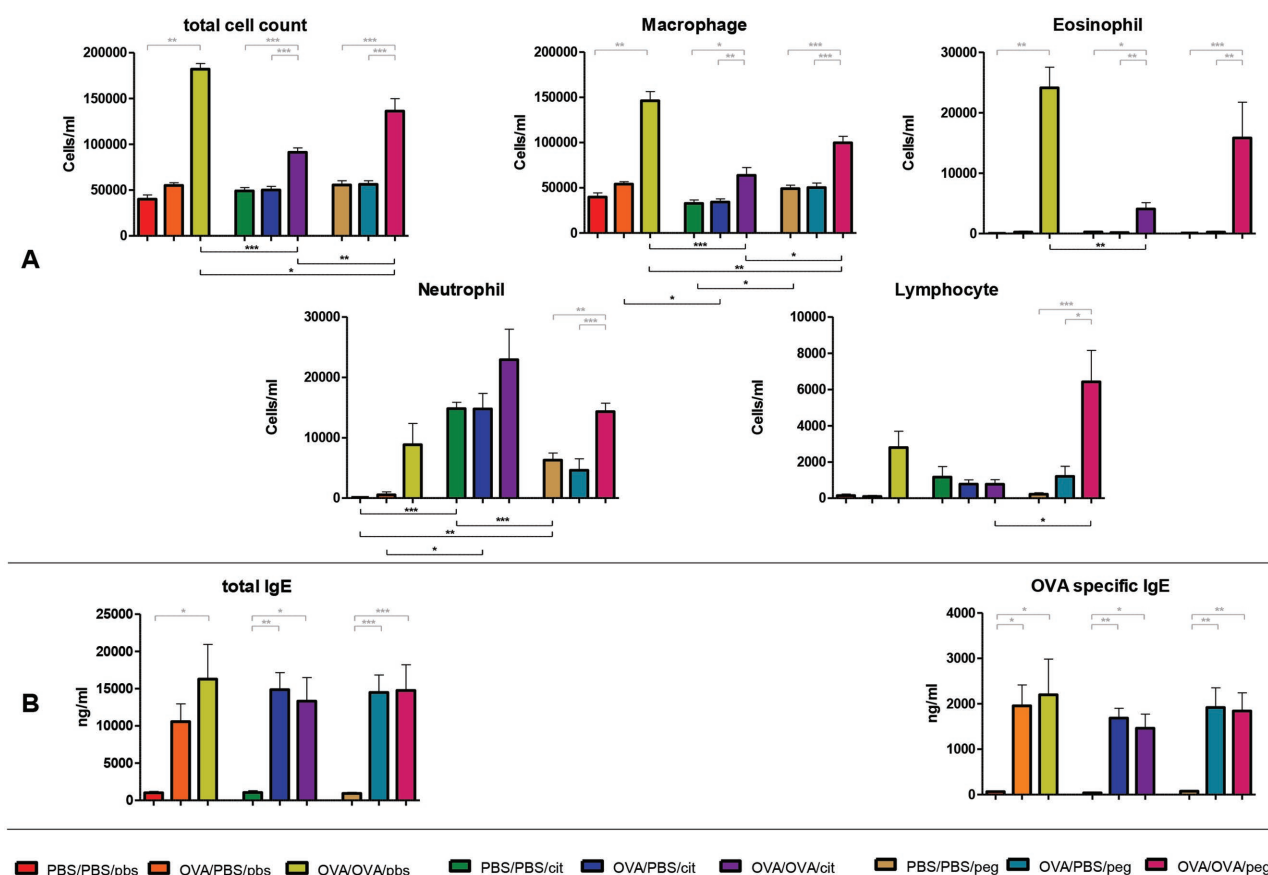


Figure 2. A) BAL cell counts demonstrated more inflammation in the OVA/OVA groups compared to the PBS/PBS and OVA/PBS groups, especially for macrophages and eosinophils. In total cell count, OVA/OVA/pbs ranged significantly ($p < 0.01$) higher than PBS/PBS/pbs; OVA/OVA/cit and OVA/OVA/peg were highly significantly ($p < 0.001$) above PBS/PBS/cit and PBS/PBS/peg. Both citrated and PEGylated gold nanoparticles exerted an overall antiinflammatory effect and resulted in reduced total cell count numbers for OVA/OVA/cit ($p < 0.01$) and OVA/OVA/peg ($p < 0.05$) compared to OVA/OVA/pbs. The antiinflammatory effect in total cell count was significantly stronger for the citrated gold nanoparticles than for the PEGylated particles ($p < 0.01$). This trend was seen for macrophages and eosinophils, but not for lymphocytes and neutrophils, where the opposite effect, an increased cell count in the nanoparticle-treated groups, was observed. B) Total IgE and OVA specific IgE were significantly elevated for the sensitized groups (OVA/PBS and OVA/OVA) compared to the nonsensitized (PBS/PBS) groups.

It has previously been shown that airway reactivity during methacholine challenge is a good indicator of airway hyperresponsiveness, and reactivity can be measured as the slope of linearized concentration-response curves. Linearization was achieved by plotting the natural logarithm of the total respiratory resistance (Rrs) as percentage of baseline against the methacholine concentration as previously described.^[33–36] Here, response curve slopes showed a pattern similar to that seen with BAL total cell counts. The asthma group without nanoparticles' treatment (OVA/OVA/pbs) had the highest slope, while the nanoparticle-treated asthma (OVA/OVA/cit and OVA/OVA/peg) groups were only slightly above their control groups. Although the measured slope increases were smaller than expected and rather seen at the lower methacholine concentrations, the overall trend is consistent with the observed cell counts for macrophages and eosinophils, which were also highest in the asthma group without nanoparticle treatment.

There are two previous conflicting studies about the local effects of gold nanoparticles applied to the respiratory tract of asthmatic mice. Barreto et al. investigated the effect of 12 nm citrated gold nanoparticles on an OVA

asthma model in Swiss-Webster and A/J mice and found that gold nanoparticle treatment reduced OVA-induced inflammation and airway hyperresponsiveness, similar to our results.^[20] In the second study, Hussain et al. used 40 nm citrated gold nanoparticles in toluene diisocyanate (TDI)-induced asthma in BALB/c mice and reported that both the inflammation and airway hyperresponsiveness increased due to gold nanoparticle treatment.^[21] The gold nanoparticles in these studies had comparable surface chemistry to the citrated gold nanoparticles in our study but were of different size. One explanation for the contradictory effects of nanosized gold could be different inflammation profiles in the TDI and OVA asthma models, as suggested by Barreto et al., or the different nanoparticle sizes. This latter hypothesis is supported by a recent study, which demonstrated that gold nanoparticles caused a size-dependent reduction in IL-1 β -dependent inflammatory responses in human myeloid leukemia cells. Gold nanoparticles with 5 nm diameter had the strongest inhibitory effect, 15 nm gold nanoparticles were less inhibitory, while 35 nm gold nanoparticles had no significant effect.^[19] Since the particles used by Hussain et al. were even larger than 35 nm, it is possible that the

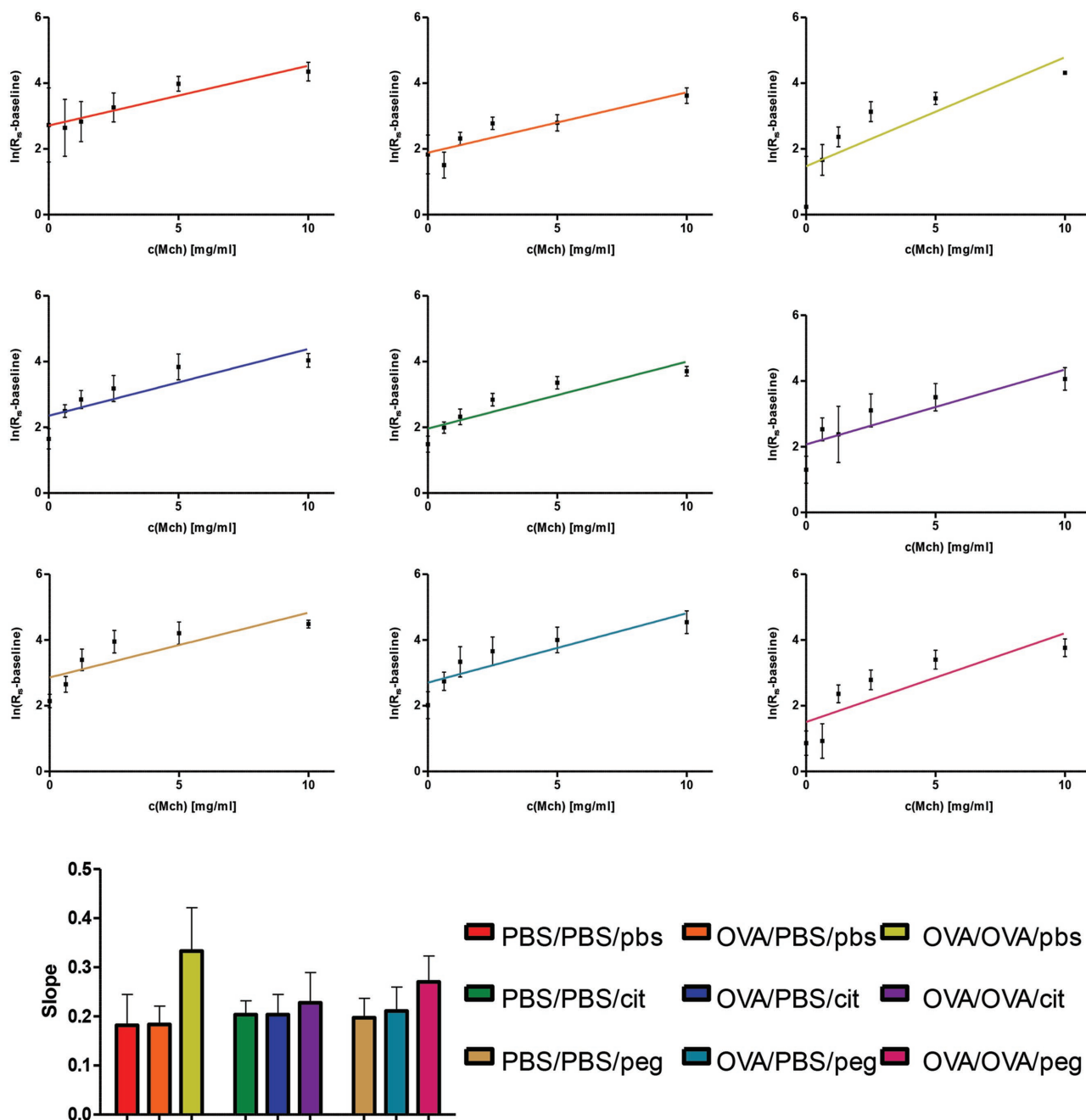


Figure 3. The reactivity is shown as the slope of the linearized concentration response curves in the airway resistance measurement with methacholine challenge. The column diagram compares the slope values between all groups. The highest slope was seen for the asthma group without nanoparticle treatment (OVA/OVA/pbs). The asthma groups with gold nanoparticle treatment (OVA/OVA/cit and OVA/OVA/peg) were only slightly increased. This resembles the antiinflammatory effect seen for BAL total cell count in Figure 2.

antiinflammatory mechanism was outcompeted by another proinflammatory effect.

2.2. Detection of Gold Nanoparticles in Lung Tissue Sections

To understand the mechanism by which gold nanoparticles suppress inflammation and airway hyperresponsiveness, we next localized them in lung tissue sections. Light microscopy analysis revealed black cytosolic agglomerations in both the citrated and PEGylated gold nanoparticle groups, which were

absent in the lungs of pbs-treated mice. Silver staining by auto-metallography enhanced the black agglomerations, suggesting that these agglomerations were formed from gold nanoparticles since only gold, silver, and some metal sulfides and selenides are positively stained by this method.^[37] However, to definitively prove that the black agglomerations were indeed gold nanoparticles, agglomerations were further analyzed by electron microscopy with energy dispersive X-ray spectroscopy (EDX) (**Figure 4**). The gold peaks detected by scanning electron microscopy (SEM)-EDX were found at the same locations as the black agglomerations seen by light microscopy.

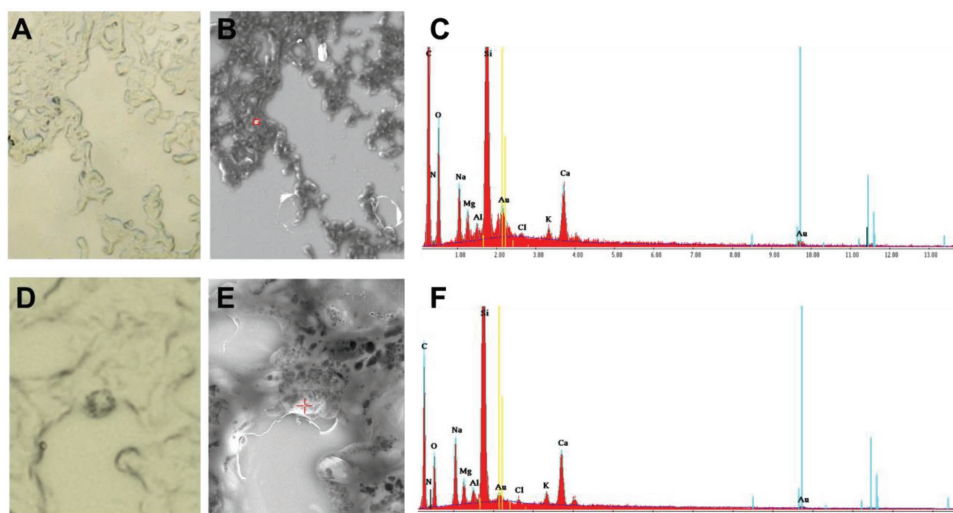


Figure 4. To prove that the black precipitates in alveolar macrophages in A) light microscopy images were citrated gold nanoparticles, the same area of the same section was subjected to B) electron microscopy. At the marked position in (B), a local EDX scan for chemical elements was performed. C) The EDX spectrum showed strong gold signals, which were only found at locations containing black agglomerations and proving that the black spots in the alveolar macrophages were citrated gold nanoparticles. D–F) show the same for the PEGylated nanoparticles.

Immunofluorescence staining (**Figure 5**) was next used to evaluate which cell types contained the gold precipitates. No differences were seen between citrated and PEGylated nanoparticles (data not shown). The nanoparticle-containing cells were CD11c positive, F4/80 positive, inducible Nitric Oxide Synthase (iNOS) positive, and Major Histocompatibility Complex II (MHCII) low, the expected pattern for

alveolar macrophages.^[38–40] Other cell populations were also identified: Siglec-F positive and CD11c negative cells (presumably eosinophils), Ly6G positive cells with fragmented nucleus (presumably neutrophils), CD3ε positive cells (presumably T-Lymphocytes), and CD45r positive cells (presumably B-Lymphocytes). Of those cells, only the Siglec-F positive and CD11c negative cells (eosinophils)

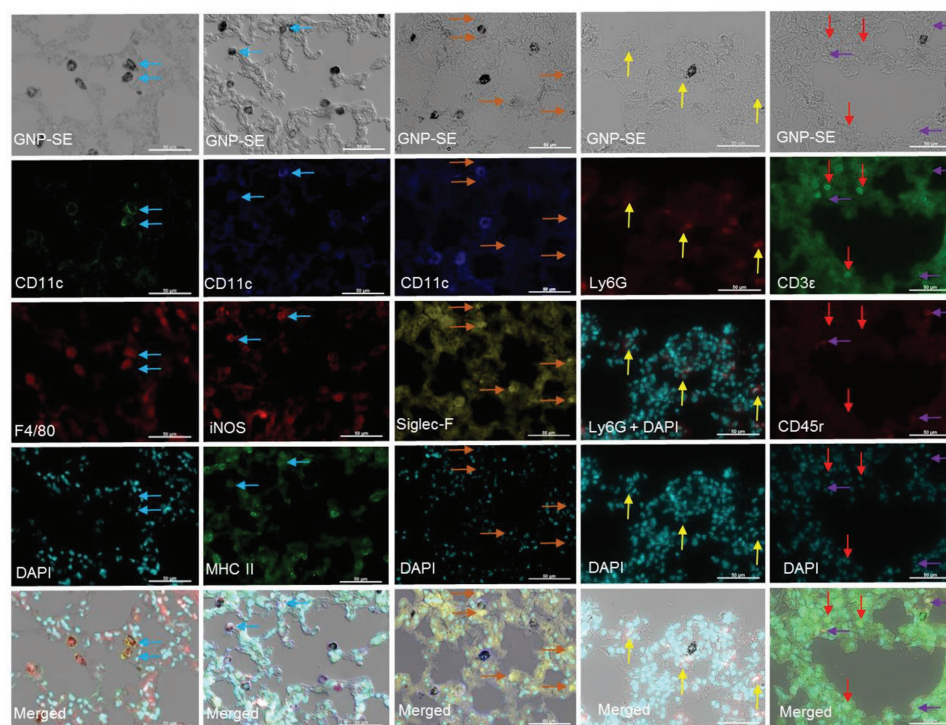


Figure 5. Immunofluorescence staining of lung sections showed that cells containing black precipitates (stained with silver enhancement (SE)) were CD11c positive, F4/80 positive, iNOS positive, and MHCII low. Therefore, these cells were likely to be alveolar macrophages (blue arrows). Small amounts of black precipitates were also seen in Siglec-F positive and CD11c negative cells (orange arrows, presumably eosinophils). Other cells, such as Ly6G positive cells (yellow arrows, presumably neutrophils), CD3ε positive (red arrows, presumably T-Lymphocytes), and CD45r positive (magenta arrows, presumably B-Lymphocytes) did not show visible amounts of gold nanoparticles.

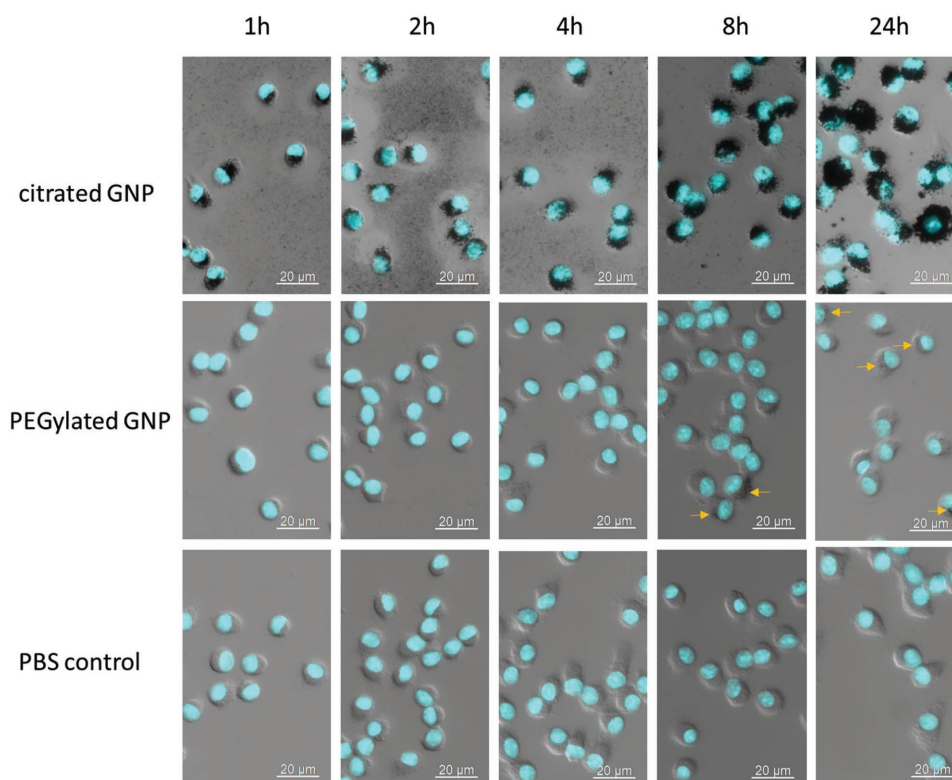


Figure 6. Adherent mouse alveolar macrophage cultures were treated with 80 μL of either citrated gold nanoparticles (0.4 mg mL^{-1}), PEGylated gold nanoparticles (0.4 mg mL^{-1}), and PBS. After different time periods, the cells were 4, 6-diamidino-2-phenylindole (DAPI) stained and analyzed by microscope. In the wells treated with the citrated gold nanoparticles, nanoparticle aggregates were found intra- and extracellular at all time points. There are halos in the extracellular precipitates around the macrophages, which suggests phagocytosis of extracellular nanoparticle aggregates. The PEGylated gold nanoparticles showed no extracellular agglomeration. After 8 h, aggregates of the PEGylated gold nanoparticles started to form inside the macrophages. Even more aggregates were visible after 24 h, although much less than in the wells with citrated particles. There were no aggregates in the PBS control. The cell culture medium caused agglomeration of the citrated gold nanoparticles prior to phagocytosis. In case of the PEGylated gold nanoparticles, agglomeration started later and only inside the macrophages. The absence of extracellular aggregates suggests that those particles were phagocytosed in nonagglomerated form.

showed gold precipitates, albeit at much smaller amounts than the alveolar macrophages. Eosinophils are described in the literature as capable of phagocytosis.^[41] It is remarkable that the cell populations where nanoparticle phagocytosis was observed (macrophages and eosinophils) were also those where the strongest reduction in BAL count due to nanoparticle treatment was seen.

In contrast to the citrated gold nanoparticles, it was surprising to find PEGylated gold nanoparticles agglomerated inside alveolar macrophages at quantities high enough to be detectable by light microscopy. Vacuum evaporation showed (Figure S2, Supporting Information) that the PEGylated gold nanoparticles were extremely resistant to agglomeration and tolerated salt concentrations above physiological level. In addition, mixing with $1\times$ PBS and murine bronchial mucus *in vitro* did not induce agglomeration of the PEGylated particles, while it agglomerated the citrated gold nanoparticles.

We next analyzed phagocytosis of PEGylated and citrated particles over time in macrophage cell cultures (Figure 6). Citrated gold nanoparticle aggregates were found in almost all cells at all time points. There were also agglomerates that precipitated outside of the macrophages. Agglomeration most likely happens prior to phagocytosis, directly after the nanoparticles get into contact with

the cell culture medium. The halos with less precipitation around the macrophages seem to be the result of phagocytosis activity. In contrast, PEGylated nanoparticles formed no extracellular aggregates. After 8 h they agglomerated only inside the macrophages but to a lesser degree than the citrated with further accumulation after 24 h. This suggests that macrophages can internalize nonagglomerated PEGylated 5 nm gold nanoparticles and induce intracellular agglomeration over extended time periods. This is consistent with the *in vivo* observations in mouse lung sections representing seven days of uptake from first nanoparticle exposure to the end of the experiment. Other studies suggest that a low lysosomal pH and proteases destabilize and destroy polymeric nanoparticle coatings to promote intracellular agglomeration.^[42–44]

2.3. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Analysis

We next examined systemic uptake of the nanoparticles. Previous studies have shown that nanoparticles applied via the respiratory tract can cross the blood–air barrier, providing an easy route for systemic administration.^[45,46]

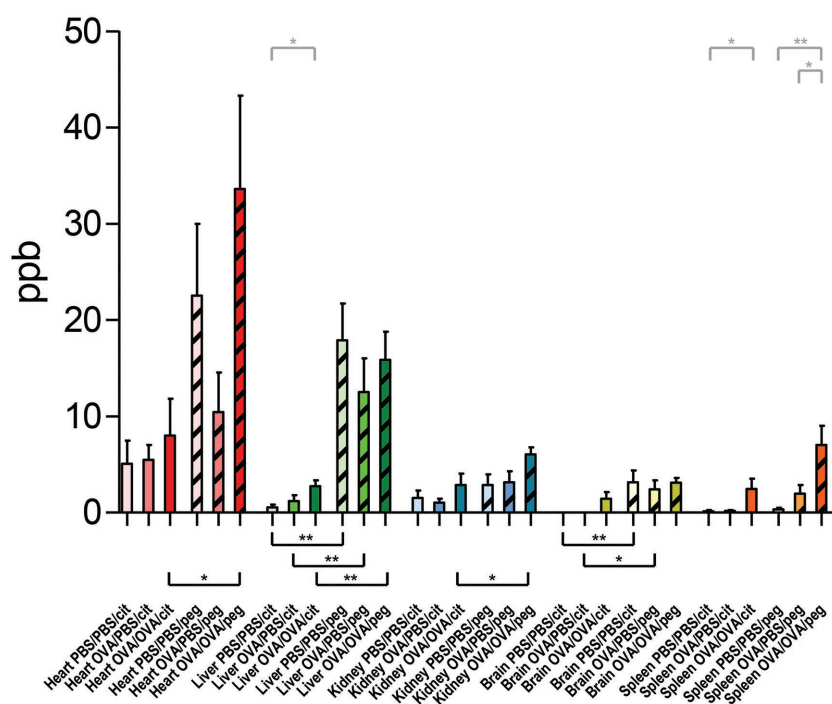


Figure 7. Gold content (mass gold/mass organ in ppb) in different organs measured by ICP-MS for citrated (no pattern) and PEGylated (cross-hatched) gold nanoparticles. Overall, gold nanoparticle uptake was greatest in the heart and lowest in the brain. Black bars indicate significant differences in uptake between PEGylated and citrated particles. The gray bars represent significant differences in uptake between asthma (OVA/OVA) and nonasthma groups (PBS/PBS and OVA/PBS). For all organs, accumulation of gold nanoparticles was greater for PEGylated nanoparticles, most obviously for the liver (significant at $p < 0.01$). In addition, there was an overall trend of higher gold nanoparticle uptake for mice with asthma, especially visible in the spleen (significant at $p < 0.05$ for cit and $p < 0.01$ for peg nanoparticles). The groups without nanoparticle treatment (PBS/PBS/pbs, OVA/PBS/pbs, and OVA/OVA/pbs) had no detectable gold levels in ICP-MS and are therefore not shown in this graph.

Small and negatively charged nanoparticles appear to show higher uptake across the air–blood barrier. One study reported an inversely proportional relationship between gold nanoparticle core diameter and air–blood barrier crossing.^[12] In another study with polymeric nanoparticles, only negatively charged nanoparticles with a hydrodynamic diameter less than 34 nm showed high pulmonary uptake.^[47] In light of these findings, we decided to use small gold nanoparticles, and transmission electron microscopy (TEM) measurements confirmed a 5 nm core diameter of our nanoparticles. By dynamic light scattering (DLS), citrated and PEGylated nanoparticles had hydrodynamic diameters of 9 nm (polydispersity of 28.5%) and 6 nm (polydispersity of 21.8%), respectively, both well below the 34 nm threshold. In addition, zeta potential measurements verified that both our citrated (−51 mV) and PEGylated (−43 mV) nanoparticles had a negative surface charge, which is not only an important factor for crossing the air–blood barrier but also increases blood circulation times after intravenous injection.^[48]

Using ICP-MS (**Figure 7**), we were able to detect gold nanoparticle uptake to extrapulmonary organs in nanoparticle-treated mice. As expected, no detectable gold was found in the control groups without nanoparticle treatment (PBS/PBS/pbs, OVA/PBS/pbs, and OVA/OVA/pbs). Overall, the

gold concentrations in extrapulmonary organs were in the ppb range and were several magnitudes smaller than the concentrations detected in the lungs. The mean extrapulmonary concentrations were less than 0.01% of the lung concentration for citrated particles and less than 0.1% for PEGylated particles. In addition, there was unequal distribution of the gold nanoparticles to different extrapulmonary organs. The highest gold levels were found in the heart for both citrated (5–8 ppb range) and PEGylated gold nanoparticles (10–34 ppb range), while the kidney, brain, and spleen had overall lower gold concentrations. However, for the liver, the uptake of citrated gold nanoparticles was also relatively low (0.5–3 ppb range) while uptake of PEGylated nanoparticles was closer to that found in the heart (12–18 ppb range).

Kidney tissue of mice with the high gold levels in ICP-MS was also taken for SEM and TEM measurements, where no glomerular nanoparticle depositions could be found (**Figure 8**). Therefore, it seems those detected gold levels are rather a consequence of normal excretory process and not caused by glomerular nanoparticle deposition.

For all organs, there was an overall trend toward higher nanoparticle uptake in mice with OVA-induced inflammation. This trend was most visible in the spleen, which showed very low gold levels in

the nonasthma groups (0.1 ppb for cit and 0.3 ppb for peg) and significantly ($p < 0.05$ and $p < 0.01$, respectively) more gold in the asthma groups for both citrated (2.5 ppb) and PEGylated nanoparticles (7 ppb). The higher nanoparticle uptake in asthma animals could be due to barrier defects and increased lung blood flow. Although this is the first study of the influence of OVA-induced asthma on the translocation of nanoparticles through the lung, one other study tested the effect of lipopolysaccharide (LPS)-induced lung inflammation on the uptake of 40 nm citrated gold nanoparticles.^[13] However, these results are not directly comparable because LPS-induced inflammation has a different immune cell profile similar to a neutrophilic pneumonia, and as noted above different nanoparticle sizes can have a large impact on behavior. Nevertheless, both our experiments^[13] show that inflammation of the respiratory tract most affects the spleen, which shows the greatest increase in nanoparticle uptake compared to other organs. Since inflammation-induced barrier defects do not explain such behavior, we hypothesize that there is increased migration of nanoparticle-loaded immune cells to the spleen or increased phagocytosis of blood borne nanoparticles by active immune cells in the spleen in respiratory inflammation. This requires further investigation.

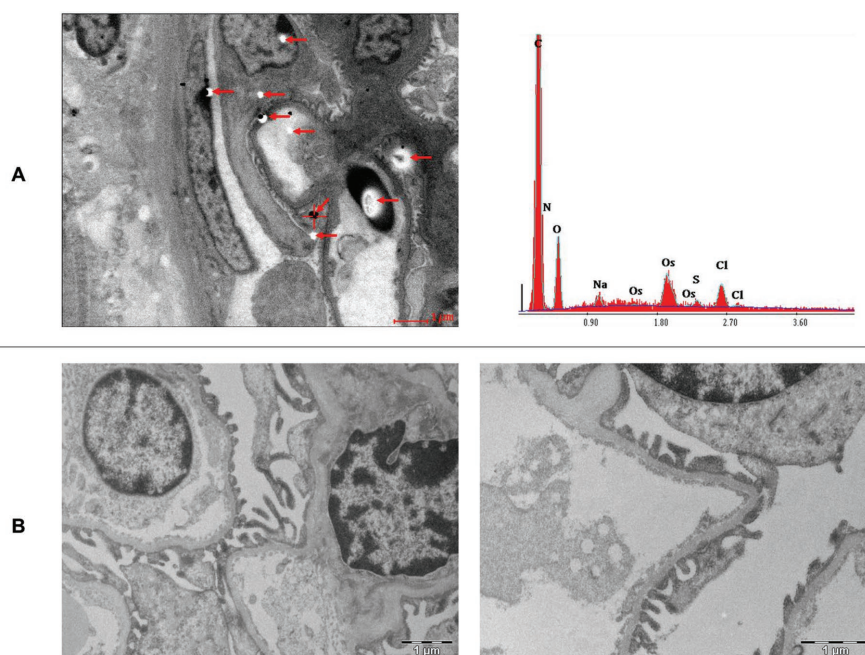


Figure 8. SEM and TEM images of kidney samples with high nanoparticle content have been taken to look for glomerular accumulations. A) In SEM, black agglomerations were found, but EDX scans (locations marked by arrows, spectrum exemplarily shown for + revealed that those were artifacts with no gold content, caused by fixation with osmium tetroxide. B) In a second step, nonosmicated but double stained (uranyl acetate and lead citrate) ultrathin sections have been visualized by TEM. There, no accumulations that could have been caused by gold nanoparticles were seen in the glomeruli.

Our data also show that the surface coating had a stronger influence on nanoparticle uptake than the asthma status. Greater uptake of PEGylated gold nanoparticles compared to citrated gold nanoparticles was found in all organs but was most significant ($p < 0.01$) in the liver with an increase in uptake of more than 10 ppb. This could be due to higher stability of PEGylated nanoparticles against agglomeration or, as previously described, enhanced mucus penetration due to PEGylation.^[17,18] We showed above that the citrated nanoparticles more strongly suppressed local features of asthma than the PEGylated nanoparticles, perhaps due to more local interactions as a result of earlier and more extensive agglomeration of citrated particles. This would result in fewer particles being available for systemic uptake. Our results seem to contrast with another study on radioactively labeled gold nanoparticles and non-PEGylated particles with two different PEG sizes (750 Da and 10 kDa chains), which reported that PEGylation had no influence on nanoparticle uptake from the airways.^[15] Citrate nanoparticles stabilized with bis(p-sulfonatophenyl)phenylphosphine were used as the non-PEGylated control. In our opinion, this might explain the small differences between PEGylated and non-PEGylated groups, because another recent publication showed that bis(p-sulfonatophenyl)phenylphosphine is more effective at preventing agglomeration caused by high salt concentrations than citrate.^[49] Further, in our TEM measurement, the PEGylated nanoparticles showed much higher agglomeration resistance during solvent evaporation than citrated particles. Together, these data support our assumption that the changed agglomeration behavior of the PEGylated gold

nanoparticles could be responsible for the higher observed nanoparticle uptake.

3. Conclusion

Here we performed intranasal instillation of citrated and PEGylated gold nanoparticles in BALB/c-mice with and without an asthma-like airway inflammation. This is the first description of pulmonary nanoparticle uptake to extrapulmonary organs in the context of asthma. There was increased nanoparticle uptake in mice with an asthmatic precondition, especially to the spleen. This suggests that the host factor of airway inflammation needs to be considered when evaluating potential nanoparticle toxicity. There was also greater systemic uptake of PEGylated gold nanoparticles than citrated nanoparticles after respiratory application, with the influence of the coating greater than that of asthma on gold nanoparticle uptake. We suggest that different agglomeration kinetics of citrated and PEGylated gold nanoparticles are responsible for this observed effect. Citrated gold nanoparticles agglomerated rapidly in physiological fluids, while PEGylated particles remained stable. Nevertheless, PEGylated gold nanoparticle agglomerates were still observed in alveolar macrophages. Moreover, we demonstrated an antiinflammatory effect of gold nanoparticles (particularly citrated) on OVA-induced airway inflammation, which was reflected in the number of immune cells in the airways and in lung function tests. Our results suggest that gold nanoparticle-based asthma drugs have therapeutic potential. However, systemic uptake of gold nanoparticles through the respiratory tract is

increased by asthma and must be considered to avoid potential adverse effects.

4. Experimental Section

Nanoparticle Preparation: Suspensions of PEG 3000-coated and carboxylic-acid-functionalized gold nanoparticles and citrate and tannic acid coated gold nanoparticles in 0.1×10^{-3} M PBS, both with a core size of 5 nm, were obtained from Sigma-Aldrich (St Louis, MI). Nanoparticles were characterized by DLS and zeta-potential measurements (Smoluchowski limit) and TEM. For DLS, number-weighted size distributions were obtained (scattering angle 173° , wavelength 633 nm, Zeta Sizer Nano ZSP, Malvern Instruments). For TEM, the nanoparticle suspensions were dried on pioloform TEM grids and analyzed with a FEI Tecnai 12 Biotwin TEM setup.

To obtain equal concentrations of samples for intranasal application, PEGylated nanoparticle suspensions were diluted by 8.33 with 0.6×10^{-3} M PBS and citrated nanoparticles were concentrated sixfold by vacuum evaporation, yielding a gold concentration of 0.4 mg mL^{-1} . The quality of each nanoparticle sample was controlled by ultraviolet–visible spectroscopy on the day of application prior to administration (Figure S3, Supporting Information). Frozen nanoparticle samples were later diluted to $40 \mu\text{g mL}^{-1}$ and tested for LPS with a commercial endpoint chromogenic Limulus Amebocyte Lysate (LAL) assay (QCL-1000 kit, Lonza, Verviers, Belgium). This assay was chosen because it was shown to work well in the presence of nanoparticles.^[50] The PEGylated nanoparticles showed absorption at 407 nm which had to be subtracted from the samples, while the citrated nanoparticles had no relevant absorption at that wavelength because of nanoparticle degradation as a result of the freezing and unfreezing process. In both nanoparticle samples, the LPS content was below detection limit (0.1 EU mL^{-1}).

Animals: Female wild-type BALB/c-mice (6–8 weeks old) were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and kept in regular 12 h dark/light cycles at 22°C with laboratory food and tap water ad libitum. Mice were acclimatized for at least two weeks prior to study. All animal experiments were performed in strict accordance with German animal protection laws with approval from the appropriate governmental authority.

OVA Mouse Model and Nanoparticle Application: BALB/c mice were sensitized to the allergen OVA by ip injection with aluminum-hydroxide-adsorbed OVA (2 mg AlOH_3 with 20 μg ovalbumin) on days 1, 14, and 21. Control animals received PBS. On days 26 and 27, a proportion of BALB/c mice were challenged with 20 min exposure to nebulized 1% (w/w) OVA in PBS and a control group was nebulized with PBS (Figure S4, Supporting Information).

Nanoparticles were applied on days 20 and 27: mice were narcotized with isoflurane and 50 μL of nanoparticle suspension was applied intranasally to each test mouse. One-third of the mice received PEGylated gold nanoparticles, one-third citrated gold nanoparticles, and the remainder PBS as a control.

Airway Resistance Assay: On day 28, mice were anaesthetized with an ip injection of ketamine hydrochloride (100 mg kg^{-1}) and xylazine hydrochloride (8 mg kg^{-1}). They were then tracheostomized with an 18 gauge cannula and connected to a SCIREQ flexiVenti device, which ventilated the mice with a tidal volume of

10 mL kg^{-1} at a frequency of 150 breaths per minute. The flexiWare software was used to measure the total respiratory resistance (Rrs). A nebulizer synchronized to inspiration was connected to the flexiVent setup. PBS was nebulized for the first measurement, after which five more measurements with methacholine nebulization at increasing concentrations (0.625 – 10 mg mL^{-1}) were performed.

Tissue Sampling and ICP-MS Analysis: Mice were killed by bleeding after airway resistance measurements. Their organs were then carefully removed, taking care to thoroughly clean tweezers and scalpels between removal of each organ to prevent cross-contamination with gold residues. Lung tissue was obtained for histological analysis and BAL was conducted for cell-counting studies. For ICP-MS screening for gold, portions of several organs were cut, weighed, and dissolved in 5 mL concentrated ultrapure HNO_3 followed by addition of a 4% (v/v) solution of ultrapure HCl to a final volume of 10 mL.

After several days at room temperature, the samples were completely dissolved and were analyzed for gold (Au-197) using an Agilent 7500cx (Agilent Technologies, Santa Clara, CA). Holmium 165 was used as an internal standard. Based on a NIST protocol,^[51] a diluent containing 1.5% (v/v) HNO_3 and 4% (v/v) HCl was prepared and used.

Staining, Histological Analysis, and Light Microscopy: Lung tissue cryosections obtained from the different experimental groups were examined with a Zeiss Axio Imager.M2 microscope (Carl Zeiss AG, Oberkochen, Germany). In addition to H&E and PAS staining, immunofluorescence (IF) staining for CD11c, F4/80, MHC II, iNOS, Siglec-F, Ly6G, CD3 ϵ , and CD45r was conducted. For IF, cryosections were dried at 20°C for 15 min, rehydrated for 5 min in PBS, and incubated with 5% serum to reduce nonspecific binding. Sections were then incubated with primary antibodies (antimouse CD11c (Biolegend, San Diego, CA), antimouse F4/80 (eBioscience, San Diego, CA), antimouse MHC II (Biolegend), antimouse iNOS (Novus Biologicals, Littleton, CO), antimouse Siglec-F (eBioscience, San Diego, CA), antimouse Ly6G (Abcam, Cambridge, UK), antimouse CD3 ϵ (Biolegend), and antimouse CD45r (eBioscience, San Diego, CA)) for 1 h at 20°C and then incubated overnight at 4°C . Sections were then rinsed twice with 0.1 M PBS before incubation with secondary fluorescein-conjugated antibodies (donkey antirat cyanine Cy3, donkey antirat IgG Alexa Fluor 488, goat anti-Armenian hamster Dylight 649, goat anti-Armenian hamster IgG Alexa Fluor 488, donkey antirabbit IgG cyanine Cy3, donkey antirabbit IgG Alexa Fluor 488) for 2 h at room temperature. Sections were counterstained with 100 μL DAPI ($0.5 \mu\text{g mL}^{-1}$, Carl Roth, Karlsruhe, Germany) for 15 min at 20°C , washed twice with 0.1 M PBS and once with double-distilled water, mounted with fluorescent mounting medium Fluoroshield (Sigma-Aldrich, St Louis, MI), and coverslipped. A silver enhancement kit (Sigma-Aldrich, St Louis, MI) was used to visualize small gold nanoparticle accumulations.

BAL: 1 mL ice-cold PBS was instilled into the lungs as described previously.^[25,52] Total cell counts were performed. After centrifugation ($320\times g$, 10 min, 4°C), supernatants were removed and the pellets were resuspended in 1 mL PBS. For differential cell counting, cytopots were prepared and stained with Diff-Quik.

SEM-EDX: Lung sections of interest identified by light microscopy were scanned for gold particles with a SEM-EDX electron microscope (FEI/Philips XL 30 FEG ESEM) after removing the coverslips of sections prepared for light microscopy. Kidney tissue, fixed

in 2.5% glutardialdehyde and dehydrated in an ascending series of ethanol, was treated with 2% osmium tetroxide and coated with carbon were also evaluated with this setup.

TEM: Kidney tissue was fixed in 2.5% glutardialdehyde and dehydrated in an ascending series of ethanol. After dehydration, the samples were embedded in Embed 812 resin. No osmium tetroxide was used. Ultrathin sections were prepared and contrasted with uranyl acetate and lead citrate. Measurements were done with a FEI Tecnai 12 Biotwin TEM setup.

IgE Measurement: Total IgE and OVA specific IgE were tested with Elisa. Mouse IgE ELISA Ready-SET-Go! (eBioscience, San Diego, CA) and Anti-Ovalbumin IgE (mouse) ELISA Kit (Cayman Chemical, Ann Arbor, MI) were used according to the instructions.

In vitro Experiments: Nanoparticle phagocytosis was examined over time in mouse macrophage cell cultures. Alveolar macrophages were isolated from BALB/c BALs and grown as adherent cultures in RPMI medium in eight-chamber culture dishes at 37 °C. Macrophages were treated with 80 µL gold nanoparticle dispersions at the same concentrations as received by the mice. Samples were fixed, stained, and cells counted 1, 2, 4, 8, and 24 h after treatment.

Statistical Analysis: Statistical analysis was performed with GraphPad Prism 5.02 (GraphPad Software, Inc., La Jolla, CA). Data are presented as means ± SEM. The Kolmogorov–Smirnov test was used to test for Gaussian distribution. Differences between groups were tested with unpaired t-test and ANOVA for Gaussian distributed groups and with the Mann Whitney test and Kruskal–Wallis test for non-Gaussian distributed groups. Grubbs' test ($p < 0.05$) was used to detect and eliminate outliers. p -values < 0.05 (*) and < 0.01 (**) were considered significant and p -values < 0.001 (***) were considered highly significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

J.N. and Q.T.D. contributed equally to this work. This project was supported by the foundation "Stiftung Bergmannshilfswerks Luisenthal" to Prof. Q. T. Dinh. The authors also thank Sarah Schmidt for her assistance in DLS and zeta potential measurements.

- [1] S. D. Brown, P. Nativo, J. A. Smith, D. Stirling, P. R. Edwards, B. Venugopal, D. J. Flint, J. A. Plumb, D. Graham, N. J. Wheate, *J. Am. Chem. Soc.* **2010**, *132*, 4678.
- [2] S. K. Libutti, G. F. Paciotti, A. A. Byrnes, H. R. Alexander Jr., W. E. Gannon, M. Walker, G. D. Seidel, N. Yuldasheva, L. Tamarkin, *Clin. Cancer Res.* **2010**, *16*, 6139.
- [3] J. Hrkach, H. D. Von, A. M. Mikkaram, E. Andrianova, J. Auer, T. Campbell, W. D. De, M. Figa, M. Figueiredo, A. Horhota, S. Low, K. McDonnell, E. Peeke, B. Retnarajan, A. Sabnis, E. Schnipper, J. J. Song, Y. H. Song, J. Summa, D. Tompsett, G. Troiano,

- H. T. Van Geen, J. Wright, P. LoRusso, P. W. Kantoff, N. H. Bander, C. Sweeney, O. C. Farokhzad, R. Langer, S. Zale, *Sci. Transl. Med.* **2012**, *4*, 128ra39.
- [4] D. D. Von Hoff, M. M. Mita, R. K. Ramanathan, G. J. Weiss, A. C. Mita, P. M. LoRusso, H. A. Burris 3rd, L. L. Hart, S. C. Parsons, D. M. Low, S. E. Zale, J. M. Summa, H. Youssoufian, J. C. Sachdev, *Clin. Cancer Res.* **2016**, *22*, 3157.
- [5] J. G. Morton, E. S. Day, N. J. Halas, J. L. West, *Methods Mol. Biol.* **2010**, *624*, 101.
- [6] J. M. Stern, V. V. Solomonov, E. Sazykina, J. A. Schwartz, S. C. Gad, G. P. Goodrich, *Int. J. Toxicol.* **2016**, *35*, 38.
- [7] N. Khlebtsov, L. Dykman, *Chem. Soc. Rev.* **2011**, *40*, 1647.
- [8] E. Seydoux, L. Rodriguez-Lorenzo, R. A. Blom, P. A. Stumbles, A. Petri-Fink, B. R. Rutishauser, F. Blank, C. von Garnier, *Nano-medicine* **2016**, *12*, 1815.
- [9] N. R. Jacobsen, P. Moller, K. A. Jensen, U. Vogel, O. Ladefoged, S. Loft, H. Wallin, *Part. Fibre Toxicol.* **2009**, *6*, 2.
- [10] J. H. Sung, J. H. Ji, J. D. Park, M. Y. Song, K. S. Song, H. R. Ryu, J. U. Yoon, K. S. Jeon, J. Jeong, B. S. Han, Y. H. Chung, H. K. Chang, J. H. Lee, D. W. Kim, B. J. Kelman, I. J. Yu, *Part. Fibre Toxicol.* **2011**, *8*, 16.
- [11] I. Gossens, J. A. Post, L. J. de la Fonteyne, E. H. Jansen, J. W. Geus, F. R. Cassee, W. H. de Jong, *Part. Fibre Toxicol.* **2010**, *7*, 37.
- [12] W. G. Kreyling, S. Hirn, W. Moller, C. Schleh, A. Wenk, G. Celik, J. Lipka, M. Schaffler, N. Haberl, B. D. Johnston, R. Sperling, G. Schmid, U. Simon, W. J. Parak, M. Semmler-Behnke, *ACS Nano* **2014**, *8*, 222.
- [13] S. Hussain, J. A. Vanoirbeek, S. Haenen, V. Haufröid, S. Boland, F. Marano, B. Nemery, P. H. Hoet, *BioMed. Res. Int.* **2013**, *2013*, 923475.
- [14] S. Takenaka, W. Moller, M. Semmler-Behnke, E. Karg, A. Wenk, O. Schmid, T. Stoeger, L. Jennen, M. Aichler, A. Walch, S. Pokhrel, L. Madler, O. Eickelberg, W. G. Kreyling, *Nanomedicine* **2012**, *7*, 855.
- [15] J. Lipka, M. Semmler-Behnke, R. A. Sperling, A. Wenk, S. Takenaka, C. Schleh, T. Kissel, W. J. Parak, W. G. Kreyling, *Biomaterials* **2010**, *31*, 6574.
- [16] M. Semmler-Behnke, W. G. Kreyling, J. Lipka, S. Fertsch, A. Wenk, S. Takenaka, G. Schmid, W. Brandau, *Small* **2008**, *4*, 2108.
- [17] A. Ibricevic, S. P. Guntzen, K. Zhang, R. Shrestha, Y. Liu, J. Y. Sun, M. J. Welch, K. L. Wooley, S. L. Brody, *Nanomedicine* **2013**, *9*, 912.
- [18] J. S. Suk, A. J. Kim, K. Trehan, C. S. Schneider, L. Cebotaru, O. M. Woodward, N. J. Boylan, M. P. Boyle, S. K. Lai, W. B. Guggino, J. Hanes, *J. Controlled Release* **2014**, *178*, 8.
- [19] V. V. Sumbayev, I. M. Yasinska, C. P. Garcia, D. Gilliland, G. S. Lall, B. F. Gibbs, D. R. Bonsall, L. Varani, F. Rossi, L. Calzolari, *Small* **2013**, *9*, 472.
- [20] E. Barreto, M. F. Serra, R. V. Dos Santos, C. E. Dos Santos, J. Hickmann, A. C. Cotias, C. R. Pao, S. G. Trindade, V. Schmidt, C. Giacomelli, V. F. Carvalho, Rodrigues E. Silva PM, R. S. Cordeiro, M. A. Martins, *J. Biomed. Nanotechnol.* **2015**, *11*, 1038.
- [21] S. Hussain, J. A. Vanoirbeek, K. Luyts, V. De Vooght, E. Verbeken, L. C. Thomassen, J. A. Martens, D. Dinsdale, S. Boland, F. Marano, B. Nemery, P. H. Hoet, *Eur. Respir. J.* **2011**, *37*, 299.
- [22] P. S. Foster, S. P. Hogan, A. J. Ramsay, K. I. Matthaei, I. G. Young, *J. Exp. Med.* **1996**, *183*, 195.
- [23] J. M. Drazen, J. P. Arm, K. F. Austen, *J. Exp. Med.* **1996**, *183*, 1.
- [24] R. A. Joachim, L. B. Cifuentes, V. Sagach, D. Quarcoo, E. Hagen, P. C. Arck, A. Fischer, B. F. Klapp, Q. T. Dinh, *Clin. Exp. Allergy* **2006**, *36*, 1001.
- [25] U. Herz, A. Braun, R. Ruckert, H. Renz, *Clin. Exp. Allergy* **1998**, *28*, 625.
- [26] K. Ebino, R. Lemus, M. H. Karol, *Inhalation Toxicol.* **1999**, *11*, 171.
- [27] S. Jonasson, A. Gustafsson, B. Koch, A. Bucht, *Inhalation Toxicol.* **2013**, *25*, 179.
- [28] M. R. Shurin, N. Yanamala, E. R. Kisin, A. V. Tkach, G. V. Shurin, A. R. Murray, H. D. Leonard, J. S. Reynolds, D. W. Gutkin, A. Star,

- B. Fadeel, K. Savolainen, V. E. Kagan, A. A. Shvedova, *ACS Nano* **2014**, *8*, 5585.
- [29] S. U. Frick, N. Bacher, G. Baier, V. Mailander, K. Landfester, K. Steinbrink, *Macromol. Biosci.* **2012**, *12*, 1637.
- [30] E. J. Park, S. Y. Oh, S. J. Lee, K. Lee, Y. Kim, B. S. Lee, J. S. Kim, *Environ. Res.* **2015**, *143*, 138.
- [31] N. Shershakova, E. Baraboshkina, S. Andreev, D. Purgina, I. Struchkova, O. Kamysnikov, A. Nikonova, M. Khaitov, *J. Nanobiotechnol.* **2016**, *14*, 8.
- [32] M. Mahdavi, F. Mavandadnejad, M. H. Yazdi, E. Faghfuri, H. Hashemi, S. Homayouni-Oreh, R. Farhoudi, A. R. Shahverdi, *J. Infect. Public Health* **2016**, *22*, 1876.
- [33] M. Novali, K. H. Shalaby, A. Robichaud, A. Benedetti, L. Fereydoonzad, T. K. McGovern, T. F. Schuessler, J. G. Martin, *Respir. Physiol. Neurobiol.* **2015**, *218*, 11.
- [34] G. Truchetti, E. Troncy, A. Robichaud, L. Gold, T. Schuessler, S. Maghezzi, L. Bassett, S. Authier, *J. Pharmacol. Toxicol. Methods* **2014**, *70*, 48.
- [35] J. K. Walker, M. Kraft, J. T. Fisher, *Front. Physiol.* **2012**, *3*, 491.
- [36] K. L. Evans, R. A. Bond, D. B. Corry, F. R. Shardonofsky, *J. Appl. Physiol.* **2003**, *94*, 245.
- [37] G. Danscher, *Histochemistry* **1984**, *81*, 331.
- [38] R. Zaynagetdinov, T. P. Sherrill, P. L. Kendall, B. H. Segal, K. P. Weller, R. M. Tighe, T. S. Blackwell, *Am. J. Respir. Cell Mol. Biol.* **2013**, *49*, 180.
- [39] A. V. Misharin, L. Morales-Nebreda, G. M. Mutlu, G. R. Budinger, H. Perlman, *Am. J. Respir. Cell Mol. Biol.* **2013**, *49*, 503.
- [40] M. Williams, B. N. Lambrecht, H. Hammad, *Mucosal Immunol.* **2013**, *6*, 464.
- [41] R. Shamri, J. J. Xenakis, L. A. Spencer, *Cell Tissue Res.* **2011**, *343*, 57.
- [42] W. G. Kreyling, A. M. Abdelmonem, Z. Ali, F. Alves, M. Geiser, N. Haberl, R. Hartmann, S. Hirn, D. J. de Aberasturi, K. Kantner, G. Khadem-Saba, J. M. Montenegro, J. Rejman, T. Rojo, I. R. de Larramendi, R. Ufartes, A. Wenk, W. J. Parak, *Nat. Nanotechnol.* **2015**, *10*, 619.
- [43] S. J. Soenen, U. Himmelreich, N. Nuytten, T. R. Pisanic 2nd, A. Ferrari, M. De Cuyper, *Small* **2010**, *6*, 2136.
- [44] H. Chen, P. Zou, J. Connarn, H. Paholak, D. Sun, *Nano Res.* **2012**, *5*, 815.
- [45] M. Geiser, W. G. Kreyling, *Part. Fibre Toxicol.* **2010**, *7*, 2.
- [46] A. Nemmar, P. H. Hoet, B. Vanquickenborne, D. Dinsdale, M. Thomeer, M. F. Hoylaerts, H. Vanbilloen, L. Mortelmans, B. Nemery, *Circulation* **2002**, *105*, 411.
- [47] H. S. Choi, Y. Ashitate, J. H. Lee, S. H. Kim, A. Matsui, N. Insin, M. G. Bawendi, M. Semmler-Behnke, J. V. Frangioni, A. Tsuda, *Nat. Biotechnol.* **2010**, *28*, 1300.
- [48] S. G. Elci, Y. Jiang, B. Yan, S. T. Kim, K. Saha, D. F. Moyano, G. Yesilbag Tonga, L. C. Jackson, V. M. Rotello, R. W. Vachet, *ACS Nano* **2016**, *10*, 5536.
- [49] P. Jin, Z. Dai, T. Chang, The role of bis(p-sulfonatophenyl) phenylphosphine in stabilizing gold nanoparticle, presented at AASRI International Conference on Industrial Electronics and Applications, **2015**.
- [50] S. Smulders, J. P. Kaiser, S. Zuin, K. L. Van Landuyt, L. Golanski, J. Vanoirbeek, P. Wick, P. H. Hoet, *Part. Fibre Toxicol.* **2012**, *9*, 41.
- [51] L. L. Yu, L. J. Wood, S. E. Long, Determination of Gold in Rat Tissue with Inductively Coupled Plasma Mass Spectrometry, http://ncl.cancer.gov/NCL_Method_PCC-8.pdf (accessed: Feb, 2010).
- [52] R. A. Joachim, V. Sagach, D. Quarcoo, T. Dinh, P. C. Arck, B. F. Klapp, *Neuroimmunomodulation* **2006**, *13*, 43.

Received: September 13, 2016
Revised: November 8, 2016
Published online:



Supporting Information

for *Small*, DOI: 10.1002/smll.201603070

Local Effects on Airway Inflammation and Systemic Uptake
of 5 nm PEGylated and Citrated Gold Nanoparticles in
Asthmatic Mice

*Albert J. Omlor, Duc D. Le, Janine Schlicker, Matthias
Hannig, Raphael Ewen, Sebastian Heck, Christian Herr,
Annette Kraegeloh, Christina Hein, Ralf Kautenburger, Guido
Kickelbick, Robert Bals, Juliane Nguyen, and Q. Thai Dinh**

Supporting Information

Local Effects on Airway Inflammation and Systemic Uptake of 5 nm PEGylated and Citrated Gold Nanoparticles in Asthmatic Mice

*Albert J. Omlor, Duc D. Le, Janine Schlicker, Matthias Hannig, Raphael Ewen, Sebastian Heck, Christian Herr, Annette Kraegeloh, Christina Hein, Ralf Kautenburger, Guido Kickelbick, Robert Bals, Juliane Nguyen, and Quoc T. Dinh**

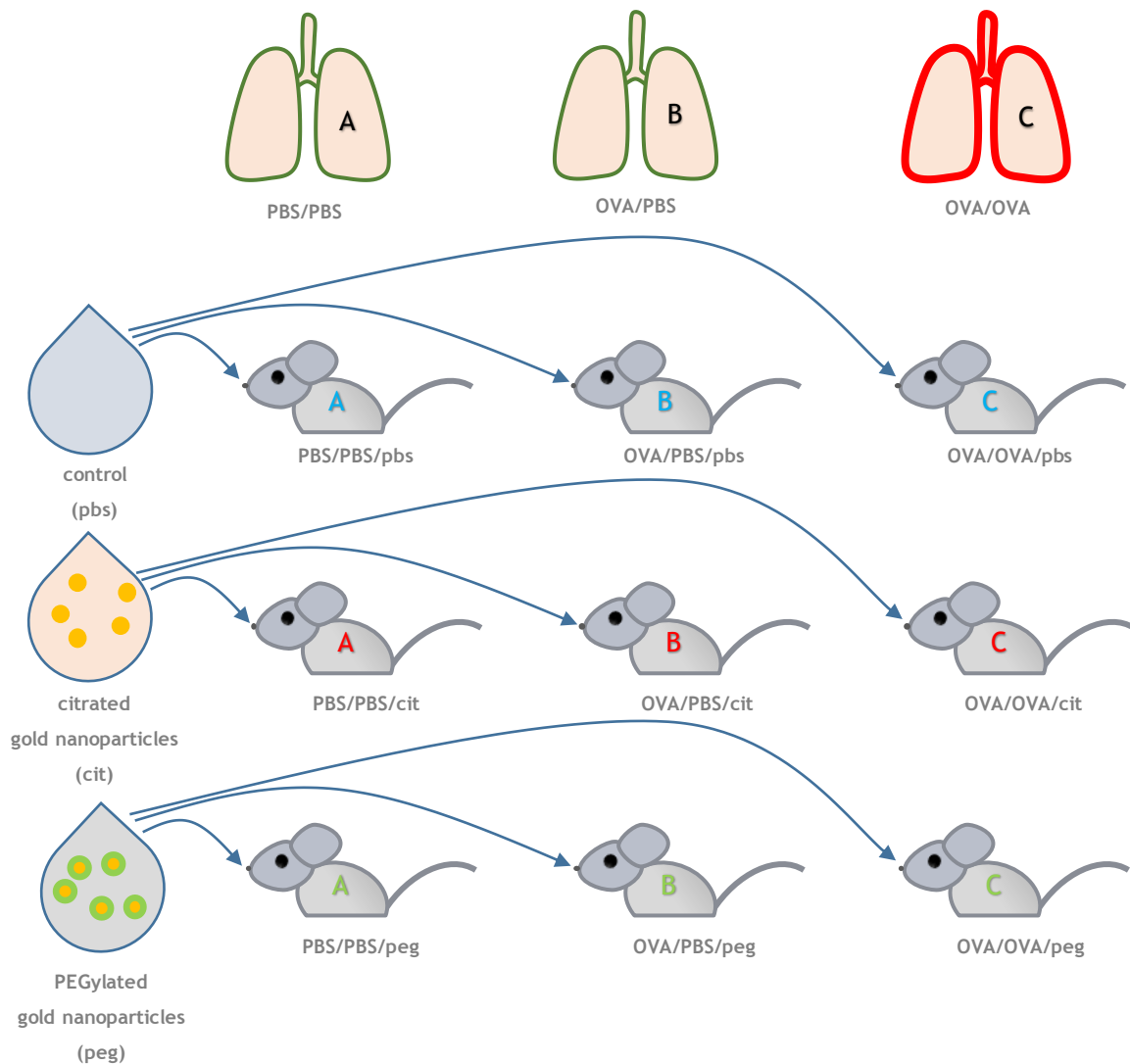


Figure S1. This study utilized nine test groups that were systematically labeled by three syllables. The first syllable described the agent used for sensitization: PBS (control) or OVA. The second defined the agent that was taken for the challenge: PBS (control) or OVA. The first two syllables together defined the asthmatic status. Groups with PBS/PBS (A) were neither sensitized nor challenged and therefore had no asthma. Groups with OVA/PBS (B) were sensitized to ovalbumin but not challenged and therefore had no asthma; this group was needed to prove that the asthma mouse model was functional. Groups with OVA/OVA (C) were both sensitized and challenged to ovalbumin and therefore had asthma. The third syllable determined the applied gold nanoparticles: pbs (control) or cit (citrated) or peg

(PEGylated). Three groups did not receive any nanoparticles (blue), while the six remaining groups did (red received citrated nanoparticles and green received PEGylated nanoparticles). The blue groups had only 5 mice in each group ($n=5$). The red and green groups had 8 mice in each group ($n=8$).

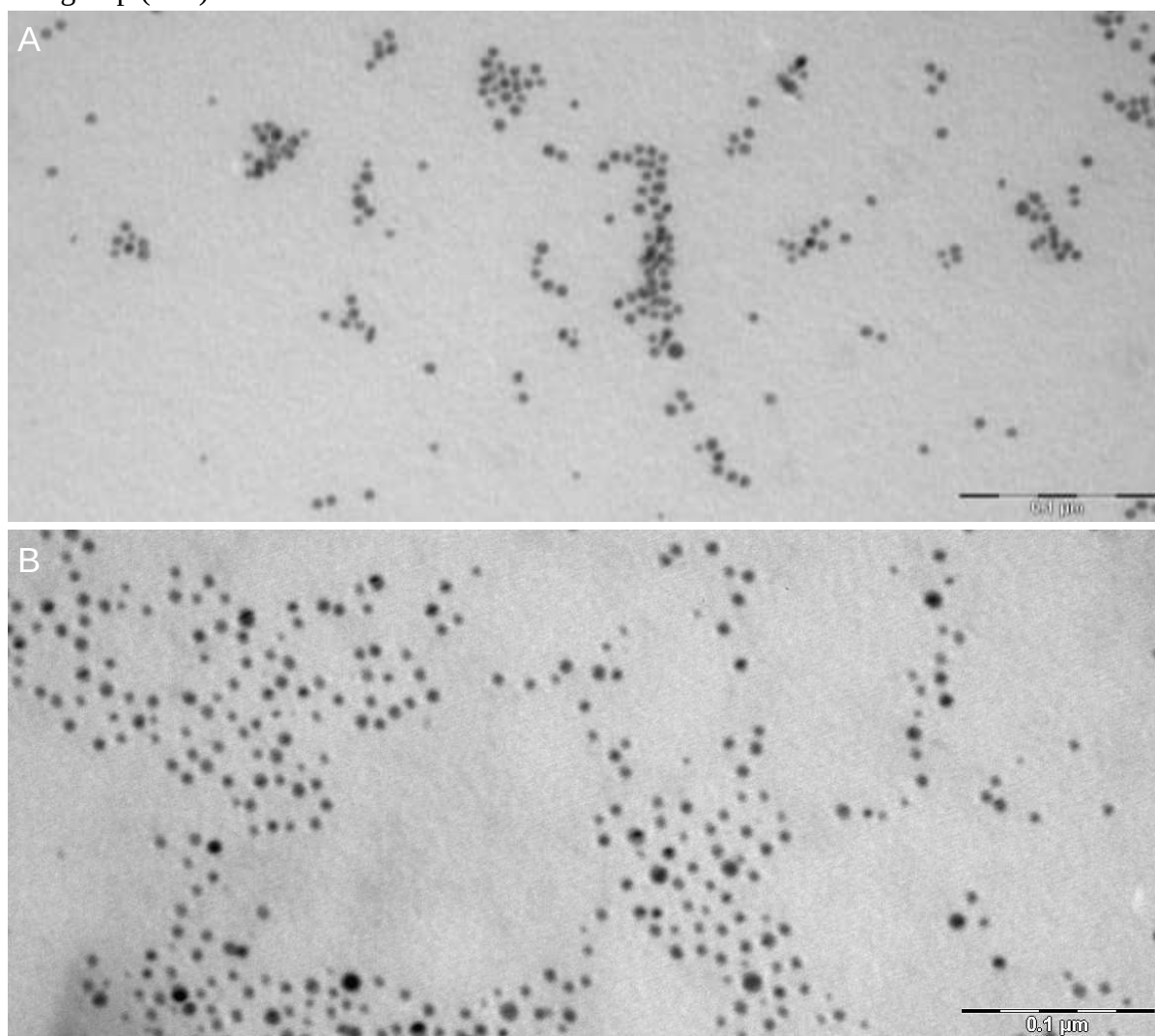


Figure S2. TEM images of citrated (A) and PEGylated (B) 5 nm gold nanoparticles. Both nanoparticle types had a core diameter of approximately 5 nm. The solvent was evaporated to leave only particles on the TEM grid; evaporation gradually increases both the nanoparticle and salt concentration and therefore decreases the nanoparticle suspension stability. The citrated particles aggregated at some point during the evaporation of solvent, which ultimately led the irreversible formation of chains and aggregates on the grid. In contrast, the PEGylated nanoparticles remained stable during the entire evaporation process and maintained a certain distance between each other on the grid. In contrast to the citrated particles, the PEGylated particles could be successfully re-suspended in water by sonification. The enhanced stability of the PEGylated gold nanoparticles was also seen *in vitro*, when the nanoparticles were mixed with PBS or murine bronchoalveolar lavage. While the citrated gold nanoparticles agglomerated in both cases, the PEGylated particles were neither affected by the physiological salt concentration nor by proteins and polysaccharides in the bronchial mucus.

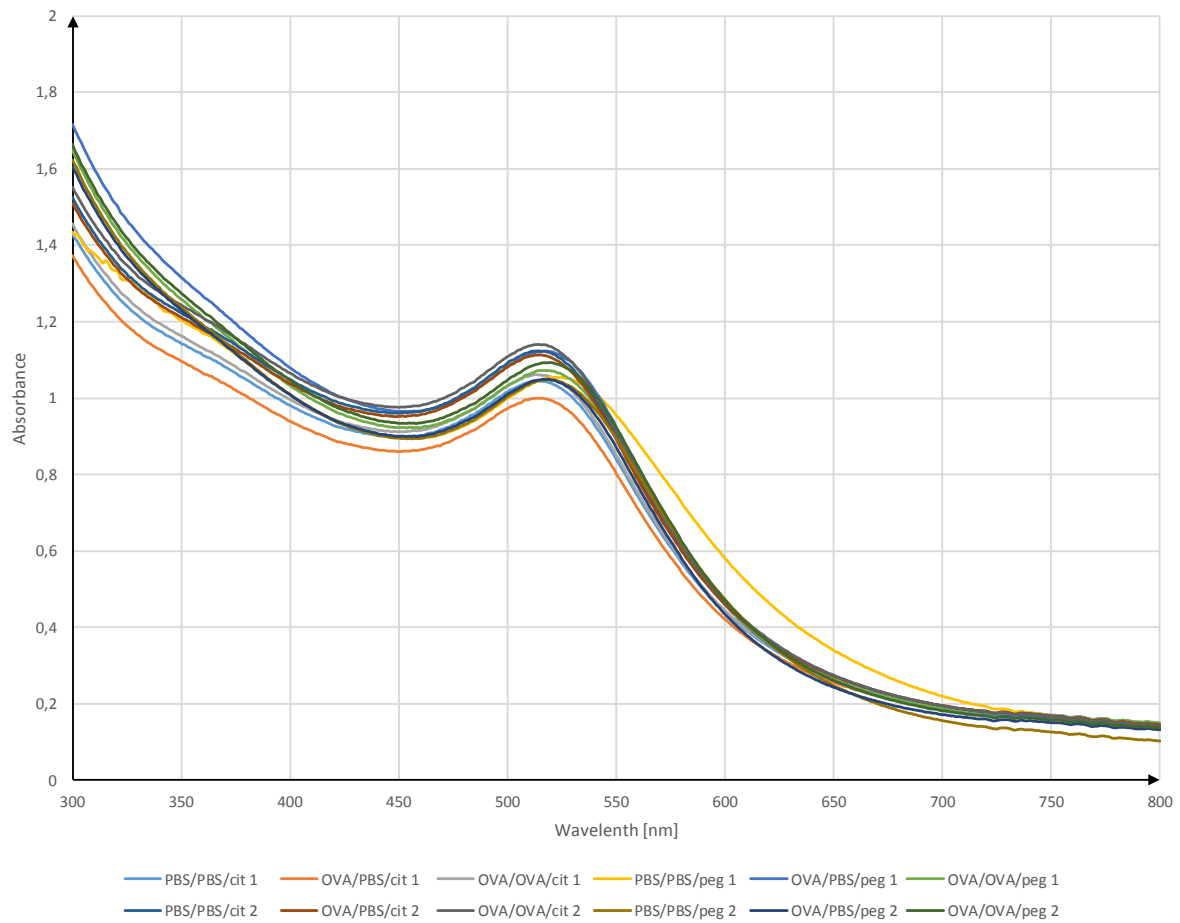


Figure S3. The quality of the applied nanoparticles was evaluated by UV-Vis spectroscopy of six-fold diluted aliquots on the same day prior to application. UV-Vis spectroscopy is ideal for this purpose, because, for gold nanoparticles, the spectra are not only dependent on the concentration but also directly related to the particle size distribution. According to the manufacturer, both the citrated and the PEGylated 5 nm gold nanoparticles possess a molar extinction coefficient of $1.10 \times 10^7 \text{ M}^{-1}\text{cm}^{-1}$ and an absorption maximum between 515 nm and 520 nm. The similarity of the graphs shows that the applied nanoparticle dispersions had comparable concentrations and size distributions.

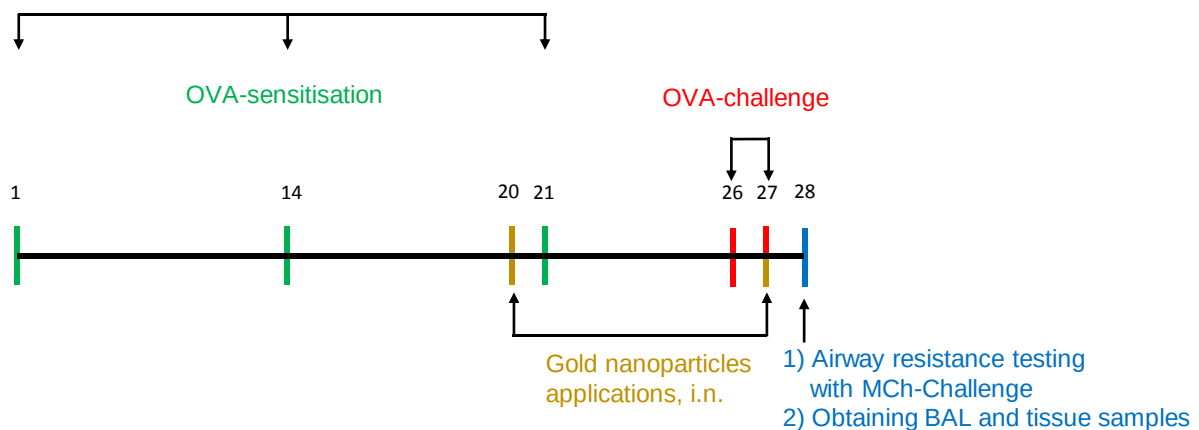


Figure S4. Mice received an intraperitoneal injection of aluminum hydroxide-adsorbed OVA (2 mg AlOH_3 with 20 μg ovalbumin) in the intervention groups and PBS in the control groups on day 1, 14, and 21. On days 26 and 27, mice were exposed to nebulized ovalbumin or PBS

for 20 min depending on the group. On days 20 and 27, mice were treated intranasally with PBS, citrated gold nanoparticles, or PEGylated gold nanoparticles. Day 28 was the final study endpoint. Airway resistance measurements were performed with methacholine challenge under anesthesia. A bronchoalveolar lavage was taken for cell counting. Afterwards, the mice were euthanized and tissue samples taken for histologic analysis and ICP-MS testing.