Biomaterials 34 (2013) 8333–8343

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Systemic and immunotoxicity of silver nanoparticles in an intravenous 28 days repeated dose toxicity study in rats

Wim H. De Jong*, Leo T.M. Van Der Ven, Annemarie Sleijffers, Margriet V.D.Z. Park, Eugene H.J.M. Jansen, Henk Van Loveren, Rob J. Vandebriel

Centre for Health Protection, National Institute for Public Health and The Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM), PO Box 1, 3720 BA Bilthoven, The Netherlands

ARTICLE INFO

Article history:
Received 18 June 2013
Accepted 23 June 2013
Available online 22 July 2013

Keywords:
Nanosilver
Nanotoxicity
Intravenous
28 Days repeated dose study

ABSTRACT

Because of its antibacterial activity nanosilver is one of the most commonly used nanomaterials. It is increasingly used in a variety of both medical and consumer products resulting in an increase in human exposure. However, the knowledge on the systemic toxicity of nanosilver is relatively limited. To determine the potential systemic toxicity of silver nanoparticles (Ag-NP) with different sizes (20 nm and 100 nm) a 28-days repeated dose toxicity study was performed in rats using intravenous administration. The toxic effect of the 20 nm Ag-NP was performed using the bench mark dose (BMD) approach.

Treatment with a maximum dose of 6 mg/kg body weight was well tolerated by the animals. However, both for 20 nm and 100 nm Ag-NP growth retardation was observed during the treatment. A severe increase in spleen size and weight was present which was due to an increased cell number. Both T and B cell populations showed an increase in absolute cell number, whereas the relative cell numbers remained constant. At histopathological evaluation brown and black pigment indicating Ag-NP accumulation was noted in spleen, liver, and lymph nodes. Ag-NP was also detected incidentally in other organs. Clinical chemistry indicated liver damage (increased alkaline phosphatase, alanine transaminase, and aspartate transaminase) that could not be confirmed by histopathology. Hematology showed a decrease in several red blood cell parameters.

The most striking toxic effect was the almost complete suppression of the natural killer (NK) cell activity in the spleen at high doses. Other immune parameters affected were: decreased interferon-γ and interleukin (IL)-10 production by concanavalin-A stimulated spleen cells, increased IL-1β and decreased IL-6, IL-10 and TNF-α production by lipopolysaccharide stimulated spleen cells, increase in serum IgM and IgE, and increase in blood neutrophilic granulocytes. For the spleen weight a critical effect dose of 0.37 mg/kg body weight (b.w.) could be established. The lowest critical effect dose (CED) for a 5% change compared to control animals was observed for thymus weight (CED05 0.01 mg/kg b.w.) and for functional immune parameters, i.e. decrease in NK cell activity (CED05 0.06 mg/kg b.w.) and LPS stimulation of spleen cells (CED05 0.04 mg/kg b.w.). These results show that for nanosilver the most sensitive parameters for potential adverse responses were effects on the immune system.

1. Introduction

Silver nanoparticles (Ag-NP) are frequently used in consumer and medical products because of their antimicrobial activity [1–7]. Despite the rapidly growing presence of silver-containing nanoproducts on the market [5–8], there is only limited information on the possible risks of exposure to silver nanoparticles. In recent reviews evaluating the risk assessment of silver nanoparticles, different knowledge gaps were identified, including toxicokinetics [4,9,10]. Similar data gaps were identified for the possible registration of nanosilver as a substance under the EU REACH regulation [11]. Previously we identified the spleen and liver as main target organs for various nanomaterials after intravenous administration [12–14]. This can be explained by the fact that these organs are part of the reticulo-endothelial system (RES) that has removing foreign agents from the circulation as one of its functions. *In vitro* studies demonstrated that Ag-NP are cytotoxic by their

* Corresponding author.
E-mail address: wim.de.jong@rivm.nl (W.H. De Jong).

0142-9612
http://dx.doi.org/10.1016/j.biomaterials.2013.06.048

effect on cellular metabolism and membrane integrity, and inhibit embryonic stem cell differentiation [15].

In repeated dose toxicity inhalation studies animals were exposed for 28 and 90 days in a whole body exposure chamber using a nanoparticle generator for the production of silver nanoparticles [16–18]. In the first evaluation of the 28-days toxicity study with 12–15 nm Ag-NP general toxicity parameters, i.e. body weight, hematology and blood clinical chemistry values, did not indicate systemic toxicity at the doses investigated [16]. At histopathological examination only incidentally some liver alterations (cytoplasmic vacuolisation or hepatic necrosis) were observed. Indications for local reactions in the lung were absent [16]. The tissue concentration of silver as determined with atomic absorption spectrometry showed a dose dependent increase in the lung and low concentrations in liver and brain. In the 90-days repeated dose inhalation toxicity study lung function was impaired, and indications for lung inflammation were noted by cellular and protein content of the bronchoalveolar lavage fluid (BALF) and lung histopathology [17]. In a 28-days oral toxicity study with 60 nm silver nanoparticles by Kim et al. [19], a dose dependent increase in silver content was observed in all tissues investigated (testis, kidneys, liver, brain, lung, stomach, blood). There was no effect on body weight although some blood biochemistry parameters (alkaline phosphatase, cholesterol) indicated some liver damage [19]. In a recent 28-days oral toxicity study Van Der Zande et al. [20] found that the silver concentration in organs was highly correlated with the $Ag+$ ion content of the nanosilver suspension. The highest concentrations were observed in liver and spleen [20]. In general the studies using either inhalation or oral exposure did not show severe systemic toxicity, probably because of the relatively low systemic exposure to nanosilver that is due to low absorption of the nanosilver from the lung and gastrointestinal tract (GI-tract).

To avoid limited systemic exposure due to the cellular barriers present in lung and GI-tract, we used intravenous administration of nanosilver to evaluate its potential systemic toxicity. We investigated the *in vivo* toxicity of two sizes of Ag-NP (20 nm and 100 nm) in a repeated dose toxicity study after intravenous administration for 28 days. Special emphasis was on the effects of the spleen as part of the immune system as our previous results showed accumulation of Ag-NP in the spleen [13].

2. Materials and methods

2.1. Animals

Male and female Wistar derived WU rats, 8 weeks of age, obtained from Harlan Nederland BV, Horst, The Netherlands, were used. Animals were bred under SPF conditions and barrier maintained during the experiment. Drinking water and conventional feed were provided *ad libitum*. Husbandry conditions were maintained according to all applicable provisions of the national laws, Experiments on Animals Decree and Experiments on Animals Act. The experiment was approved by an independent ethical committee prior to the study according to the Dutch legislation.

2.2. Chemicals

BioPure silver nanoparticles, 20 nm diameter and 100 nm diameter in 2 mM phosphate buffer were obtained from NanoComposix, San Diego, CA, USA. The nanosilver dispersions provided were characterized with minimal agglomeration or aggregation. The nanoparticle characteristics are presented in Table 1.

2.3. Experimental design

The present study was performed according to the general principles of OECD guideline 407 ("Repeated dose 28-day oral study in rodents") with some adjustments. The animals were exposed to nanosilver particles via intravenous administration. Instead of the usual design of three dose groups (low, mid and high dose) and one control group, we increased the number of dose groups, at the expense of the group sizes, i.e. while keeping the total number of animals the same. With this design, and by applying the bench mark dose (BMD) approach [21], an improved characterization of the dose response is obtained without increasing the number of animals.

Table 1
Characteristics of BioPure silver nanoparticles.[a]

Parameter	20 nm CTH1359	100 nmCTH1409
Size ± SD (nm)	21.0 ± 2.6	107 ± 7.6
Coefficient of variation (%)	12.2	7.1
Size range (min–max diameter)	12.4–27.9	92.8–128.4
Number of particles (ml^{-1})	3.9×10^{13}	3.8×10^{11}
Surface area per particle (nm^2)	1.40×10^{3}	3.62×10^{4}
Surface area (nm^2/ml)	5.49×10^{16}	1.37×10^{16}
Silver concentration (mg/ml)	2	2.6
Zeta potential (mV)	−40.8	−38.7

[a] Information provided by manufacturer nanoComposix, San Diego, USA.

Because of the known accumulation in the spleen [13] additional tests with spleen cells were performed for possible immunotoxicological properties of nanosilver. In addition not all organs indicated in OECD 407 were evaluated by histopathology.

Animals were divided in 11 groups and were intravenously injected (tail vein) once a day for 28 days to either 1 ml nanosilver dispersion or vehicle control (phosphate buffer). Dose levels and experimental design are presented in Table 2. The experiment was performed in two phases, the first being the 20 nm particle treatments followed by the 100 nm particle treatments.

Rats were weighed prior to and weekly during the experiment. Individual body weights (BW) of the rats were used to calculate the individual dose levels.

2.4. Histopathology

At 24 h after the last injection, rats were anaesthetized with isoflurane (Isoflu®, AST Pharma, Oudewater, The Netherlands) in oxygen and subsequently euthanized by drawing blood from the abdominal aorta. After collecting blood, rats were evaluated macroscopically for gross lesions. The following organs were examined and sampled: adrenals, brain, bone marrow, small intestines (duodenum, jejunum, ileum), large intestines (caecum, colon, rectum), heart, kidney, liver, lung, lymph nodes (mesenteric and popliteal), esophagus, pituitary, spleen, stomach, testis (or ovaria), and thymus. Organ weights were determined except for bone marrow, pituitary and gastrointestinal tract.

All tissue samples were fixed in 4% neutral buffered formaldehyde (10% formalin) and routinely processed (Hematoxylin and Eosin staining) for histopathology.

Routine histopathology was performed for the following treatments: 20 nm Ag-NP 6 mg/kg b.w. (3 male and 3 female animals) and 2 mg/kg b.w. (3 male and 3 female animals), 100 nm Ag-NP 6 mg/kg b.w. (4 male and 4 female animals), and phosphate buffer control treated animals (6 male and 6 female animals).

2.5. Hematology

Hematology and clinical chemistry was performed on blood samples obtained at autopsy. Blood was collected in EDTA-coated tubes. Hematological parameters included white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), hemoglobin distribution width (HDW), platelet (PLT) count, and mean platelet volume (MPV). All hematology parameters in the blood samples were determined in an Advia 120 Hematology Analyzer (Siemens, Germany). In addition, blood smears were prepared for visual evaluation.

2.6. Clinical chemistry

After collection of blood serum and storage at −20 °C the following parameters were determined: albumin (ALB), alkaline phosphatase (ALP), alanine

Table 2
Dose levels and experimental design of 28 days repeated dose toxicity study with Ag-NP.

Treatment	Dose (mg/kg b.w. per day)	*n* (M–F)
Phosphate buffer	0	2–2
Phosphate buffer	0	2–2
20 nm nanosilver	0.0082	2–2
20 nm nanosilver	0.0025	2–2
20 nm nanosilver	0.074	2–2
20 nm nanosilver	0.22	3–3
20 nm nanosilver	0.67	3–3
20 nm nanosilver	2	3–3
20 nm nanosilver	6	3–3
Phosphate buffer	0	2–2
100 nm nanosilver	6	4–4

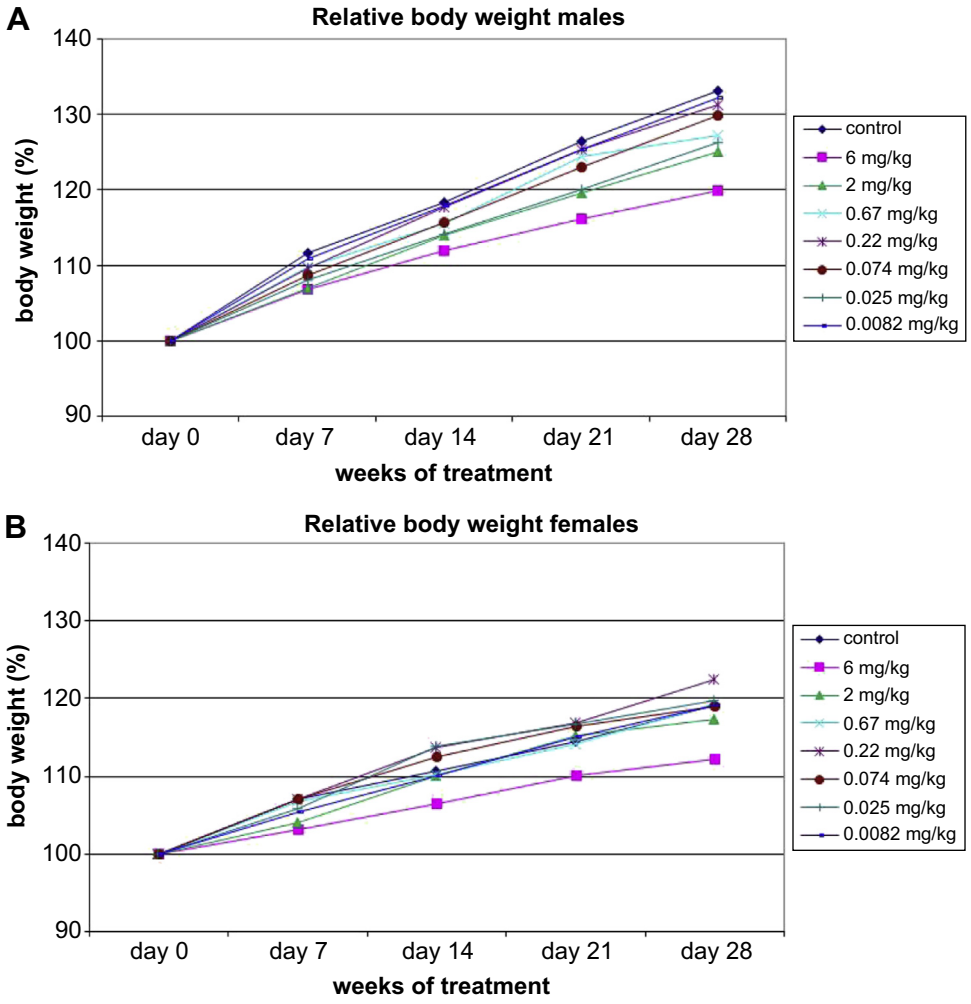

Fig. 1. A) Body weight of male rats presented as a percentage of the weight at the start of the experiment at day 0 (=100%). Animals were treated intravenously for 28 days with different doses of 20 nm Ag-NP. B) Body weight of female rats presented as a percentage of the weight at the start of the experiment at day 0 (=100%). Animals were treated intravenously for 28 days with different doses of 20 nm Ag-NP. C) Dose response analysis (Hill equation) of body weight as a function of intravenous administration for 28 days of 20 nm Ag-NP. Body weight was determined on day 29 and expressed as weight increase versus day 0. The confidence interval for the CED05 of 3.9 mg/kg body weight was (2.8, 6.1) mg/kg body weight. ○ females, Δ males.

aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), amylase (AMY), creatinin kinase (CK), lactate dehydrogenase (LDH), sodium (Na), potassium (K), glucose (GLU), urea, creatinin (CRE), cholesterol (CHOL) and total protein (TP).

2.7. Bone marrow

Cells were collected by flushing 4 ml Impuls Cytometer Fluid through the femur. The concentration of nucleated cells was determined in a Coulter Counter.

2.8. Determination of immune parameters

2.8.1. Spleen cell distribution

The spleen was weighed and a weighed part of the spleen was collected in tissue culture medium of which spleen cells were isolated. Relative spleen cell distribution was determined by fluorescence-activated cell sorter (FACS) analysis as described by Tonk et al. [22]. Spleen single-cell suspensions were prepared and evaluated for subset distribution by 3-color flow cytometry. The following monoclonal antibodies were used: allophycocyanin (APC)-conjugated mouse anti-rat CD3 (clone 1F4, T lymphocytes), R-phycoerythrin (PE)-conjugated mouse anti-rat CD8a (clone OX-8, T-suppressor/cytotoxic cells), fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD4 (clone OX-35, T helper cells), FITC-conjugated mouse anti-rat CD45RA (clone OX-33, B lymphocytes), and PE-conjugated mouse anti-rat CD161a (clone 10/78, NK cells); all from Pharmingen, San Diego, CA, USA. Single-cell suspensions were incubated with the conjugated mAbs for 30 min at 4 °C in the dark. The cells were washed twice with wash buffer (5% BSA in PBS), resuspended in 0.1% paraformaldehyde in PBS, and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). A total of 10, 000 events were recorded per sample and analyzed.

2.8.2. Natural killer (NK) cell activity

Spontaneous cytotoxicity of spleen cell populations was evaluated in an *in vitro* ^{51}Cr-release assay for determination of NK cell activity as described previously [22,23]. Spleen single-cell suspensions were adjusted in a standard medium containing 10% FBS. The YAC target cells (1×10^6 cells/100 μl) were labeled with 3.7 MBq $Na_2^{51}CrO_4$ (Amersham, Buckinghamshire, UK) for 45 min at 37 °C, washed 5×, and resuspended in standard medium containing 10% FBS at 1×10^5 viable cells/ml. The NK cell activity assays were performed in 96-well polystyrene U-bottom microplates (Costar, Cambridge, MA, USA) for each sample in triplicate. Aliquots of 1×10^4 target cells were added to each well containing the appropriate number of effector cells to give effector cell (E) to target cell (T) ratios (E:T) of 200:1, 100:1, 50:1, and 25:1, to a final volume of 200 μl. Controls included a spontaneous ^{51}Cr-release control (target cells + medium) and a maximum ^{51}Cr-release control (target cells + 1% triton). Plates were centrifuged for 5 min at 1200 rpm and incubated at 5% CO_2, 37 °C for 4 h. Radioactivity in the cell supernatants was measured using a gamma counter (Packard, Tilburg, The Netherlands) as counts per minute (cpm). Percentage of cytotoxicity was calculated as:

$$\%\text{Cytotoxicity} = (\text{sample cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm}) \times 100$$

2.8.3. Mitogen stimulated lymphocyte proliferation

Lymphocyte proliferation responses of spleen cells to mitogens (lipopolysaccharide, LPS and concanavalin A, ConA) were evaluated with the [^{3}H]-thymidine

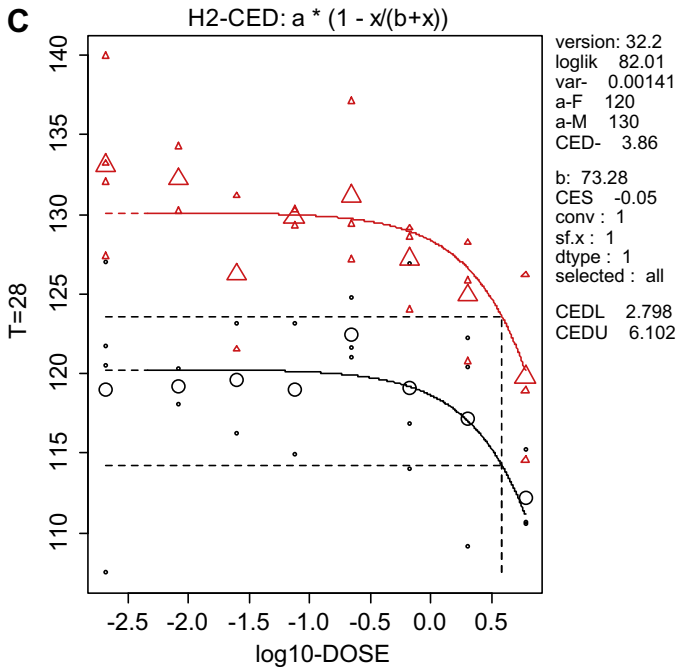

Fig. 1. *(continued)*

incorporation assay. Spleen single-cell suspensions of 4×10^6 cell/ml were seeded in triplicate in a U-bottom 96-well plate and stimulated with 15 μg/ml LPS (Sigma–Aldrich, Zwijndrecht, The Netherlands) or 5 μg/ml Concanavalin A (ConA; ICN Biochemicals, Aurora, OH, USA) for 48 and 72 h, respectively. ^{3}H-thymidine (Amersham, Buckinghamshire, UK) was added for the last 20–22 h of incubation. The cells were harvested on glass fiber filters (LKB-Wallac, Turku, Finland) using a multiple cell culture harvester (LKB-Wallac). The ^{3}H-thymidine activity was determined using a liquid scintillation counter (1205 Betaplate TM; LKB-Wallac, Turku, Finland). For further calculations, the median of the triplicates was used.

2.8.4. Cytokine production of spleen cells

Supernatants of mitogen stimulated cells (see above) were harvested for LPS stimulated cells at 24 h incubation (37 °C, 5% CO_2), and for Concanavalin A stimulated cells at 48 h incubation. Interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-17, IL-13, interferon (IFN)-γ, TNF-α and granulocyte–macrophage colony-stimulating factor (GM-CSF) were measured in the collected supernatants using a MILLIPLEX Map Kit (Millipore, Billerica, MA, USA).

2.9. Immunoglobulins

After collection of blood serum and storage at −20 °C immunoglobulin levels were determined in an enzyme linked immunosorbant assay (ELISA) using mouse monoclonal antibodies against rat IgG, IgM and IgE, respectively.

2.10. Statistical analysis

Data are presented as means with their standard deviation. Treatment effects of 20 nm particles were evaluated using the bench mark dose (BMD) approach, by fitting a nonlinear regression model to the data of all individual animals. The choice of the model for evaluating the BMD (also called CED, (critical effect dose), for continuous endpoints) follows from a procedure of applying likelihood ratio tests on the members of the following nested family of models.

Model 1: $y = a$
Model 2: $y = a \exp(bx)$
Model 3: $y = a \exp(bx^d)$
Model 4: $y = a(c - (c-1)\exp(bx))$
Model 5: $y = a(c - (c-1)\exp(bx^d))$,

where y is the response, and x denotes the applied concentration. The parameter a represents the level of response at concentration zero, and b can be considered as the parameter reflecting the potency of the agent. At high doses, models 4 and 5 level off to the value ac, so the parameter c can be interpreted as the maximum relative change compared to the background. Parameter d can be interpreted as the 'steepness' of the curve (i.e., rate of change in response for a percent change in dose). All these models are nested to each other, except models 3 and 4, which both have 3 parameters. Therefore, these two models cannot be (formally) compared to each other by a likelihood-ratio test.

For each data set (end point measured), one of these models was selected by applying the likelihood ratio test to establish whether extension of the model by increasing the number of parameters resulted in a statistically improvement of the fit to the dose–response data. The selected model was used to estimate the BMD (CED) and the associated 90%-confidence interval (see results). For these endpoints a critical effect size (CES) or bench mark response (BMR) was chosen The confidence interval was determined using a (parametric) boot strap method [24], as follows. Once a model is selected for describing the dose–response data, this fitted model is used as a basis for generating 200 artificial data sets (according to the Experimental design) by Monte Carlo sampling. For each generated data set, the CED is re-estimated. Taking all these CEDs together results in a distribution representing the uncertainty associated with the CED estimate. The 5th and 95th percentiles of this empirical distribution were determined, serving as a two sided 90% confidence interval (c.i.) of the estimated CED [25]. The results of the BMD experimental design were analyzed using PROAST software version 32.2 (http://www.rivm.nl/en/Library/Scientific/Models/PROAST), RIVM, Bilthoven, The Netherlands)). The critical effect size (CES) or benchmark response (BMR) for general and specific toxicity endpoints was chosen at 5% deviation of the control values, In this study consensus default BMR values were used as proposed by the European Food Safety Authority [21], 5% for continuous data and 10% (extra risk) for quantal data. Based on the regression model a maximal response as a percentage of control animals was calculated.

Statistical analysis of the effects of the administration of 100 nm Ag-NP was performed with SPSS software (SPSS Inc., Chicago, IL, USA).

Table 3
Critical Effect Doses of toxicological endpoints after 28 days IV administration of 20 nm silver nanoparticles.

End point	BMD, mg/kg b.w.	Confidence interval, (5th%–95th%) mg/kg b.w.	Maximal response (% of control)
Body weight (day 28)	5.26	2.89–23.69	−5.7
Body weight (d28/d0)	3.89	2.80–6.13	−7.6
Spleen weight	0.37	0.12–1.04	+132
Relative spleen weight	0.25	0.14–0.64	+150
Thymus weight	0.01	0.001–0.14	−17.4
Relative thymus weight	0.02	0.002–0.14	−15.2
Liver weight	2.20	1.39–4.87	+14.5
Relative liver weight	1.57	1.18–2.21	+21.3

The bench mark dose (BMD) at 5% change compared to controls was calculated as a geometric mean of the exponential and Hill models used in the regression analysis.

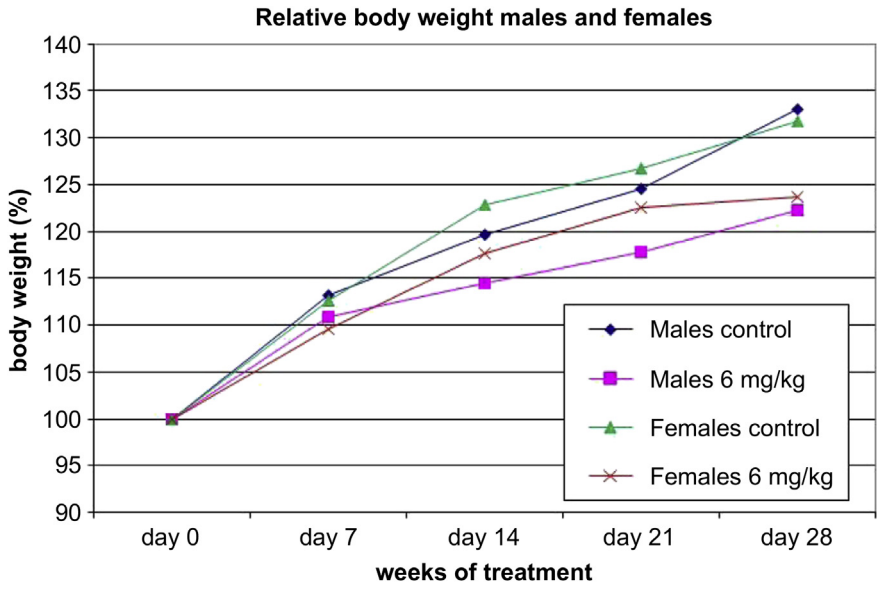

Fig. 2. Body weight of male and female rats during 28 days of intravenous treatment with 100 nm Ag-NP presented as a percentage of the weight at day 0 (=100%).

3. Results

3.1. Animal observation and effect on body weights

The treatment efficacy was well above 90% of the intended doses. The total dose administered for the various treatment groups varied between 93% and 99% of the intended dose (data not shown).

No clinical signs for toxicity were observed during the 28-days exposure period for treatment with either 20 nm Ag-NP or 100 nm Ag-NP. Both male and female rats in the highest dose group (6 mg/kg body weight) of the 20 nm Ag-NP showed lower body weights after 4 weeks of intravenous administration of the 20 nm nanosilver (Fig. 1). The BMD analysis showed that males and females did not differ statistically significantly in the dose–response for body weight (apart from background weight). The CED05 (dose at 5% deviation from control) for a decrease in BW was 5.26 mg/kg b.w. and resulted in a confidence interval of (2.9, 24) mg/kg b.w., but when evaluating the body weight gain as a percentage of the weight at day 0 it reduced to a CED05 of 3.89 mg/kg b.w. with a confidence interval of (2.8, 6.1) mg/kg b.w. (Fig. 1, Table 3). The maximal response calculated from the regression models indicates a minor weight difference of 6–8% compared to control animals.

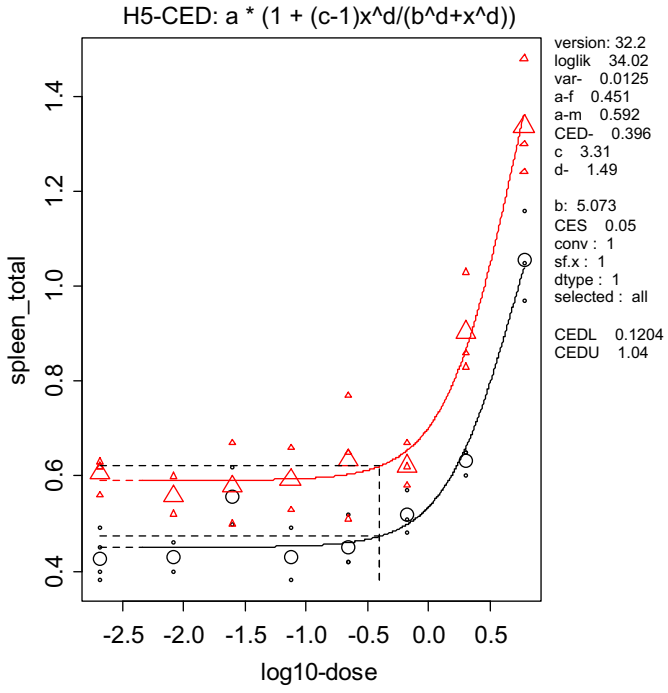

Fig. 3. Dose response analysis (Hill equation) of spleen weight as a function of intravenous administration for 28 days of 20 nm Ag-NP. Spleen weight was determined on day 29. The confidence interval of the CED05 of 0.39 mg/kg body weight was (0.12, 1.04) mg/kg body weight. ○ females, Δ males.

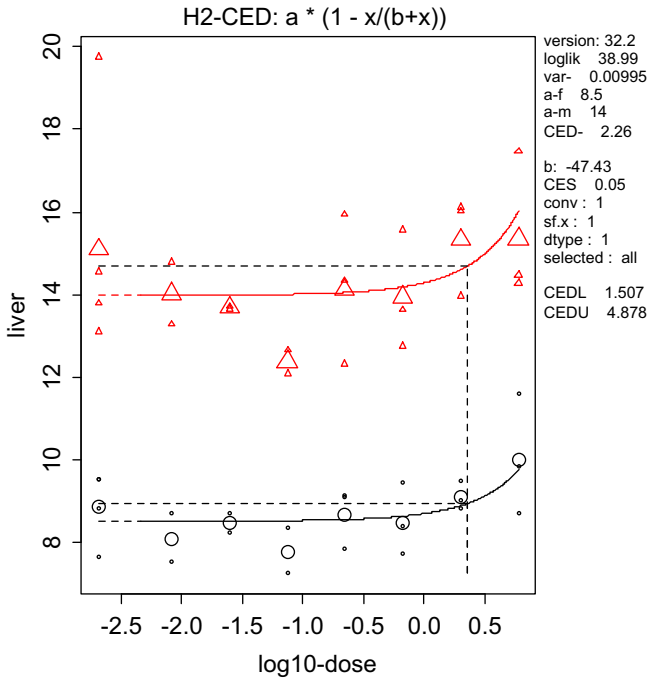

Fig. 4. Dose response analysis (Hill equation) of liver weight as a function of IV administration for 28 days of 20 nm Ag-NP. Liver weight was determined on day 29. The confidence interval for the CED05 of 2.20 mg/kg body weight was (1.39, 4.88) mg/kg body weight. ○ females, Δ males.

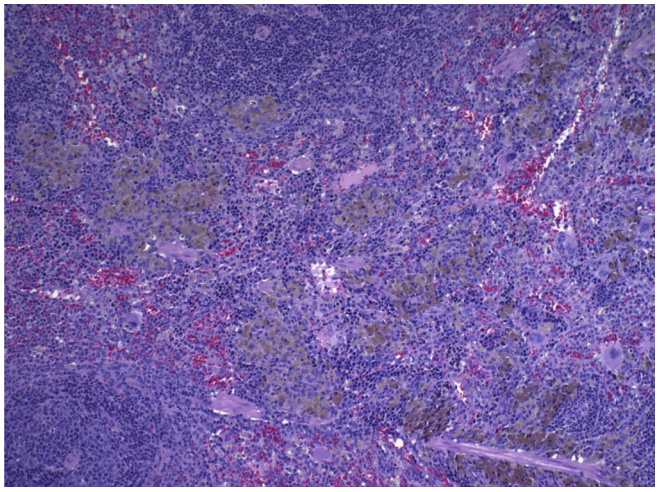

Fig. 5. Presence of brownish pigment in red pulp of the spleen after 28 days intravenous administration of 20 nm Ag-NP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

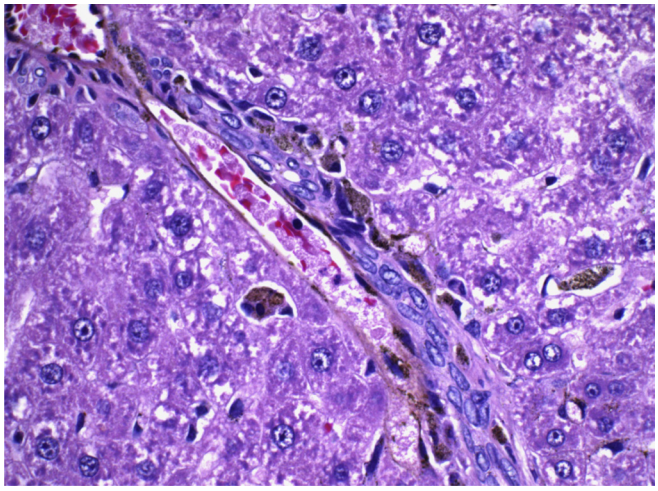

Fig. 6. Presence of brown pigment in Kupffer cells of the liver after 28 days intravenous administration of 20 nm Ag-NP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

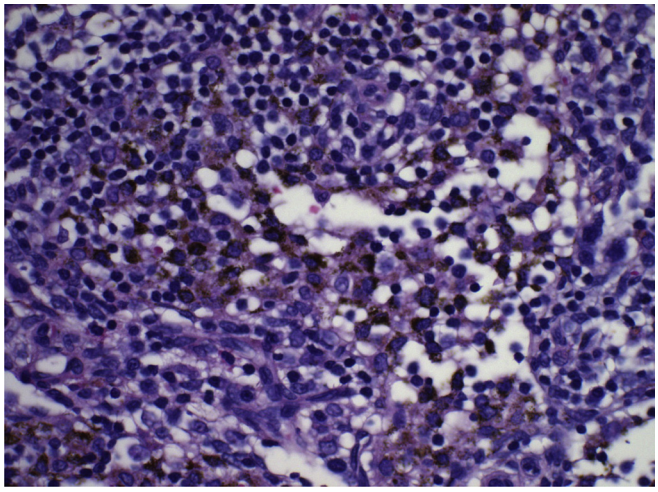

Fig. 7. Presence of pigment in sinusoids of lymph node after 28 days intravenous administration of 20 nm Ag-NP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

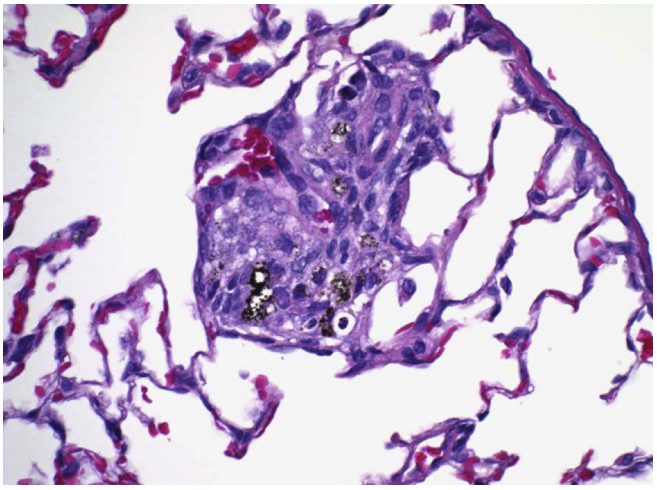

Fig. 8. Presence of pigment (nanoparticles) in granuloma of the lung after 28 days intravenous administration of 100 nm Ag-NP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For the 100 nm Ag-NP a delay in growth in both male and female animals was observed as well (Fig. 2).

3.2. Macroscopic evaluation and effect on organ weights

Macroscopic examination of the rats revealed enlarged brownish colored spleens and livers, and enlarged and dark colored lymph nodes in the highest dose groups.

Heart, kidneys, adrenals, brain, testes and epididymis were weighed and showed no differences in the different dose groups

Table 4
BMD analysis of clinical chemistry and hematology parameters.

Clinical chemistry Blood	BMD, mg/kg b.w.	Confidence interval, (5th%–95th%) mg/kg b.w.	Maximal response, (% of control)
ALB	2.60	1.66–5.28	−11
ALP	0.99	0.41–1.22	+81
ALT	0.62	0.43–0.91	+69
AMY	1.36	0.93–2.24	+25
AST	0.73	0.47–1.22	+56
GLU	1.24	0.64–4.38	−21
LDH	0.60	0.29–1.80	−37
TP	3.97	2.76–7.06	+7.7
UREA	3.04	1.66–15.2	+10
CK	0.66	0.31–4.02	−32
Hematology	**BMD, mg/kg b.w.**	**Confidence interval, (5th%–95th%) mg/kg b.w.**	**Maximal response, (% of control)**
Bone marrow WBC ($\times 10^9$/l)	0.98	0.47–3.94	−26
RBC ($\times 10^{12}$/l)	NS		
Hb (mmol/l)	2.75	0.97–4.94	−9.1
Ht (l/l)	4.22	2.75–8.42	−7.0
MCV (fl)	3.54	2.82–4.62	−8.3
MCH (fmol)	2.75	2.26–3.44	−10.4
MCHC (mmol/l)	13.40	8.45–31.92	−2.3
RDW (%)	5.03	3.29–10.6	+6.0
HDW (mmol/l)	1.37	0.73–1.59	−76
PLT ($\times 10^9$/l)	NS		
MPV (fl)	1.84	1.29–2.98	−17.8
Reticulocytes ($\times 10^9$/l)	0.88	0.58–1.48	+43
Reticulocytes (%)	0.90	0.58–1.63	+41
Blood WBC ($\times 10^9$/l)	NS		

For abbreviations see Materials and methods. The bench mark dose (BMD) at 5% change compared to controls was calculated as a geometric mean of the exponential and Hill models used in the regression analysis.

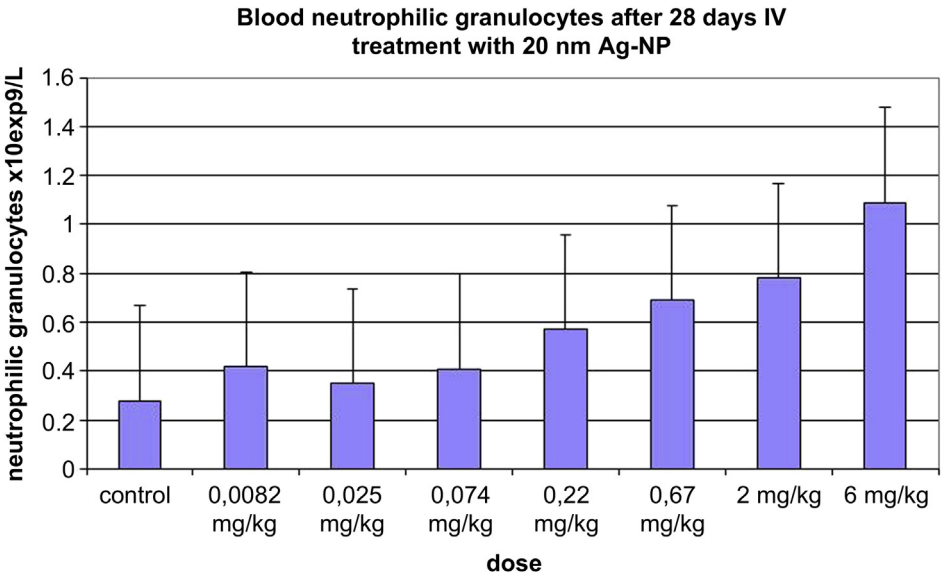

Fig. 9. Blood neutrophilic granulocytes after 28 days IV treatment with 20 nm Ag-NP. Significant increase in response $p < 0.05$ (ANOVA on all groups).

(Ag-NP 20 nm) compared to the control, whereas for liver a weight increase and for the thymus a weight decrease was observed (Table 3). Both for the absolute and relative spleen weight an increase was observed with a CED05 of 0.37 mg/kg b.w. (confidence interval 0.12, 1.04) and 0.25 mg/kg b.w. (confidence interval 0.14, 0.64), respectively (Table 3). The dose response analysis of the spleen weight increase is presented in Fig. 3. After treatment for 28 days with 100 nm Ag-NP (6 mg/kg b.w.) in both male and female animals spleen weight was similarly increased compared to control (phosphate treated) animals (Supplementary data Table S1). BMD analysis showed for the liver an increase in weight for males and females after treatment with 20 nm Ag-NP (Fig. 4), CED05 2.20 mg/kg b.w. (c.i. 1.39, 4.88), and for female animals exposed to 100 nm Ag-NP (Supplementary data Table S1B).

3.3. Histopathology

In the histopathological evaluation pigment was observed in various organs including spleen, liver, and lymph nodes (Figs. 5–7). The pigment itself was not further identified but could be ascribed

Table 5
BMD analysis of immune parameters.

Immune parameters antibodies in blood	BMD, mg/kg b.w.	Confidence interval, (5th%–95th%) mg/kg b.w.	Maximal response, (% of control)
IgM	0.15	0.02–0.77	+372
IgG	NS		
IgE	0.02	0.00–0.25	+3430
Immune parameters in spleen cells	**BMD, mg/kg b.w.**	**Confidence interval, (5th%–95th%) mg/kg b.w.**	**Maximal response, (% of control)**
Spleen cellularity (10^7)	0.28	0.01–0.52	+104
NK: % release/culture (100:1)	0.06	0.04–0.11	−85
NK activity/spleen (100:1)	0.12	0.04–0.29	−67
CD8 T-cytotoxic-cell/10^7 spleen cells	0.43	0.29–0.63	+124
CD4 T-helper-cell/10^7 spleen cells	0.40	0.27–0.59	+141
CD3 T-cell/10^7 spleen cells	0.42	0.30–0.60	+127
CD161a NK-cell/10^7 spleen cells	0.71	0.45–1.26	+58
CD45RA B-cell/10^7 spleen cells	0.14	0.06–0.34	+103
CD4/CD8	NS		
T-cells:B-cells	1.83	0.99–9.78	+18
CD8 T-cytotoxic-cell/spleen	0.18	0.16–0.42	+341
CD4 T-helper-cell/spleen	0.17	0.15–0.41	+377
CD3 T-cell/spleen	0.12	0.04–0.23	+374
CD161a NK-cell/spleen	0.25	0.20–0.51	+216
CD45RA B-cell/spleen	0.12	0.04–0.27	+314
ConA stimulation cpm/culture	0.26	0.02–1.26	+43
ConA stimulation cpm/spleen	0.07	0.03–0.17	+185
LPS stimulation cpm/culture	0.04	0.0001–0.29	−24
LPS stimulation cpm/spleen	0.74	0.44–1.56	+55
LPS spleen cells IL-1β	0.54	0.14–1.19	+94
LPS spleen cells IL-6	0.56	0.30–1.19	−39
LPS spleen cells IL-10	1.08	0.51–5.29	−24
LPS spleen cells TNF-α	0.28	0.21–0.66	−50
Con A spleen cells IL-10	0.81	0.38–3.18	−30
Con A spleen cells IFN-γ	0.59	0.32–1.26	−37

The bench mark dose (BMD) at 5% change compared to controls was calculated as a geometric mean of the exponential and Hill models used in the regression analysis.

to the presence of silver nanoparticles in the various organs. After administration of 20 nm Ag-NP the pigment in the spleen was mainly observed in the red pulp, while after 100 nm Ag-NP the pigment was observed in both the red and white pulp of the spleen. The pigment was also observed in the sinusoids of the lymph nodes evaluated (mesenteric and popliteal lymph node). In the liver the pigment (nanoparticles) was present in the Kupffer cells lining the walls of the venous sinusoids. The pigment was also observed in venous endothelial cells and in small foci of inflammatory cells. These foci are also present (without pigment) in control animals and are generally considered background histopathology in the rat strain used. Incidentally pigment was also observed in the kidney, adrenal glands and in most of the intestinal tract (jejunum, ileum, caecum, colon and rectum).

In the lung small granulomas were observed partly caused by the injection technique as indicated by the presence of hair fragments. Some granulomas contained black pigment after injection of 100 nm Ag-NP (Fig. 8).

3.4. Hematology and clinical chemistry

In general minor alterations were observed in clinical chemistry parameters (Supplementary data Table S2). Several clinical chemistry parameters showed a dose related effect including albumin (ALB), alkaline phosphatase (ALP), alanine transaminase (ALT), amylase AMY, aspartate transaminase (AST), creatinin kinase (CK), glucose (GLU), lactate dehydrogenase (LDH), total protein (TP), and urea (Table 4). The increased ALP, ALT and AST are indicative for liver damage. For blood several parameters associated with red blood cell generation showed a significant decrease in the highest doses administered which could be confirmed by dose response analysis with a CES of 5% (Table 4). The hematocrit (Ht), mean corpuscular cell volume (MCV), mean corpuscular cell hemoglobin (MCH), and hemoglobin (Hb) level in red blood cells showed a limited decrease after treatment with the 20 nm Ag-NP. The number of reticulocytes was increased indicating red blood cell production which might be associated with the decreases observed for the hemoglobin parameters. After treatment with 100 nm Ag-NP a similar decrease was observed that was, however, not significant for all four parameters (data not shown). Although the total number of WBC was not increased, the absolute number of blood neutrophilic granulocytes showed a significant dose related increase with the 20 nm Ag-NP ($p < 0.05$ ANOVA, Fig. 9). For the treatment with 100 nm Ag-NP similar effects on the blood parameters were observed (data not shown). For bone marrow no differences in cell count were observed after treatment with 20 nm or 100 nm Ag-NP (data not shown).

3.5. Immunotoxicity

An increase in IgM and IgE serum levels was seen after treatment with both 20 nm and 100 nm Ag-NP (Table 5, Supplementary data Table S3). In the spleen T cell, B cell and NK cell populations showed an increase after treatment with 20 nm Ag-NP (Table 5). Similarly, for 100 nm Ag-NP an increase in T, B and NK cell populations was observed (Fig. 10). This increase in cell numbers can be considered responsible for the weight increase of the spleen. Mitogen responses to B (LPS) and T (Concanavalin A) cell mitogens showed increased responses per spleen due to the increased cell number (Table 5) Interferon-γ, TNF-α, IL-6 and IL-10 cytokine production were decreased after treatment with 20 nm Ag-NP, whereas IL-1β production was increased (Table 5). For the 100 nm Ag-NP treatment only the IL-10 production was decreased (data not shown).

Both nanoparticles (20 nm and 100 nm Ag-NP) induced an almost complete suppression of the NK cell activity at all effector-to-target ratios (200:1, 100:1, 50:1, 25:1) investigated (Table 5, Fig. 11). The suppression of NK cell activity by the 20 nm Ag-NP started at a dose of 0.22 mg/kg b.w., but was most pronounced at doses of 2 mg/kg and 6 mg/kg body weight. The CED05 for the suppression of NK cell activity was 0.06 mg/kg b.w. with a confidence interval (0.04, 0.11) when evaluated per NK cell culture, and 0.12 mg/kg b.w. with a confidence interval of (0.04, 0.29) when evaluated per spleen.

4. Discussion

The first indication for a toxic effect of the intravenously administered 20 nm and 100 nm Ag-NP was the observed decrease in body weight gain, confidence interval CED05 (2.8, 6.1). The weight gain in the 6 mg/kg body weight treated animals was approximately 66% of the weight gain in the control animals (33% weight increase in the controls versus 19%–24% weight increase in the Ag-NP treated groups). The increase in spleen weight with a CED05 around 0.3 mg/kg b.w., and the decrease in thymus weight CED05 around 0.01 mg/kg b.w. were the most sensitive general toxicity parameters for Ag-NP toxicity. The increase in spleen weight was 2–3 fold. Also for the liver a significant weight increase was observed (Fig. 4). Brownish colored pigment was observed in various organs like spleen, liver and lymph nodes, while gross pathological lesions were not observed, the one exception being the enlarged spleen in all animals treated at the highest administered doses of both 20 nm and 100 nm Ag-NP. For the 20 nm Ag-NP a clear dose response could be observed (Fig. 3). It was remarkable that the

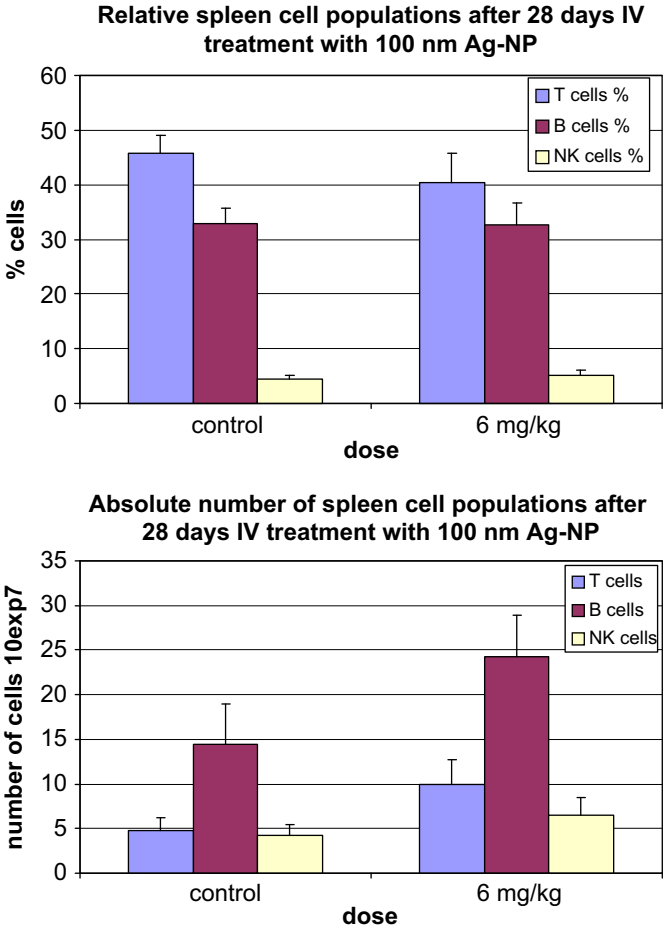

Fig. 10. Relative (top) and absolute (bottom) cellular composition of spleen cell populations after 28 days intravenous treatment with 100 nm Ag-NP.

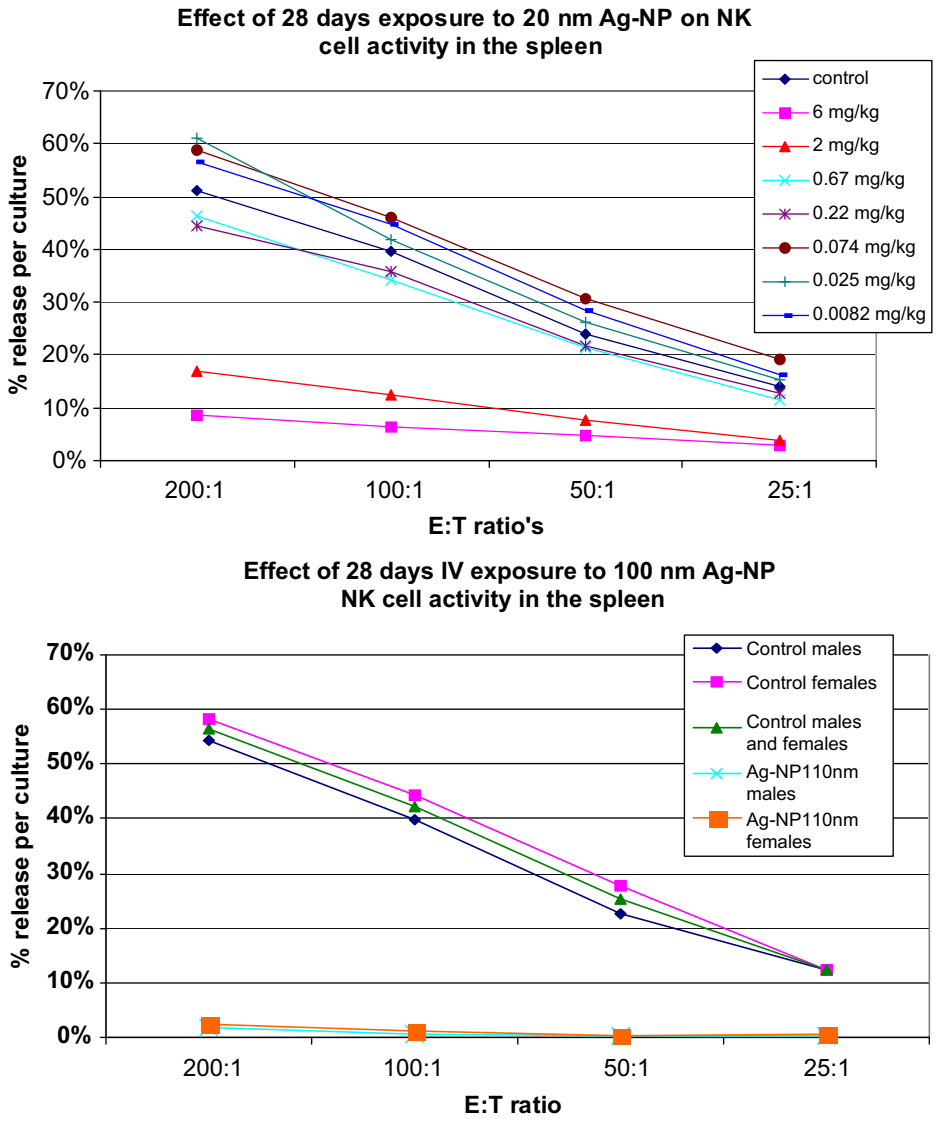

Fig. 11. NK cell activity in the spleen of rats after 28 days intravenous treatment with 20 nm or 100 nm Ag-NP.

accumulation of the Ag-NP in Kupffer cells was not accompanied with any inflammatory reaction. Also in lymph nodes the presence of Ag-NP was not accompanied by an inflammatory response. However, the increase of neutrophilic granulocytes in the blood does indicate an inflammatory response of the body (Fig. 9). Yet in the various other organs investigated no indications for an inflammation could be observed. Only in the lung isolated areas with a granulomatous inflammation could be identified. These granulomatous inflammatory reactions were partly caused by the injection technique as indicated by the presence of foreign body material in the inflammation, and partly by the Ag-NP as indicated by the presence of granular pigment in the inflammation (Fig. 8). Such granulomatous inflammation is a common side effect of prolonged repeated intravenous administrations as with every injection there is a chance of injecting some skin and/or hair material in the circulation. An overview of the results obtained in the 28 days repeated dose toxicity study with 20 nm and 100 nm Ag-NP is presented in Table 6.

In previous studies, a 28 days and 90 days inhalation study with heat generated aerosols of silver nanoparticles (size 12–15 nm (28 days study), and 19 nm (90 days study), and exposure for 6 h/day five days per week, no effects on body weight were observed [16,17]. Also in a 28 days oral toxicity study in rats conducted by the same research group no effect on body and minimal effects on organ weight (increase in brain weight in the high dose group, and in liver weight in the mid dose group) was observed at orally administered doses up to 1000 mg/kg body weight per day [19]. In a more recent study Van Der Zande et al. [20] also did not find an effect on body and organ weight in a 28 days oral toxicity study of nanosilver in rats. Also in mice the oral administration of 1 mg/kg body weight of 22 nm, 42 nm, 71 nm, and 323 nm Ag nanoparticles for 14 days did not result in an effect on body weight [26]. In our study we used the intravenous route bypassing the need for crossing cellular barriers in the lung and GI-tract to evaluate possible systemic toxic effects of Ag-NP for 20 nm and 100 nm Ag-NP. So, the whole dose administered was systemically available and the internal exposure (as presented by the intravenous dose) resulting in toxic responses could be identified using the benchmark approach. In the present study a reduction in body weight was observed at the highest dose of 6 mg/kg body weight, a dose that was most likely not obtained in the inhalation and oral toxicity studies. The BMD analysis indicated that the dose for a 5% reduction

Table 6
Overview of important toxic effects of silver nanoparticles after 28 days intravenous administration of 20 nm or 100 nm Ag-NP in rats.

20 nm Ag-NP				100 nm Ag-NP			
Effect	Sex	CED05 mg/kg b.w.	Result	Effect	Sex	Dose mg/kg b.w.	Result
General							
Body weight gain	M/F	3.89–5.26	↓	Body weight gain	M/F	6	↓
Liver weight	M/F	1.57–2.20	↑		F	6	↑
Spleen							
Spleen weight	M/F	0.25–0.37	↑↑↑	Spleen weight	M/F	6	↑↑↑
Spleen cell number	M/F	0.28	↑↑	Spleen cell number	M/F		↑↑
T cells spleen	M/F	0.12	↑↑	T cells spleen	M/F		↑↑
B cells spleen	M/F	0.12	↑↑	B cells spleen	M/F		↑↑
NK cells spleen	M/F	0.25	↑	NK cells spleen	M/F		↑
NK cell activity	M/F	0.06–0.12	↓↓↓	NK cell activity	M/F		↓↓↓
IL-10 production	M/F	0.28–0.58	↓	IL-10 production	M/F		↓
IFN-γ production	M/F	0.59	↓	IFN-γ production	M/F		–
Thymus							
Thymus weight	M/F	0.01–0.02	↓	Thymus weight	M/F		–

in body weight was somewhere between 3 and 6 mg/kg b.w. (Table 3). In both the 28 days and 90 days inhalation study no effect was seen on spleen weight in contrast to the present intravenous study [16,17]. Also in the rat oral studies no effect on spleen weight was observed [19,20]. In the mouse oral study spleen weight was not reported [26]. Our BMD analysis showed that 20 nm Ag-NP induced a 5% increase in spleen weight already at around 0.3 mg/kg body weight. In both the 28 days and 90 days inhalation study some minimal histopathology was noted in the liver, whereas in the 90 days inhalation study also minimal inflammatory responses were noted in the lung that were not seen in the 28 days inhalation study [16,17]. Our results on clinical chemistry and hematology differ from the inhalation studies as in both inhalation studies no consistent alterations were seen in the clinical chemistry of the blood and cellular blood parameters, whereas we observed a dose related effect for several clinical chemistry parameters. These differences may be due to the actual internal dose being higher in our studies to which organs like liver, spleen and bone marrow were exposed. In the blood an effect was noted on some red blood cell parameters (hematocrit, mean corpuscular cell volume, mean corpuscular hemoglobin, and blood hemoglobin). The alterations in the red blood cells may indicate an effect of the nanoparticles on hemoglobin syntheses during red blood cell maturation/formation in the bone marrow. The increase in reticulocytes in the blood indicates an increased possibly compensatory production of red blood cells. A 28 days oral toxicity study in mice found liver toxicity as indicated by an increase in liver enzyme levels of alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) in serum [26]. Similarly, we found an increase in these liver enzyme levels in serum of 20 nm Ag-NP treated rats. However, although the presence of Ag-NP could be observed in the liver Kupffer cells, alterations indicating liver histopathology were not observed.

In addition to common toxicological endpoints like body and organ weight, blood clinical chemistry and hematology, and histopathology, immune toxicity was evaluated as in our previous study to the tissue distribution of Ag-NP the spleen was one of the main target organs [13]. Previously in a 28 days oral toxicity study of nanosilver no effects on the immune system were observed [20]. In our study an increase in IgM and IgE antibody levels in serum was observed. Also in the mouse oral study [26] an increase in IgE antibodies in blood serum was found but cellular blood parameters like NK, B and T cell populations were not altered. The increase in IgM and IgE immunoglobulin levels might be explained by the increased number of B cells in the spleen. As antibodies play a prominent role in the defense against various infectious agents it needs to be determined what effects the treatment of Ag-NP would have in a microbial infection or antigen challenge model. Several models, e.g. immunization with keyhole limpet hemocyanin (KLH), bacterial or viral infectious agents are available to study the effect of xenobiotics on both the cellular and humoral immunity [27].

In the spleen indications were found for effects of Ag-NP treatment on T cell activity, as indicated by a decrease in the production of IFNγ and IL-10 after Concanavalin A stimulation of the spleen cells. B cell activity was also affected as indicated by a decrease in IL-6, IL-10 and TNF-α production, and increase in IL-1β production after LPS stimulation of the spleen cells. In the mouse 28 days oral study an increase in IL-10 in serum was noted [26]. In our study the most pronounced toxic effect of the treatment with Ag-NP was noted for the activity for natural killer (NK) cells in the spleen. The NK cell activity was almost completely suppressed after treatment of high doses with both 20 nm and 100 nm Ag-NP. Spleen NK cell activity was found to be the most sensitive parameter of the functional immune parameters investigated. The demonstration of the severe NK cell suppression needs to be further investigated in immune challenge models to evaluate the functional impact of such severe suppression in terms of resistance against outside challenges.

For the toxicity of Ag-NP there is still the question whether the toxicity is due to the nanoparticles themselves or due to the release of ions from the nanoparticles. Recently it was shown that the ion content of an Ag-NP preparation influences the toxicity of the nanoparticle preparation with preparations of a higher ion content being more toxic for cells *in vitro* [28]. The total toxicity could be ascribed to both the ion content and the nanoparticles in the preparation as in this *in vitro* study also cytotoxicity of the silver nanoparticles themselves was demonstrated. *In vivo* after oral administration the silver concentration in organs was highly correlated with the $Ag+$ ion of the nanosilver suspension [20].

5. Conclusions

The most pronounced effects of the intravenous administration of both 20 nm and 100 nm Ag-NP were the increase in spleen weight and the almost complete suppression of the natural killer (NK) cell activity in the spleen at the higher doses. As the NK cell is part of the innate immune system such suppression may have an effect on the resistance against threats from outside such as viral infections or from newly developed cancerous cells for which NK-cells are a first line of defense. The present study shows that Ag-NPs induce general toxicity in the rat at an internal exposure at a CED05 of approximately 0.3 mg/kg body weight (spleen enlargement) whereas immune toxicity (reduced thymus weight) is already induced at a CED05 dose of 0.01 mg/kg body weight. In

addition, several functional immune parameters were affected like NK cell activity with a CED05 of 0.06 mg/kg body weight, and LPS mitogen stimulation of spleen cells (CED05 0.04 mg/kg), and cytokine production by stimulated spleen cells (CED05 0.3–1.0 mg/kg body weight). This information can be used for evaluation of maximal exposure by other routes, e.g. inhalation and oral, providing the availability of appropriate kinetic models to estimate internal systemic exposure.

Disclosures

The authors have no financial conflicts of interest.

Acknowledgments

The authors would like to acknowledge the excellent technical assistance of Piet K Beekhof, Eric R Gremmer, Liset JJ De La Fonteyne, Arja De Klerk, Henny W Verharen, Bert PJ Verlaan, Jolanda Vermeulen, and Dirk Elbers, Piet van Schaaik and Hans Strootman for taking care of the animals. The histopathology was performed by Hetty Van Den Brink (NOTOX, Den Bosch, The Netherlands). Wout Slob is thanked for critically reading the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.06.048.

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