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Exposure to silver nanoparticles affects viability and function of natural killer cells, mostly via the release of ions

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Abstract Natural killer (NK) cells play a crucial role in linking innate and adaptive immune responses, especially during viral infections and tumor surveillance. They have two major effector functions: the killing of stressed/abnormal cells and the release of cytokines. Their activity is regulated via inhibitory and activating surface receptors. At the same time that the production and use of engineered nanoparticles is steadily increasing, the risk for exposure to silver nanoparticles (AgNPs) from consumer products or biomedical applications is growing. Given this, we assessed the effects of 20-nm big AgNPs on NK cells, which represent an important part of the immune system. Our study involved overnight exposure of human blood NK cells to different concentrations of AgNPs, and silver (Ag) ion controls, and analyzing them for viability, surface receptor expression, intracellular markers, cytokine release, and killing potential. Exposure to AgNPs, but not to Ag ion controls, reduced the viability and the cytotoxic potential after polyriboinosinic-polyribocytidylic acid stimulation of NK cells and increased the expression of the inhibitory receptor CD159a. Exposure to AgNPs and Ag ion controls reduced the expression of the activating receptors CD335 and of CD16 and increased the expression of the activating receptor CD314. Overall, exposure to AgNPs changes NK cells' function and phenotype and may present a risk for modulating human immune responses, which should be further investigated.

Keywords Cytotoxicity · CD16 · CD314 (NKG2D) · Engineered nanoparticles · Viral infection

Abbreviations

AgNPs	Silver nanoparticles
CD	Cluster of differentiation
NK cell	Natural killer cell
ERK	Extracellular signal-regulated kinase
IFN	Interferon
IL	Interleukin
MAPK	Mitogen-activated protein kinase
NPs	Nanoparticles
PBS	Phosphate-buffered saline

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pI:C	Polyriboinosinic-polyribocytidylic acid
PMA	Phorbol 12-myristate 13-acetate
RT	Room temperature
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
ULBP	UL16-binding protein

Introduction

Natural killer (NK) cells belong to the innate lymphoid cells (Spits et al. 2013) and link the innate and adaptive immune responses (Vivier et al. 2011) during viral infections and tumor surveillance (Vivier et al. 2008). They make up about 10% of the lung lymphocytes (Culley 2009) and represent an important cell population in the nasal (Horvath et al. 2011) and the intestinal mucosa, in the blood, in the lymph nodes, in the spleen, and other organs (Di Santo 2008; Vivier et al. 2008). Their two major effector functions are the direct, antibody-independent killing of stressed, abnormal, or infected cells and the secretion of cytokines. Cell killing can be mediated via three pathways, of which the first one is dominant: (1) release of the cytotoxic mediators perforin and granzyme B, (2) Fas-Fas ligand-mediated pathway, involving the death receptor Fas/Apol/CD95 on target cells, and (3) the cytokine-dependent pathway which cross-links tumor necrosis factor (TNF) and TNF receptors (Chavez-Galan et al. 2009). NK cells release a variety of cytokines, such as type I cytokines (most important of these, interferon (IFN)-γ), type 2 cytokines (e.g., interleukin (IL)-4), TNF-α, or IL-10. The secreted cytokines act on other cells (e.g., dendritic cells (Walzer et al. 2005), epithelial cells (Müller and Jaspers 2012), or T cells (Vivier et al. 2011)), enabling the crucial role of NK cells during immune responses.

The activity of NK cells is regulated via inhibitory and activating receptors, whose surface expression is regulated by their microenvironment (Müller and Jaspers 2012). The cytotoxic receptor cluster of differentiation (CD)16 and the cell adhesion receptor CD56 have traditionally been used to characterize more cytotoxic ($CD56^{dim}CD16^{+}$) and more cytokine-secreting ($CD56^{bright}CD16^{dim}$) subsets (Farag and Caligiuri 2006). Recently, a larger diversity of NK cell activation markers have been discovered (Vivier et al. 2011): CD314 (NKG2D) binds to upregulated ligands on stressed epithelial cells (e.g., UL16-binding protein (ULBP) 1–6 (Lanier 2008)), and CD335 (NKp46) binds directly to hemagglutinins derived from influenza or parainfluenza viruses (Mandelboim et al. 2001). Inhibitory receptors, such as CD158b (KIR2DL2/L3) or CD159a (NKG2A), bind to class I major histocompatibility complex (MHC) to ensure the inhibition of NK cells by normal autologous cells (Watzl 2003). NK cells also express the chemokine receptor CD183 (CXCR3), which binds CXCL10 (IP-10) released, among others, by epithelial cells, and plays a role in cell trafficking.

Production and use of engineered nanoparticles (NPs, particles with all three dimensions in the range from 1 to 100 nm (ISO 2015)) is continuously increasing. Silver NPs (AgNPs) are among the most frequently produced and used NPs (McGillicuddy et al. 2016; Zhang et al. 2016a). Their antibacterial, antifungal, and antiviral characteristics make them a potential alternative for antibiotics and interesting for a broader use in the medical field (Zhang et al. 2016b). Because AgNPs are already used in sunscreen, cosmetics, food packages, or textiles (Zhang et al. 2016b), the risk for exposure occurs not only during medical treatments but also during consumption or use of NP products. Additionally, there is also a risk for occupational exposure during the production or recycling of the NP products.

Studying immunomodulatory effects of engineered NPs is challenging, and methods are still under development; however, it is of high importance (Pfaller et al. 2010; Oostingh et al. 2011). Investigations of the effects of NP exposure on single cell types could contribute to our understanding of how NPs interact with the human body and, in particular, with the human immune system. Since each NP has its own characteristic and each cell type reacts differently to external influences, interactions between NPs and one cell type cannot be generalized (Mahmoudi et al. 2012). Interactions would, therefore, need to be studied for each NP-cell type combination separately. As NK cells are an important immune cell type and have been shown to be affected by environmental NPs (e.g., diesel exhaust particles (Muller et al. 2013)), potential effects of engineered NPs on NK cells are of high importance.

Our study took a look at the effects of AgNPs, on the viability and function of primary NK cells. In order to mimic the reactions of NK cells in the context of a viral infection, we also studied these effects after stimulation with the viral mimetic polyriboinosinic-polyribocytidylic acid (pI:C).

Experimental

Study design

We used an in vitro cell study design to investigate the effects of an overnight exposure to AgNPs or silver (Ag) ion controls on primary human peripheral blood NK cells' function and phenotype. The study was approved by the ethics committee "Ethikkommission Nordwest- und Zentralschweiz" (reference number 250/13), and written informed consent was obtained from all participants.

Firstly, we analyzed how the viability of NK cells was affected by the NP exposure, and excluded samples with more than 70% of dead cells; in all other samples, we analyzed the function and phenotype of all viable NK cells. We observed a high experiment-to-experiment variation resulting from the use of NK cells isolated from one donor for each experiment, and the differences in susceptibility of NK cells of the different donors to AgNP exposure. Activity and susceptibility of NK cells were not related to the age of the donors.

NK cell enrichment

NK cells were enriched from the peripheral whole blood of healthy non-smoking volunteers (total $N = 19$, average age ± standard deviation (range) = 33.5 ± 9.5 (24–64) years, body mass index = 21.7 ± 2.0 (18.8–26.2), male/female = 7:12) using a density gradient of Lymphoprep in SepMate tubes (StemCell Technologies, Grenoble, France) and the EasySep Human NK Cell Enrichment Kit (StemCell Technologies), according to the manufacturer's instructions. NK cells were resuspended in RPMI 1640 media with L-glutamine (Sigma-Aldrich, Buchs, Switzerland) completed with 10% fetal bovine serum (BioConcept, Allschwil, Switzerland) and 1% penicillin-streptomycin (Sigma-Aldrich). The purity of the enrichment was >90% (assessed by flow cytometry staining).

Particle description

The AgNPs (Ag core, mean diameter of 20.6 nm, citrate-coated, colloidal, suspended in water) are commercially available from BBI solutions (Cardiff, UK); for details about characterization, see the manufacturer's specifications and Supplementary Data (Figure S1). NPs were stored and diluted in water. To ensure similar hypotonic conditions, we adjusted the volume of water in all samples. In order to control for the effect of released Ag ions, we incubated AgNPs overnight with NK cell media, removed the NPs by centrifugation (1 h, 10,000*g*, 4 °C), and collected the media.

We also performed the same experiments with gold NPs (Au core, average diameter of 20 nm, polyethylene glycol (PEG)-COOH coated, negatively charged, colloidal, suspended in water; previously described in Rodriguez-Lorenzo et al. (2014); concentrations of 0, 20, or 100 μg/ml). However, we did not find major effects (data not shown) and thus decided not to present the data within this study.

Particle exposure and NK cell stimulation

NK cells were incubated at a concentration of 10^6 cells/ml with AgNPs (0, 1, 5, or 10 μg/ml). The AgNP dilutions were prepared using sterile, deionized water. The same volume of AgNP suspensions was added to the NK cells in complete cell culture medium (RPMI media with L-glutamine, 10% fetal bovine serum, 1% penicillin-streptomycin). pI:C (Sigma-Aldrich) was added at a concentration of 10 μg/ml and recombinant IL-12 (Tonbo Biosciences, ordered from LuBioScience GmbH, Lucerne, Switzerland) at 10 ng/ml, both simultaneously to the administration of the NPs. NK cells were incubated overnight (18–20 h).

Viability, surface receptors, and intracellular markers of NK cells—assessed by flow cytometry

All samples were first stained with viability dye (Fixable Viability Dye eFluor 450; eBioscience), according to the supplier's instructions, and resuspended in flow staining buffer (phosphate-buffered saline (PBS) without Ca^{2+}, Mg^{2+} (Sigma-Aldrich), 1% heat-inactivated FBS, 0.9% sodium azide (Sigma-Aldrich)).

Samples for surface marker staining were incubated with antibodies (Table 1) for 20 min at room temperature (RT), subsequently fixed with 2% paraformaldehyde (Electron Microscopy Sciences, ordered from Lucerna Chem, Lucerne, Switzerland), and resuspended in flow staining buffer for acquisition.

For intracellular analysis, the Golgi apparatus was blocked with Brefeldin A (eBioscience, Vienna, Austria), and the cells were subjected to non-specific stimulation with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Acros Organics, ordered from Fisher

Table 1 Antibody cocktails for flow cytometry stainings

	Tube								
	Alexa Fluor 700	APC	APC-Cy7	Brilliant Violet 605	FITC	PE	PE-Cy7	PE-TR
Surface marker	CD159a (NKG2A)[a]	CD314 (NKG2D)[b]	CD45[c]	CD183 (CXCR3)[b]	CD335 (NKp46)[b]	CD158b[d]	CD16[b]	CD56[e]
Surface marker isotype control	IgG2a[a]	IgG1[b]	IgG1[c]	IgG1[b]	IgG1[b]	IgG2[d]	IgG1[b]	IgG2[e]
Intracellular maker		IFN-γ[f]	CD45[c]		Granzyme B[d]	IL-4[f]		

APC allophycocyanin, *APC-Cy7* allophycocyanin-cyanine 7, *FITC* fluorescein isothiocyanate, *PE* phytoerythrin, *PE-Cy7* phytoerythrin-cyanine 7, *PE-TR* phytoerythrin-Texas red

[a] Antibodies were purchased from R&D Systems (Bio-Techne, Zug, Switzerland)

[b] Antibodies were purchased from BioLegend (Lucerna Chem)

[c] Antibodies were purchased from Tonbo (LuBioScience)

[d] Antibodies were purchased from BD Biosciences

[e] Antibodies were purchased from Invitrogen (Life Technologies)

[f] Antibodies were purchased from eBioscience

Scientific, Reinach, Switzerland) and ionomycin (1 μg/ml; MP Biomedicals, ordered from Lucerna Chem), for 4 h. Subsequently, samples were first stained for the CD45 surface marker (incubation with antibody for 20 min at RT) and then for intracellular markers using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience), according to the manufacturer's instructions. Cells were incubated with antibodies (Table 1) for 30 min at RT and resuspended in flow staining buffer.

All samples were acquired with a BD LSR Fortessa (BD Biosciences) within 24 h and analyzed using the FlowJo software, version 10.

Cell morphology—analysis by transmission electron microscopy

Samples were fixed with Karnofsky paraformaldehyde (3%) and glutaraldehyde (0.5%) in PBS (10 mM pH 7.4) for 1 h and washed with PBS. After a second fixation (reduced osmium tetroxide (1%), potassium ferrocyanide (1.5%); 40 min) and a third fixation (osmium tetroxide (1%); 40 min), samples were washed with water and dehydrated with ethanol (10 min at 50%, 10 min at 70%, 60 min at 70% plus 2% uranyl acetate, 10 min at 90%, 10 min at 100%). For the embedding, samples were treated for 10 min with acetone, 1–2 h with epon/acetone (1:1) mixture, 2–4 h with pure epon, and finally embedded in epon (24–48 h, 60 °C). Samples were cut into 60-nm sections with a microtome Ultracut E from Leica and contrasted (60 min, uranyl acetate (6%); 2 min, lead acetate). A Morgagni FEI 80-kV microscope was used for the images. Transmission electron microscopy (TEM) analysis was used to investigate potential morphological changes.

Cytokine release of NK cells

The release of IL-8, IL-10, and TNF-α by NK cells was assessed using a custom-made MILLIPLEX® Multiplex Assay (Merck Millipore, Schaffhausen, Switzerland) using a XMAP Technology Luminex machine according to the manufacturer's instructions.

Killing potential of NK cells

NK cells' killing potential was analyzed with the 7-AAD/CFSE Cell-Mediated Cytotoxicity Assay Kit (Cayman Chemical, ordered from Biozol, Eching, Germany) using the K562 human chronic myelogenous

leukemia cell line as target cells. Target cells were added at a ratio of NK cells/target cells = 5:1 for 3 h, as previously described (Muller et al. 2013). Samples were measured on a BD LSR Fortessa immediately after staining and analyzed using the FlowJo software, version 10.

In order to ensure that K562 cells were not killed by direct NP contact alone, we performed control experiments incubating K562 cells with AgNPs or Ag ions for 3 h. No cytotoxicity of K562 cells was found (data not shown). The addition of IL-12 to the NK cells increased the killing potential from 45 to 70% of killed target cells (Supplementary Data Figure S5), showing the potential of NK cells to regulate the killing activity.

Statistical analysis

Experiments were performed in blocks using NK cells of one individual donor for all conditions of AgNPs or Ag ion controls, respectively. The number of repetition (n) was between 4 and 6. Viability data were compared applying the Kruskal-Wallis test using raw values to ensure that other endpoints were not affected due to low viability. For the analysis of surface, intracellular, and killing potential data, corresponding controls (either media only, pI:C, or IL-12 stimulated) were subtracted from the samples and the difference was compared to the hypothetical median of 0 (representing no difference to the corresponding controls) using the Wilcoxon signed-rank test. A result of $p < 0.05$ was considered statistically significant.

Results

Viability of NK cells

Exposure upon AgNPs was associated with a dose-dependent killing of NK cells in media alone, which was even more pronounced in combination with pI:C stimulation. Exposure to Ag ion controls did not affect the viability of the NK cells (Fig. 1).

Changes in morphology

The TEM images did not show any major changes in NK cell morphology due to AgNP exposure (Fig. 2). The cell structures and membrane integrity seemed to be maintained. However, NK cells stimulated with pI:C and simultaneously exposed to AgNPs showed holes in the cell membranes, shrunken cytoplasm, and unstructured cell nuclei (Fig. 2D (1 and 2)).

Surface receptor expression of NK cells

Exposure to AgNPs was associated with reduced expression of the cytotoxic marker CD16, as well as of the natural cytotoxicity receptor CD335, and increased expression of the activating receptor CD314, and the inhibitory receptor CD159a. The chemokine receptor CD183, the adhesion receptor CD56, and the inhibitory receptor CD158b were not affected by AgNP exposure (Fig. 3). The differences to the media control were significantly altered from 0 for AgNP exposure in media. For pI:C stimulation, the data showed similar trends but did not reach statistical significance due to the lower repetition number ($n = 4$, exclusion of two experiments due to low viability). Exposure to the Ag ion controls induced almost the same changes in surface marker expression as did exposure to AgNPs: significantly reduced CD16 and CD335, and increased CD314 expression, but unchanged expression of CD56, CD159a, CD158b, and CD183 (Fig. 4).

Intracellular markers of NK cells

AgNP exposure showed no effect on the IFN-γ production but showed some slight tendencies for enhanced IL-4 and decreased granzyme B production (Supplementary Figure S2).

Cytokine release of NK cells

The cytokine release by NK cells was not affected by exposure to AgNPs. Exposure to the Ag ion controls in combination with pI:C stimulation increased slightly the release of TNF-α, while the release of IL-8 and IL-10 was not affected in biologically relevant extent (Supplementary Figure S3).

Killing potential of NK cells

Exposure to AgNPs was associated with a dose-dependent reduction of the killing potential of NK cells in the context of pI:C and IL-12 stimulation. Incubation with the Ag ion controls did not affect the killing potential of NK cells significantly. However, it did show a

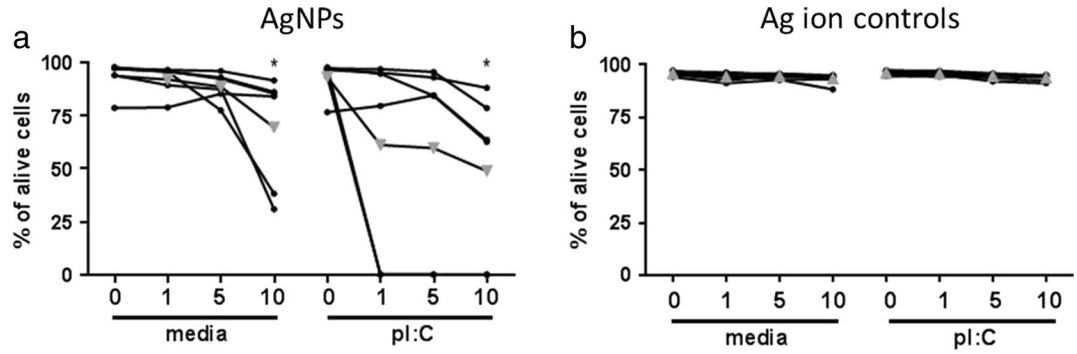

Fig. 1 Viability of NK cells after exposure to AgNPs (**a**) and Ag ion controls (**b**). NK cells were exposed to different concentrations of AgNPs (0–10 μg/ml and corresponding Ag ion controls). Data are shown as percent of living cells. *Gray triangles* represent mean values. $N = 6$ for all conditions; $*p < 0.05$, tested with Wilcoxon signed-rank test compared to the corresponding control (0 μg/ml of AgNPs). *Numbers on the x-axis* represent the NP concentrations in micrograms per milliliters

trend towards reduction in the context of IL-12 stimulation (Fig. 5).

Discussion

We studied the effects of AgNPs, on human primary peripheral blood NK cells. Ag ion controls were used to elucidate whether the observed effects were ionic or particular. Exposure to AgNPs or Ag ions was associated with major changes: exposure to AgNPs killed NK cells in a concentration-dependent manner; reduced expression of the cytotoxic receptor CD16, the activating receptor CD335, the production of granzyme B, and the killing potential of NK cells; and increased the expression of the inhibitory receptor CD159a and the activating receptor CD314. The exposure to Ag ion controls reduced the CD16 and CD335 and increased CD314 expression.

The effects of engineered NPs have already been investigated in various cell models but, to our knowledge, not yet in a pure human NK cell population. AgNPs have been shown to increase oxidative stress and cytokine production in monocytes, to increase

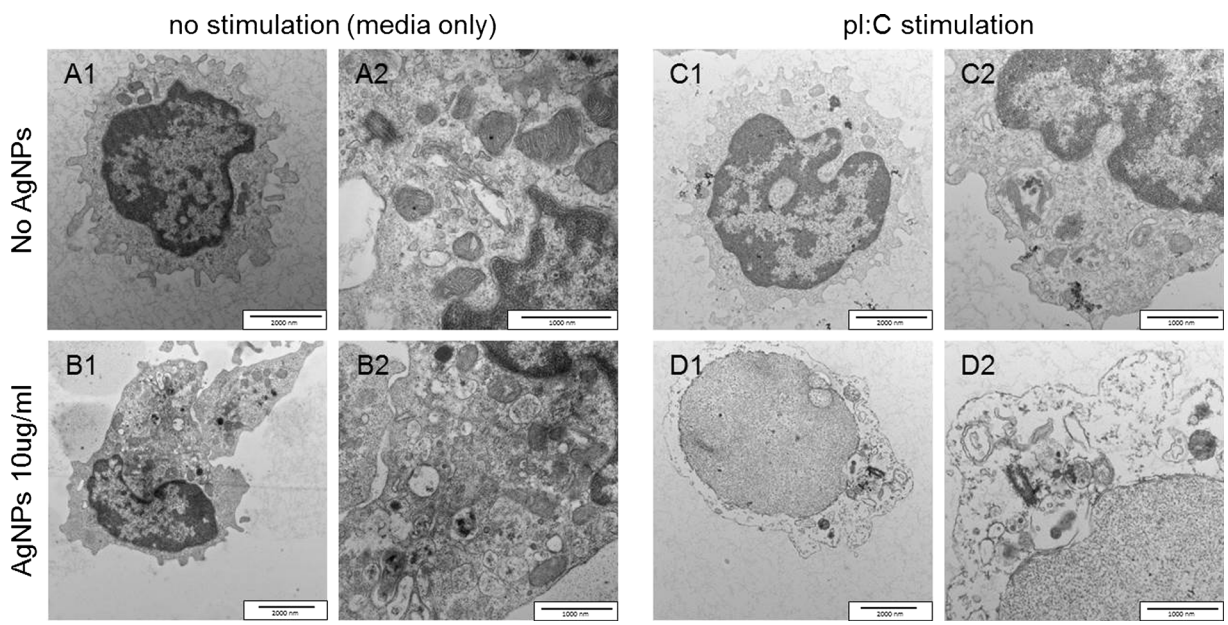

Fig. 2 Morphological TEM analysis of unstimulated and pI:C-stimulated NK cells exposed to AgNPs. Only images of NK cells incubated with the highest AgNP concentration are shown. The *left images* (*A–D*, *1*) show an overview of a whole cell and the *right images* (*A–D*, *2*) a detailed image of a subpart of the cell

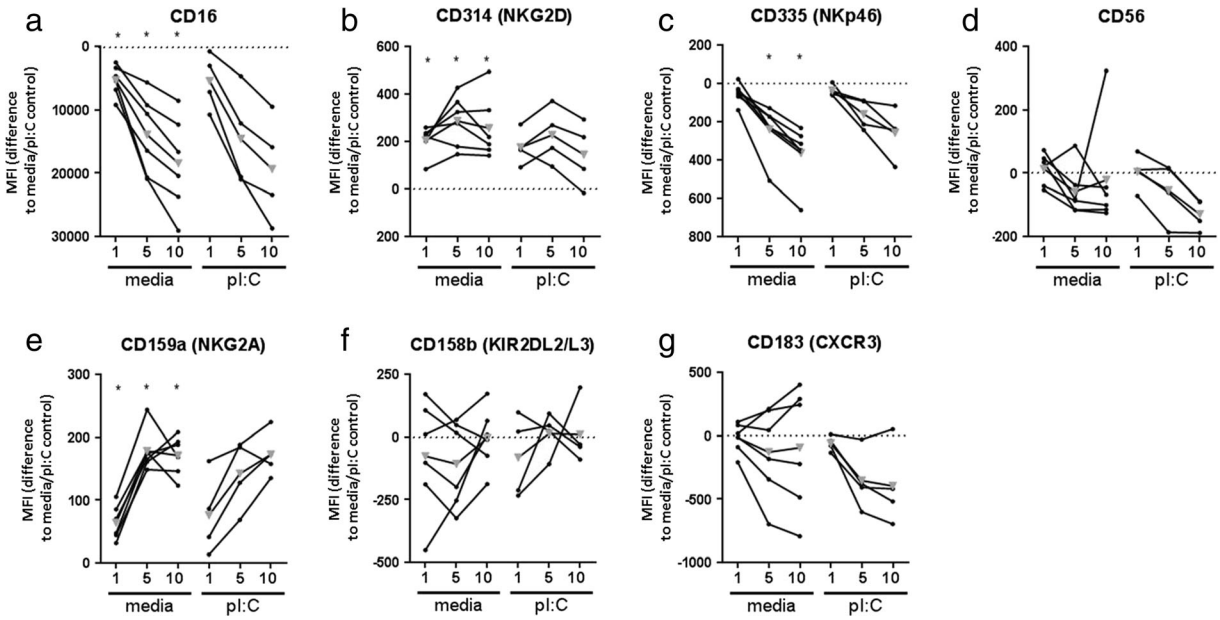

Fig. 3 **a**–**g** Surface receptor expression of NK cells after AgNP exposure. Data of NK cell surface marker expression after AgNP exposure are shown as the difference to the corresponding control (media only or pI:C stimulation, values not shown, represented by the *dotted line at 0*). *Gray triangles* represent mean values. *$p < 0.05$, tested with Wilcoxon signed-rank test compared to the corresponding controls

inflammatory cytokines in lung macrophages and dendritic cells, and to enhance production of reactive oxygen species, DNA damage, and apoptosis in the human lung epithelium (summarized in Petrarca et al. (2015)). However, the same AgNPs as in our study did not induce oxidative stress or inflammation in the abovementioned

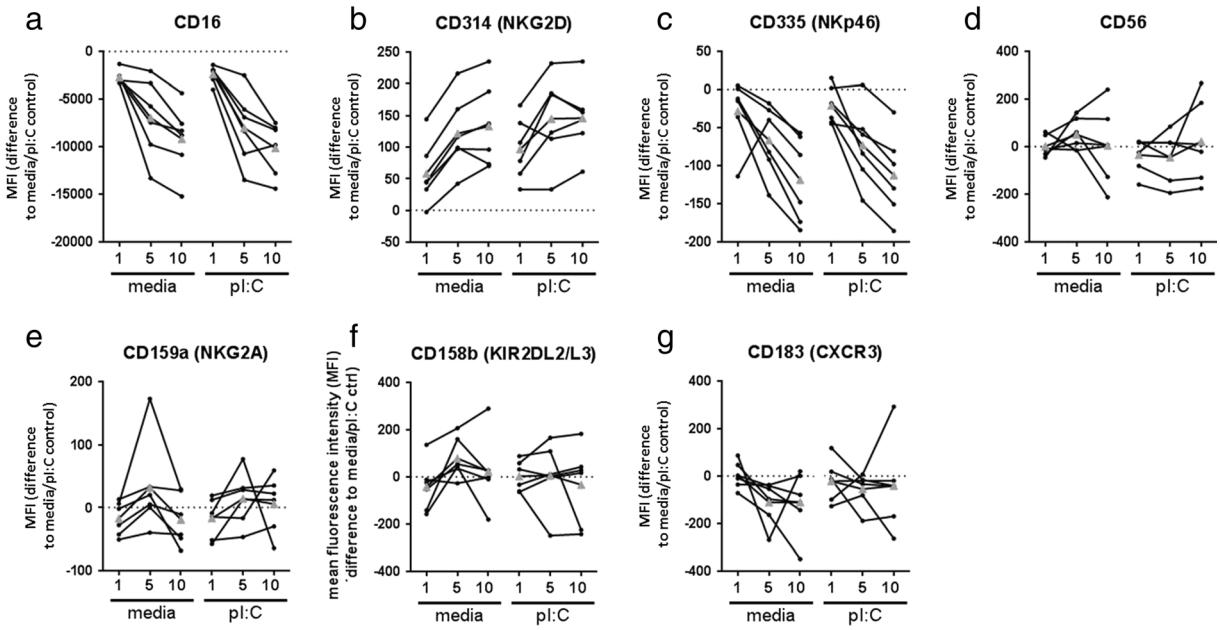

Fig. 4 **a**–**g** Surface receptor expression of NK cells after exposure to Ag ion controls. Data of NK cell surface marker expression are shown as the difference to the corresponding control (media only or pI:C stimulation, values not shown, represented by the *dotted line at 0*). *Gray triangles* represent mean values. *$p < 0.05$, tested with Wilcoxon signed-rank test compared to the corresponding controls

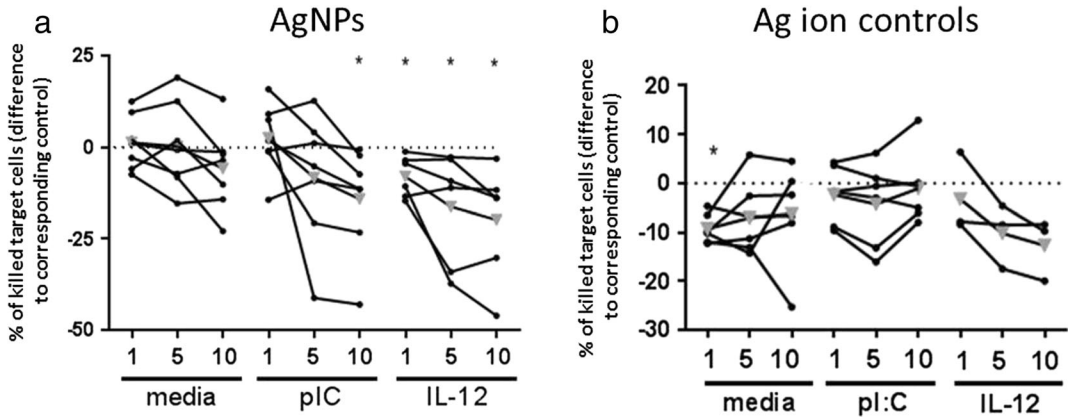

Fig. 5 Killing potential of NK cells tested with K562 target cells after AgNP and Ag ion control exposure. After overnight exposure, NK cells were incubated for 3 h with pre-labeled K562 target cells and then analyzed for percentage of killed target cells. In addition to incubation with AgNPs, NK cells were simultaneously stimulated with pI:C or IL-12. **a** NK cells' killing potential after AgNP exposure. **b** Killing potential after exposure to Ag ion controls. Data are shown as the difference to the corresponding control (media only, pI:C, or IL-12 stimulation, represented by the *dotted line at 0*). *Gray triangles* represent mean values. $*p < 0.05$, tested with Wilcoxon signed-rank test compared with the corresponding controls

triple cell co-culture model (Herzog et al. 2013). The fact that we found adverse effects of AgNP exposure in primary NK cells could mean that NK cells are more susceptible than the cells in the triple cell co-culture model. The susceptibility of NK cells upon AgNP exposure has already been shown in a rat study which found suppressed killing activity of spleen NK cells after intravenous exposure upon 6 mg/kg body weight of uncoated 20-nm AgNPs over 28 days (De Jong et al. 2013). However, another study found no impact on NK cell activity after a 28-day exposure of rats to uncoated 20-nm AgNPs after oral administration of 90 mg/kg body weight (van der Zande et al. 2012). Additionally, their control exposure to Ag ions did not impair the NK cell activity. They did, however, find high concentrations of Ag ions also in the blood of the rats exposed to AgNPs, and AgNPs, as well as Ag ions, accumulated in all organs of the rats. In summary, this demonstrates a transformation of AgNPs after oral administration compared to intravenous, which may influence the toxicity of AgNPs. As already stated by De Jong et al. (2013), the suppression of NK cell activity by exposure to AgNPs should be investigated in an in vivo immune challenge model, e.g., infection with respiratory viruses.

In addition to AgNPs, other metal NPs have been shown to affect NK cells: exposure to uncoated ZnO NPs reduced the viability and the CD16 expression of NK cells in a PBMC cell population (Hanley et al. 2009; Andersson-Willman et al. 2012), while no effects on NK cells were found upon exposure to uncoated TiO_2 NPs (Andersson-Willman et al. 2012). Taken together, the results of these studies, along with our results, show the potential risk of engineered NPs to affect NK cells in an NP type-dependent manner, and highlight the importance of testing every NP type, and especially different NP coatings, for effects on different biological systems.

The reduction of NK cells' cytotoxic potential (reduced killing potential, CD16 expression, and granzyme B production), and the reduction of CD335 expression (receptor for (para)influenza-derived hemagglutinins), may be associated with an impairment of the antiviral immune response in which NK cells are involved. It has been shown that diesel exhaust exposure increases susceptibility for viral infections (Hahon et al. 1985; Harrod et al. 2003; Jaspers et al. 2005; Ciencewicki and Jaspers 2007) and that the reduced killing activity of NK cells after diesel exhaust particle exposure (Muller et al. 2013) may play a crucial role in enhancing this susceptibility. Finding similar results for the exposure to AgNPs raises the question about a potential impairment of the antiviral response due to AgNP exposure. However, a study by Xiang et al. (2013) showed a protective effect of AgNP administration on H3N2 influenza infection in vitro and in vivo via the inhibition of the influenza virus (Xiang et al. 2013). Based on our results, we suggest the inclusion of an NK cell function analysis into future (animal) studies before a final conclusion on a potential protective or adverse effects of AgNPs on influenza infection can be drawn.

NK cells can kill target cells via three different pathways (perforin/granzyme B, Fas-Fas ligand, or cross-linking TNF and TNF receptors), of which the one involving granzyme B is most important (Chavez-Galan et al. 2009). Our results show an association of AgNP exposure with not only a reduced killing potential but also with reduced granzyme B production. Based on this, we can speculate that exposure to AgNPs may affect the pathway responsible for granzyme B production and reduce the killing potential via lower granzyme B levels. Granzyme B mobilization towards target cells is regulated via the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway (Trotta et al. 1998), which has been shown to be affected by AgNP exposure (Bohmert et al. 2015; Castiglioni et al. 2015). Further investigations, which exceeded the scope of this study, would be needed to verify this association.

We used primary human peripheral blood NK cells to test for effects of AgNPs in an in vitro study design. This allowed us to make use of exact controls, take into account the natural variability between subjects, and conduct the experiment in a more realistic in vivo situation as compared to the use of cell lines. However, peripheral blood NK cells are only directly exposed to AgNPs after either intravenous administration or translocation into the human bloodstream after oral intake or inhalation. The most realistic AgNP exposure routes for humans are either via oral ingestion or in occupational settings via inhalation. Via both exposure routes, NP surfaces would be altered by biological masking processes until they can interact with NK cells. Regarding AgNPs, the potential to release Ag ions, and the subsequent formation of Ag salts, as shown by van der Zande et al. (2012), needs to be considered. As we also found severe effects due to the release of Ag ions, the release of Ag ions from AgNPs may be the main way for the induction of changes in NK cells' function and phenotype. Thus, there may be a need for the development of AgNPs which do not release ions upon contact with biological systems. We found some of the effect for both AgNP and Ag ion control exposure. However, we found more and stronger effects after AgNP exposure. A potential explanation could be the active or passive uptake of AgNP by NK cells. This could lead to very high intracellular Ag ion concentrations due to intracellular ion release. NK cells could be more directly exposed to AgNPs via inhalation. However, the presence of surfactant proteins in the lungs and their interactions with inhaled NPs also has the potential to alter the toxicity, as has been previously shown for carbon nanotubes (Gasser et al. 2010, 2012). Additionally, it has to be considered that NK cells in the airways are different compared with blood NK cells, due to a different microenvironment. In future investigations, it would be interesting to make use of co-culture models in order to take into account cell-cell interactions. It would also be interesting to perform a human in vivo study using inhaled AgNPs and a standardized dose of live attenuated influenza vaccine, as was previously done with diesel exhaust exposure (Noah et al. 2012).

Conclusion

AgNP exposure on NK cells was associated with the induction of significant changes in NK cells' function and phenotype; most—but not all—changes were mediated by the release of Ag ions. These changes may impair the antiviral immune response of NK cells. Based on our findings, we conclude that AgNP exposure presents a possible risk for modulating human respiratory immune responses.

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Compliance with ethical standards The study was approved by the ethics committee "Ethikkommission Nordwest- und Zentralschweiz" (reference number 250/13), and written informed consent was obtained from all participants.

Conflict of interest The authors declare that they have no conflict of interests.

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