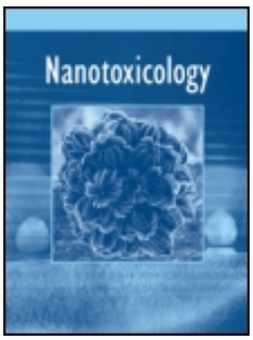

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ORIGINAL RESEARCH

Immunotoxicity and genotoxicity testing of PLGA-PEO nanoparticles in human blood cell model

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Abstract

A human blood cell model for immunotoxicity and genotoxicity testing was used to measure the response to polylactic-co-glycolic acid (PLGA-PEO) nanoparticle (NP) (0.12, 3, 15 and 75 μg/cm^2 exposure in fresh peripheral whole blood cultures/isolated peripheral blood mononuclear cell cultures from human volunteers ($n = 9$–13). PLGA-PEO NPs were not toxic up to dose 3 μg/cm^2; dose of 75 μg/cm^2 displays significant decrease in [^{3}H]-thymidine incorporation into DNA of proliferating cells after 4 h (70% of control) and 48 h (84%) exposure to NPs. In non-cytotoxic concentrations, *in vitro* assessment of the immunotoxic effects displayed moderate but significant suppression of proliferative activity of T-lymphocytes and T-dependent B-cell response in cultures stimulated with PWM > CON A, and no changes in PHA cultures. Decrease in proliferative function was the most significant in T-cells stimulated with CD3 antigen (up to 84%). Cytotoxicity of natural killer cells was suppressed moderately (92%) but significantly in middle-dosed cultures (4 h exposure). On the other hand, in low PLGA-PEO NPs dosed cultures, significant stimulation of phagocytic activity of granulocytes (119%) > monocytes (117%) and respiratory burst of phagocytes (122%) was recorded. Genotoxicity assessment revealed no increase in the number of micronucleated binucleated cells and no induction of SBs or oxidised DNA bases in PLGA-PEO-treated cells. To conclude on immuno- and genotoxicity of PLGA-PEO NPs, more experiments with various particle size, charge and composition need to be done.

Keywords

PLGA-PEO nanoparticles, immunotoxicity, genotoxicity, lymphocytes, natural killer cells, phagocytes and respiratory burst

History

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Introduction

The interaction between nanoparticles (NPs) and immunocompetent cells has been demonstrated; however, the data available are limited. Assessment of the immunotoxicity and genotoxicity of nanomaterials is essential for safe use of medical diagnostic (pharmaceutical) nanoproducts and therefore development of appropriate *in vitro* tests and cellular models is required (Dusinska et al. 2009; Oostingh et al. 2011; Lankveld et al. 2010).

Human peripheral blood is a very useful biological material for *in vitro* screening of the immunotoxic and genotoxic potential of chemicals including nanoproducts. Blood is a model for the first target in the case of intravenous administration of NPs used in medical diagnostics and therapy and a surrogate target model for other routes of exposure, giving information on the overall body response to environmental and industrial pollutants (Dusinska and Collins, 2008; Oostingh et al. 2011). Apart from easy access, the main strength of human peripheral blood as an *in vitro* testing model is, among others, the complexity of the model containing several cell components in a relatively intact environment.

A properly selected battery of *in vitro* immune and genotoxic assays might identify significant cells/molecules probably targeted by NPs. Immune function assays are the most reliable tests to monitor the action of immunocompetent cells. They are widely used to determine congenital or acquired immune disorders in clinical diagnostics. Adaptation of such tests allows evaluation of the effect of NP on specific (acquired) and non-specific (natural) immune response. In our study, specific immune response was monitored by the lymphocyte transformation test. This assay is used in clinical diagnosis of drug allergy, and modified as the local lymph node assay in mice, and it has been validated for assessment of the allergic potential of chemicals in toxicology (OECD 429, 2010). The function of phagocytes can easily be determined by measurement of the phagocytic activity of granulocytes and monocytes using fluorescein-labelled *Staphylococcus aureus* with flow cytometry. Moreover, the respiratory burst of phagocytes using hydroxyethidine can be measured in one tube simultaneously. Cytotoxicity of natural

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killer cells (NK cells) is a reliable method for monitoring of tumour defence and several papers have recommended this assay for assessment of immunotoxic potential (Lankveld et al. 2010).

In addition to the assessment of immune effect, it is very useful to measure DNA stability using the same model. Both the micronucleus (OECD 487, 2010) and the comet assays are reliable tests that can assess the genotoxic potential of NPs (Gonzalez et al. 2010; Karlsson 2010; Kirsch-Volders et al. 2011; Kazimirova et al. 2012; Magdolenova et al. 2012). Genotoxicity is the result of events that can occur at different levels of organisation of genetic material and thus markers of genotoxicity can cover a broad spectrum of damage at DNA and chromosomal levels. While the comet assay is able to recognise transient DNA lesions (strand breaks (SBs) and base damage) that could be repaired or lead to gene mutations the cytokinesis-block micronucleus (CBMN) assay can detect clastogenicity, aneugenicity and effects on cell-cycle checkpoints. Moreover, the possible interference of cytochalasin B (which acts also as an inhibitor of endocytosis) with NPs is eliminated (Magdolenova et al. 2012).

Polylactic-co-glycolic acid (PLGA-PEO)-based NPs have been selected for model testing (Figure 1). PLGA-PEO NPs are biocompatible and biodegradable and therefore have been extensively investigated as a carrier for site-specific delivery of vaccines, genes, drugs and other biomolecules in the body (Mahapatro & Singh 2011). Functionalised with either gold nanocrystals or quantum dots, they exhibited favourable features for computed tomography and optical imaging (Mieszawska et al. 2012). In relation to the immune system, NPs are utilised in a broad spectrum of vaccines (Keijzer et al. 2011; Hamdy et al. 2011). Data on immunotoxicity of PLGA NP carrier are limited, focused on key properties of particles contributing to the generation of the different types of immune response. Published results show that microparticles compared with NPs induced significant release of TNF-α and IL-1β. PLGA-PEO microparticles (5–7 μm) in contrast with NPs (389 nm) are not able to be phagocytosed by J774 macrophages, but instead they attach to cell membrane and constitute a more potent inflammatory stimulus after the uptake process (Nicolete et al. 2011). Hydrophobic particles >500 nm elicited a more robust increase in secretory IgA, interleukin-2 and interferon-γ levels compared to hydrophilic particles <500 nm. Large hydrophobic particles were more efficiently internalised by rat alveolar macrophages compared to smaller hydrophilic particles (Thomas et al. 2011). Studies of cytotoxicity indicate that the viability of cells is not or only slightly affected by NP formulations (Thomas et al. 2011; Basarkar et al. 2007; Eyles et al. 2003). Generally, information on the genotoxicity of PLGA-PEO NPs is also scant. He et al. (2009) evaluated the genotoxicity of nine kinds of selected blank PELGE and PLGA NPs in CHO cells, reporting no increase in frequencies of micronuclei (MN) but more sister chromatid exchanges in cells exposed to five kinds of PELGE NPs. The discrepancies found in the two assays suggest that these polymeric NPs may produce a weak clastogenic response. De Lima et al. (2011) performed a cytogenetic analysis of human lymphocytes exposed to PLGA NPs and showed no significant changes in the mitotic index in relation to the control, indicating that in the concentration range tested, PLGA particles used in the experimental models did not present cyto- or genotoxicity. Kazimirova et al. (2012) found that PLGA-PEO NPs did not induce DNA SBs or oxidised DNA lesions in human lymphoblastoid TK6 cells, nor did they significantly increase the level of MN in binucleated cells (BNCs).

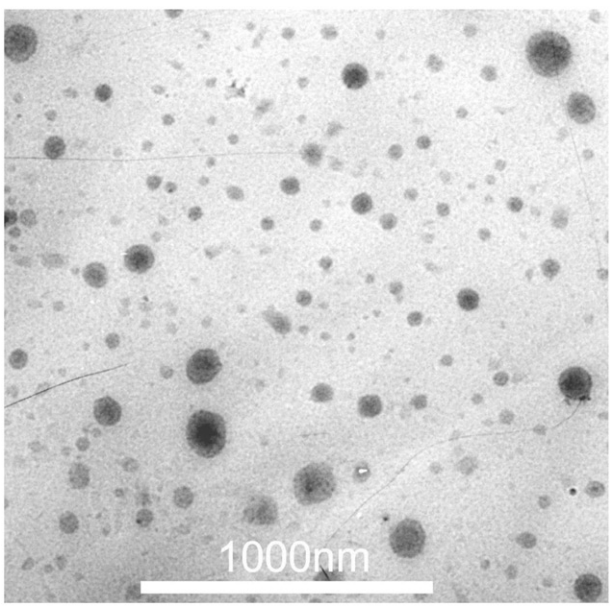

Figure 1. TEM micrograph of PLGA-PEO NPs. The particle size is within the range of 50–200 nm. The core of the particles is clearly visible.

This article summarises the assessment of immunotoxicity and genotoxicity potential of PLGA-PEO NPs as promising candidates for medical diagnostics and drug delivery in human blood cell model.

Methods

NP characterisation

The PLGA-PEO NPs were purchased from Advancell Technologies (Spain) as a suspension in water (10 mg/ml of PLGA-PEO NPs) and were characterised for their size (z-average, 143 and 180 nm) by the provider using a dynamic light-scattering (DLS) instrument Zetasizer Nanoseries, by Malvern Instruments, UK.

Transmission electron microscopy

The transmission electron microscope (TEM) JEOL 1200EX with 120 kV accelerating voltage was used for the characterisation of NPs. The TEM specimens were prepared by drying of small drop of the nano-colloid on the surface of supporting amorphous carbon foil.

Zeta potential measurements

Zeta potential was measured by a Nicomp Submicron Particle Sizer Autodilute Model 380 (Santa Barbara, CA, USA) using the electrophoretic light scattering method (for details and figure see the paper on characterisation of NPs in this special issue). As reported, zeta potential value at pH 7 resulted to be −43.4 mV.

Size distribution determination

When PLGA-PEO NPs (nominal NP concentration, 0.25 mg/ml) was added to RPMI 1640 (Sigma-Aldrich) + 10% of foetal bovine serum (FBS) (PAA), size distribution of PLGA-PEO was determined as hydrodynamic diameter by DLS at 90° detection angle with a Nicomp Submicron Particle Sizer Autodilute Model 370 (Santa Barbara, CA, USA). The employed instrument can automatically recognise, in the 0.5–6000 nm range, up to three size distributions of particles concurrently present through a patented software algorithm. PLGA-PEO NPs were vortex-shaking the tubes for a few minutes just before use. NPs in stock dispersion were added to RPMI medium with 10% FBS and then gently manually shaken. All samples were gently homogenised by a Pasteur pipette directly in DLS glass tubes just

before measurements. Size distribution after 15 min and after 24 h from addition of dispersions into medium was used as a size stability parameter.

NP dispersion

NPs were stored at 4°C and needed vortex-shaking of the tubes for a few minutes just before use. The NPs were then diluted in the appropriate cell-culture medium to obtain a stock solution equivalent to a working solution of 75 μg/cm^2. Then serial dilutions of NPs in the cell-culture medium were prepared to obtain the full concentration range of NP suspensions (0.12, 3, 15 and 75 μg/cm^2) which were immediately added to the cells.

Subjects

Thirteen volunteers (V1–V13) participated in the study (6 women, 7 men, age 40–50) not exposed to any known mutagens. Blood was collected by venipuncture from fasted subjects, and aliquoted in heparinised tubes, used for micronucleus and immunology tests and in EDTA tubes for the comet assay. All study participants signed an informed consent form. This study was approved by the Ethical Committee of the Slovak Medical University in Bratislava.

Assessment of immunotoxicity

Proliferative activity of lymphocytes

One hundred and fifty microlitres of human heparinised whole blood diluted 1:15 in complete RPMI 1640 (Sigma-Aldrich) medium containing 10% foetal calf serum (FCS, PAA), L-glutamine (Sigma-Aldrich), gentamycin (Sandoz) was dispensed in triplicate wells of a 96-well microtitre culture plate under sterile conditions. Mitogens (Sigma-Aldrich): concanavalin A (CON A) (25 μg/ml), phytohemagglutinin (PHA) (25 μg/ml), pokeweed mitogen (PWM) (2.5 μg/ml) and antigen CD3 (3 μg/ml) (Beckman Coulter). NPs were added in a volume of 25 μl in different exposure intervals (4, 24, 48 and 72 h) before the end of whole 72 h incubation period. Cyclophosphamide 40 mg/ml (CYF) (Baxter) was used as suppressive control. The plates were incubated at 37°C and 5% CO_2 for 48 h; then wells were pulsed with 1 μCi [^{3}H]-thymidine (Moravek) diluted in 20 μl medium and incubated at 37°C for additional 24 h. After incubation, cell cultures were harvested onto glass filter paper. Filters were placed into scintillation fluid (Perkin Elmer). Radioactivity was measured using a Beta Scintillation counter Microbeta 2 (Perkin Elmer). Calculations: Counts per minute (cpm)/culture were measured in triplicate for each variable.

Cytotoxic (killing) activity of NK cells

Human heparinised whole blood (diluted 1:1 with PBS) was layered on Lymphoprep (PAA) and centrifuged for 30 min (700 × *g*). Mononuclear cells were collected from interphase, washed in PBS and subsequently in RPMI 1640 complete medium. Cells were adjusted to 5 × 10^6 cells/ml (RPMI with 20% FCS) and incubated in a volume of 200 μl. NPs were added in a volume of 25 μl. Cells were exposed to NPs for 4 or 24 h. Target cells (K-562) (Glycotope) were thawed, washed in RPMI 1640 with 20% FCS and adjusted to 1 × 10^5 cells/ml. K-562 cells were added in a volume of 100 μl to mononuclear cells (effector cells) at a 1:50 E:T ratio. Interleukin-2 (200 U/ml) (Glycotope) was added in a volume of 30 μl to one tube ("high control" samples). All tubes were mixed, centrifuged for 3 min at 250 × *g* and incubated for 2.5 h. Two hundred microlitres of propidium iodide was added and incubated for another 20 min. K-562 cells alone were incubated in parallel to monitor spontaneous cell death (control sample). At the end of the incubation, tubes were placed on ice and measured within 30 min using a flow cytometer.

Phagocytic activity of granulocytes and monocytes and respiratory burst of phagocytes

One hundred and fifty microlitres of human heparinised whole blood (diluted 1:1 in RPMI 1640 medium containing 10% FCS) was dispensed into wells of a 96-well microtitre culture plate under sterile conditions. NPs were added in a volume of 25 μl. Cells were exposed to NPs for 4 or 24 h. After incubation, 30 μl of blood from each microplate well was pipetted into tube and 10 μl of hydroethidine solution (Sigma-Aldrich) was added. Samples were incubated for 15 min at 37°C. Three microlitres of fluorescein-labelled *S. aureus* bacteria (1.4 × 10^6 per test) (Molecular Probes) was added to the ''test'' tubes. All tubes were incubated for another 15 min at 37°C. Samples were put on ice and 700 μl of cold lysis solution was added. To the ''control'' tubes, the *S. aureus* were added after the lysis solution. Samples were tested in triplicates and analysed by flow cytometry within 30 min.

Assessment of genotoxicity

Cytokinesis-block micronucleus test

Cultures were set up in duplicate by adding 0.5 ml whole blood to 4.5 ml of RPMI medium with L-glutamine and $NaHCO_3$ (Sigma-Aldrich) supplemented with 10% FCS and antibiotics (penicillin and streptomycin, Gibco) in 9 cm^2 flasks.

Cultures were treated with PLGA-PEO NPs (3, 15 or 75 μg/cm^2) (G_0 exposure) for 24 h. Culture medium with NPs was discarded by centrifugation (90 × *g* for 10 min) and cells were resuspended in fresh culture medium prepared and supplemented with 0.18 mg/ml PHA to stimulate lymphocytes for 72 h at 37°C. Cytochalasin B (Sigma-Aldrich, 6 μg/ml) was added for the last 28 h to accumulate cells that had completed one nuclear division at the binucleated stage (Fenech 2007).

After the incubation period, cells were hypotonically treated in 0.075 M KCl and fixed twice with methanol/glacial acetic acid, 3:1. The fixed cells were dropped onto slides, and air dried and stained with 2% Giemsa-Romanowski solution for 10 min.

The frequencies of MN were determined in 2000 BNCs and the cytokinesis-block proliferation index (CBPI) in 500 cells. In parallel, MN were scored scoring in 1000 mononucleated cells (MNCs). CBPI was calculated as no. of MNCs + 2 × no. of BNCs + 3 × no. of multinucleate cells/total no. of cells. Scoring criteria for selection of MNCs, BNCs and MN were set according to the criteria of HUMN project (http://www.humn.org).

Untreated negative control cells and positive control cells treated with mitomycin C (0.05 μg/ml) were assayed in parallel cultures.

Comet assay

Single and double DNA SBs and oxidised DNA lesions (oxidised purines) were measured *in vitro* on human peripheral blood mononuclear cells (PBMCs) using the alkaline version of the comet assay modified with lesion-specific enzyme formamidopyrimidine DNA glycosylase (FPG) (Dusinska & Collins 1996). PBMCs were isolated by gradient centrifugation. The cell suspension (2.106 cells per ml) in RPMI medium was dispensed into a 96-well plate (100 μl per well). PLGA-PEO NPs working stock solution was diluted in complete medium to concentrations 3, 15 and 75 μg/cm^2. Twenty five microlitres of tested solution was added per well of cell suspension. Samples were treated for 24 h in RPMI medium with 20% FCS. The designated 96-well plates have a growth area of 0.32 cm^2. Cells for positive control were treated with 50 μM H_2O_2 in cold PBS for 5 min on ice.

Cells were suspended in low melting point agarose (1% (w/v) in PBS) at 37°C and 45 µl aliquots immediately pipetted onto a microscope slide (precoated with a layer of 1% (w/v) normal melting point agarose) and covered with 15 × 15 mm coverslip. Two gells per slide were prepared. The agarose was allowed to set for 5–10 min at 4°C and the slides were incubated in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, NaOH to pH 10.0 and 1% v/v Triton X-100) at 4°C for 24 h to remove cellular proteins. After lysis, the slides were washed twice with buffer F (40 mM HEPES 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0, 4°C) in a staining jar, for 8 min each. Thirty microlitres of FPG enzyme solution (or buffer alone, as control) was placed onto each gel and covered with a cut square of Parafilm. The slides were incubated at 37°C for 30 min in a moist box. After treatment with enzyme the slides were aligned in a horizontal electrophoresis tank containing electrophoresis buffer (1 mM Na_2EDTA and 0.3 M NaOH) for 20 min before electrophoresis at 25 V for 20 min (at an ambient temperature of 4°C). The slides were washed at 4°C for 8 min with PBS followed by 8 min in water and dried at room temperature. A day before image analysis (Comet IV, Perceptive Instruments) the slides were stained with SYBRGold (10,000-fold diluted in TE buffer) and 50 comets per gel were evaluated. DNA damage was expressed as % tail DNA, corresponding to SBs (SBs), or overall DNA damage representing SBs and oxidised purines (SBs FPG), or net FPG-sites (i.e. % tail DNA plus FPG, minus % tail DNA without FPG, representing oxidised purines). Data are presented as mean values ± standard error mean (SEM).

Statistical analysis

SPSS 16.0 software was used for statistical analysis. Triplicates or duplicates from each individual were averaged and used as a single value for analysis. Normality was tested by Shapiro–Wilcoxoń s test. To test for significant differences between groups the independent samples t-test (or paired-samples t-test) for normally distributed data, and the Mann–Whitney U-test (or Wilcoxon test) for non-normally distributed data were used. Differences between three groups were tested by one-way analysis of variance and by Bonferronís test if equal variances were assumed or by Tamhanés test if equal variances were not assumed. The Kruskall–Wallis test was used for non-normally distributed data. The data were expressed as mean values with standard deviation (means ± SD) or standard error of mean (means ± SEM). Differences at $p < 0.05$ were considered to be statistically significant.

Results

TEM of PLGA-PEO NPs shows that the particle size is within the range of 50–200 nm (Figure 1). PLGA-PEO NPs were additionally characterised in RPMI media with 10% FCS. PLGA-PEO showed large size distribution with two main peaks averaged as 253.1 ± 38.3 nm and 811.7 ± 202.6 (15 min); 530.6 ± 94.9 nm and 2061.2 ± 356.0 nm (24 h) (Figure 2A and B).

Assessment of immunotoxicity

Experiments were performed in triplicate or duplicate wells in 9–13 samples of human blood. Means + SEM were calculated. No interferences with the immune assays were found during the testing of NPs in experiments.

Cytotoxicity of PLGA-PEO NPs to peripheral blood cells

Cytotoxicity was measured using incorporation of [^{3}H]-thymidine into DNA of proliferating peripheral blood cells (Figure 3). The selected high dose of PLGA-PEO NPs (75 µg/cm^2) displayed a cytotoxic effect to peripheral blood cells in cultures ($n = 10$) exposed to NPs for 4 h (70% of control, $p < 0.001$) or 48 h (84% of control, $p < 0.05$). No significant difference in cell proliferation was found in the CYF cultures vs. vehicle (medium) control cultures over all time intervals.

Proliferative activity of lymphocytes

Proliferative function of T-cells was determined in peripheral blood cultures ($n = 13$) *in vitro* stimulated with mitogens PHA and Con A (Figure 4A and B). In non-cytotoxic concentrations, no effect of PLGA-PEO NPs on the proliferative response of lymphocytes was observed in cultures stimulated with PHA mitogen. Analysis of the results of the proliferative response of T-lymphocytes stimulated with Con A demonstrated a suppressive effect of a low dose of PLGA-PEO NPs, significant at the 24 h treatment period ($p < 0.05$, 93%). T-dependent B-cell lymphocytes were sensitive to the presence of PLGA-PEO NPs in cultures stimulated with PWM (Figure 4C). The proliferative

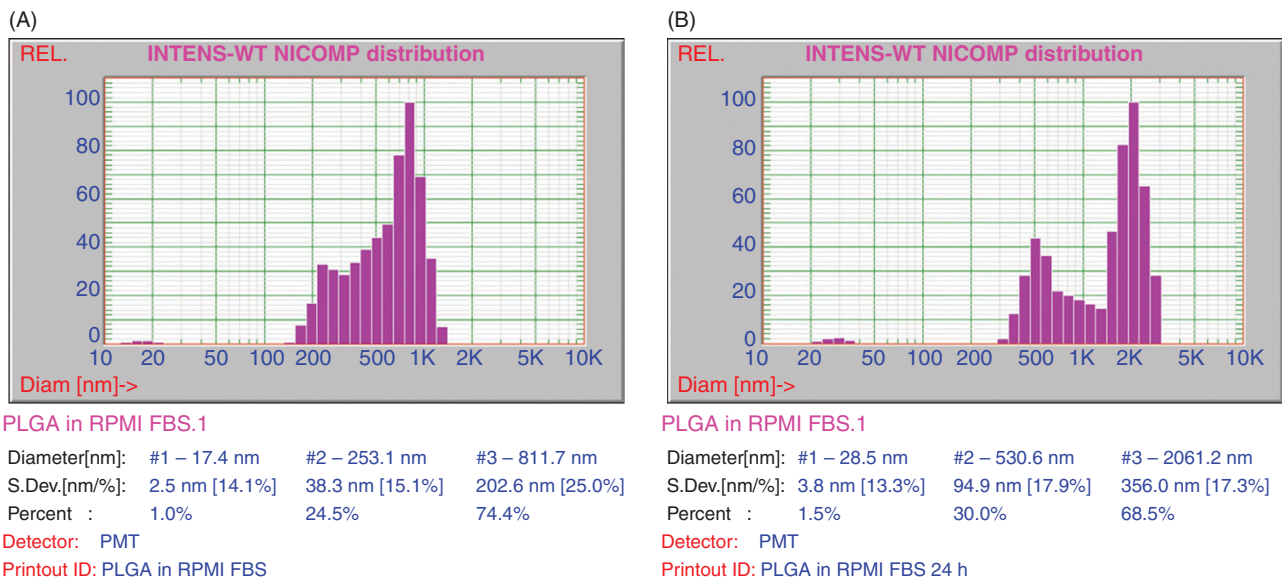

Figure 2. Hydrodynamic diameter and size distribution of PLGA-PEO NPs by DLS measured in RPMI medium with 10% of FCS after 15 min (A) and 24 h (B).

response was decreased after exposure to low dose at 4 and 24 h exposure ($p < 0.05$, 95%), but no clear dose-dependent effect was seen. The most significant suppressive effect of PLGA-PEO NPs was found in T-lymphocytes stimulated with CD3 antigen (Figure 4D), while significantly decreased proliferation in cultures treated with low and middle doses was found already after a 4 h exposure time period (4 h, 24 h, 48 h: 91–84%). Regardless of the mitogen, a significantly suppressed proliferative response of T-lymphocytes and T-dependent B-cell response to CYF was found at time intervals starting at 24 h exposure.

Killing activity of NK cells

Killing activity of NK cells was determined as percentage of propidium iodide positive dead target K-562 cells killed by peripheral blood lymphocytes. The results demonstrate a significant dose-dependent suppressive effect of middle and high doses of PLGA-PEO NPs on the killing activity of NK cells ($n = 11$) in isolated PBMCs after 4 h exposure to NPs (Figure 5) ($p < 0.05$, 92%; $p < 0.001$, 71%). No similar effect was seen after 24 h exposure. The CYF (suppressive) control displayed a highly significant suppression of killing activity of NK cells vs. vehicle (medium) control at both time intervals. On the other hand, the IL-2-stimulated (positive) control increased significantly killing effect of NK cells in comparison with medium control.

Phagocytic activity of granulocytes and monocytes and respiratory burst of phagocytes

The phagocytic function of monocytes (Figure 6A) and granulocytes (Figure 6B) was evaluated using ingestion of fluorescein-labelled *S. aureus,* and the respiratory burst (Figure 6C) was monitored using hydroxyethidine. After 4 h exposure to PLGA-PEO NPs, significant stimulation of phagocytic activity of monocytes (117% of control) was found in low-dose cultures of human peripheral blood ($n = 9$). Similarly, increase in phagocytic activity of granulocytes was observed after 4 and 48 h treatment with low and middle doses of PLGA-PEO NPs, respectively.

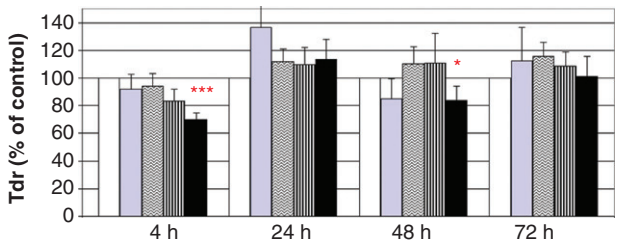

Figure 3. Cytotoxicity of PLGA-PEO NPs for peripheral blood cells determined with [^{3}H]-thymidine incorporation assay. Results are expressed as % of control (mean + SEM). Bars indicate cultures treated with different concentrations of NPs: Control (white), Cyclophosphamide (CYF) 40 mg/ml (grey); NPs: 0.12 μg/cm^2 (horizontal waves), 3 μg/cm^2 (vertical lines), 75 μg/cm^2 (black). The assay was performed after 4, 24, 48 and 72 h *in vitro* exposure of the peripheral blood cells ($n = 10$ human volunteers). Statistical analysis was performed by comparing measured cpm – counts per minute/culture in group of exposed and unexposed samples using Student's paired t-test; Significance: *$p < 0.05$, ***$p < 0.001$.

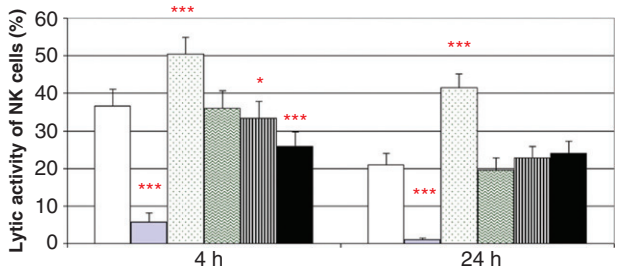

Figure 5. Killing (lytic) activity of peripheral blood NK cells determined against tumour cell line K-562 measured using flow cytometry. Results are expressed as percentage of lytic activity (mean + SEM). Bars indicate mean group lytic activity in blood cultures *in vitro* treated with different concentrations of NPs: Control (white), CYF 40 mg/ml (grey), interleukin-2 (6 U/well) (spotted bars), NPs: 0.12 μg/cm^2 (horizontal waves), 3 μg/cm^2 (vertical lines), 75 μg/cm^2 (black). The assay was performed after 4 and 24 h *in vitro* exposure of the peripheral blood cells ($n = 11$ human volunteers). Statistical analysis: Student's paired t-test; Significance: *$p < 0.05$, ***$p < 0.001$.

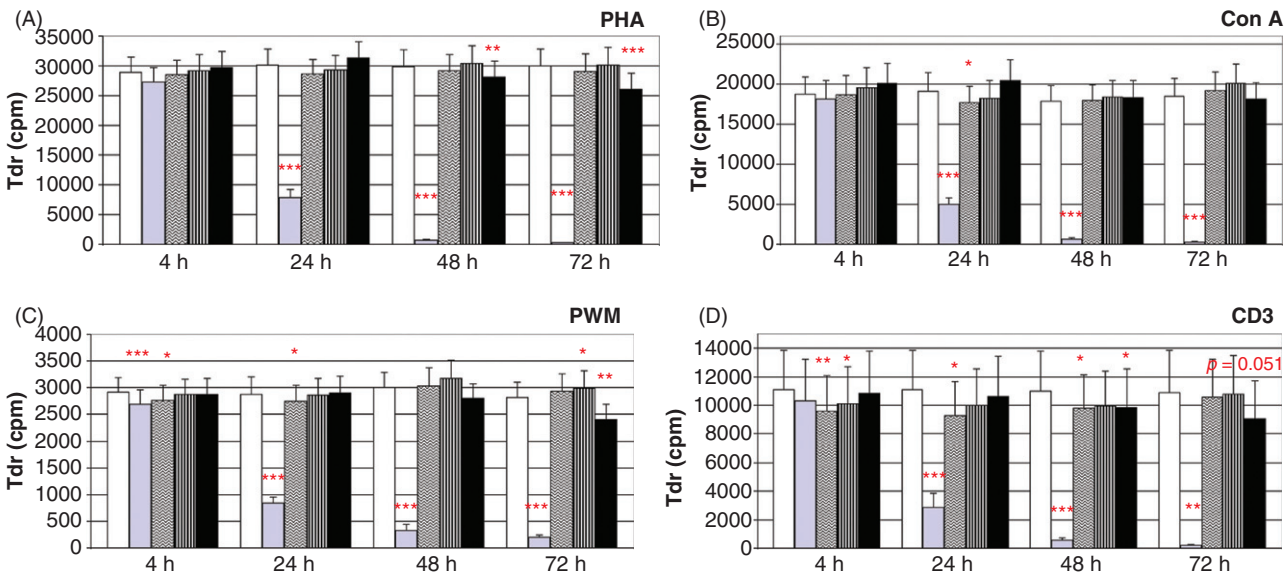

Figure 4. Proliferative response of human peripheral blood T-lymphocytes and T-dependent B-cell response measured as incorporation of [^{3}H]-thymidine into replicating cells. Results are expressed as cpm – counts per minute/culture (mean + SEM). Bars indicate mean group values of cpm. Cell cultures were *in vitro* stimulated with mitogens: phytohemagglutinin – A, concanavalin A – B, pokeweed – C and antigen (monoclonal antibody anti-CD3) – D and treated with NPs: Control (white), CYF 40 mg/ml (grey); 0.12 μg/cm^2 (horizontal waves), 3 μg/cm^2 (vertical lines), 75 μg/cm^2 (black). The assay was performed after 4, 24, 48 and 72 h *in vitro* exposure of the peripheral blood cells ($n = 13$ human volunteers). Statistical analysis: Student's paired t-test; Significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

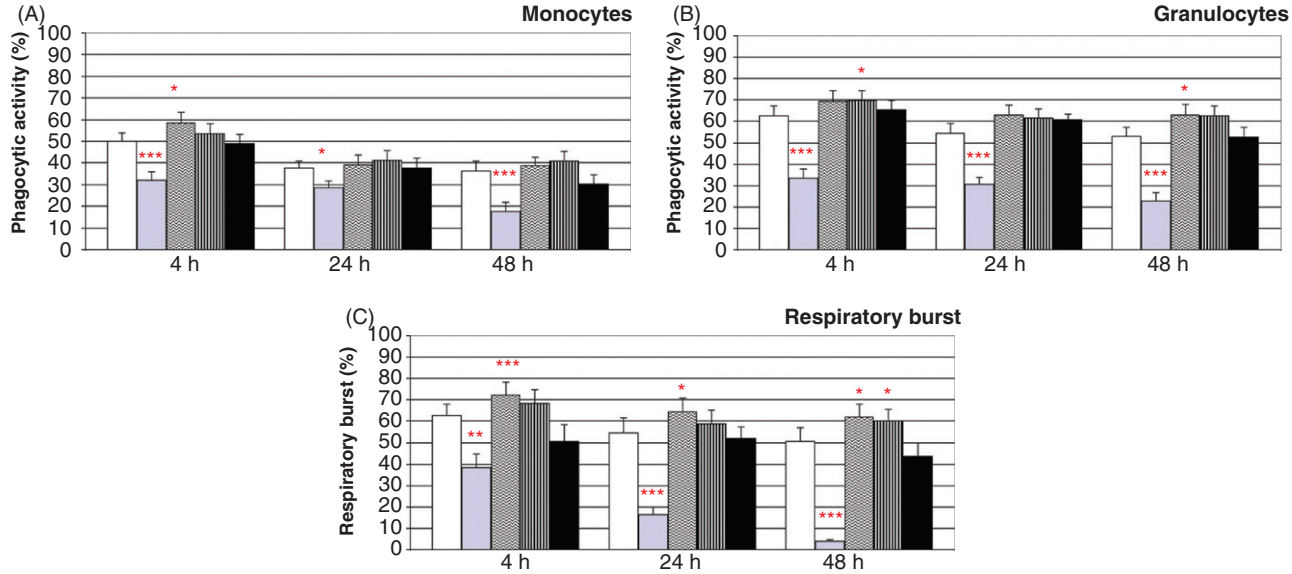

Figure 6. Phagocytic activity of monocytes (A) and granulocytes (B) evaluated using ingestion of fluorescein-labelled *S. aureus,* and the respiratory burst (C) monitored using hydroxyethidine by flow cytometry. Results are expressed as percentage of phagocytic activity and respiratory burst (mean + SEM). Bars indicate mean group activity in peripheral blood cultures *in vitro* treated with different concentrations of NPs: Control (white), CYF 40 mg/ml (grey), NPs: 0.12 μg/cm^2 (horizontal waves), 3 μg/cm^2 (vertical lines), 75 μg/cm^2 (black). The assay was performed after 4 and 24 h *in vitro* exposure of the peripheral blood cells ($n = 9$ human volunteers). Statistical analysis: Student's paired t-test; Significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

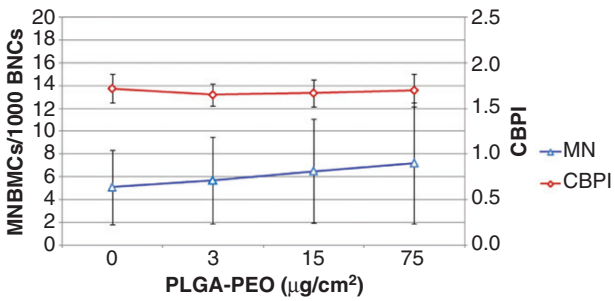

Figure 7. Frequency of MNBNCs per 1000 BNCs and CBPI in peripheral lymphocytes of volunteers after exposure to PLGA-PEO NPs (3, 15 and 75 μg/cm^2) for 24 h. The symbols show means ± SD.

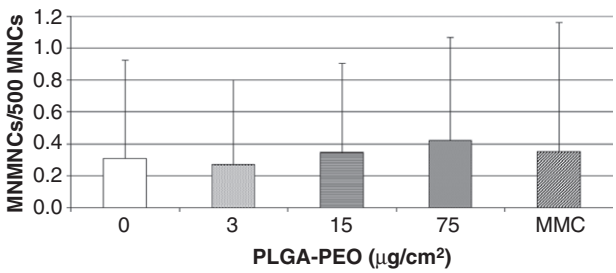

Figure 8. Frequency of MNMNCs per 500 MNCs in peripheral lymphocytes of volunteers after exposure to PLGA-PEO NPs (3, 15 and 75 μg/cm^2) and positive control Mitomycin C (0.05 μg/ml) for 24 h. The bars show means + SD.

Significant stimulatory changes in respiratory burst were also pronounced, visible mostly after treatment with low dose of tested NPs in all time periods of exposure. The phagocytic activity of granulocytes and monocytes as well as the respiratory burst of phagocytes were significantly decreased when exposed to CYF at all time periods tested.

Micronucleus frequencies

PLGA-PEO NPs did not increase the number of micronucleated binucleated cells (MNBNCs) at any of the tested doses compared with the negative control. Cell proliferation measured as CBPI was comparable to that of the negative control (Figure 7).

Mitomycin C (0.05 mg/ml) used as a positive control clearly increased the frequency of MNBNCs to 50.3 MNBNCs/1000 BNCs ($p < 0.001$).

PLGA-PEO NPs did not increase the number of micronucleated mononucleated cells (MNMNCs) at any of the tested doses, nor did Mitomycin C compared with the negative control (Figure 8).

DNA SBs and oxidised base lesions

Results presented in Figure 9 show no induction of SBs or oxidised DNA bases (net FPG sites) in cells exposed to PLGA-PEO NPs for 24 h in the doses (3, 15 and 75 μg/cm^2) compared to the background level of damage in untreated cells (negative control). As positive control DNA damaging agent, H_2O_2 (50 μM 5 min on ice) was used; it gave a significant induction of SBs (average value 23.16% tail intensity $p < 0.001$).

Correlations between genotoxic and immunologic parameters

Statistical analysis of possible correlations between genotoxic and immune parameters was performed in a group of 10 people with complete datasets of both parameters exposed to the same doses of PLGA-PEO NPs (control, 3 and 75 μg/cm^2) for 24 h. To avoid false-positive results, only those correlations appearing repeatedly were considered.

In control untreated cells as well as in cells treated with a low dose of NPs (3 μg/cm^2), the number of MN correlated negatively with proliferative response of T- and B-lymphocytes *in vitro* stimulated with different mitogens CON A ($n = 7$; $p = 0.028$; $r = -0.807$; $p = 0.045$; $r = -0.765$) and PWM ($n = 7$; $p = 0.027$; $r = -0.810$; $p = 0.02$; $r = -0.834$) and after 3 μg/cm^2 also with PHA ($n = 7$; $p = 0.021$; $r = -0.829$).

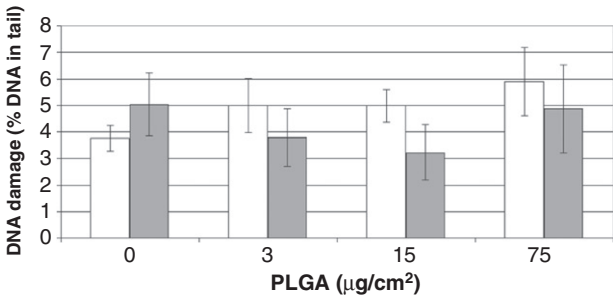

Figure 9. DNA SBs (white) and oxidised purines (netFPG) (grey) measured with the comet assay, modified with lesion-specific enzyme FPG in PBMCs of volunteers after exposure to PLGA-PEO NPs (3, 15 and 75 μg/cm^2) for 24 h. Data are presented as mean values ± SEM.

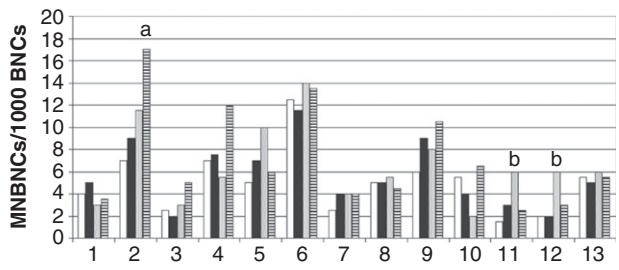

Figure 10. Individual frequencies of MNBNCs per 1000 BNCs in peripheral lymphocytes of 13 volunteers after exposure to PLGA-PEO NPs: Control (white), 3 μg/cm^2 (black); 15 μg/cm^2 (spotted), 75 μg/cm^2 (striped) for 24 h. Data are presented as means. $^a p = 0.004$, $^b p < 0.05$.

SBs FPG in cells exposed to 3 μg/cm^2 PLGA-PEO NPs correlated negatively with proliferative response of T-lymphocytes to CD3 antigen (CD3) ($n = 6$; $p = 0.050$; $r = -0.811$). Similarly an association of net FPG-sites with CD3 was found. Especially, in cells exposed to the highest concentration 75 μg/cm^2, an inverse correlation with CD3 ($n = 6$; $p = 0.005$; $r = -0.941$) was observed.

The level of oxidised bases (net FPG sites) in controls as well as after exposure to 3 and 75 μg/cm^2 of PLGA-PEO NPs correlated positively with background and induced levels of overall DNA damage (SBs FPG) ($n = 7$; $p = 0.001$; $r = 0.867$; $n = 9$; $p = 0.001$; $r = 0.894$; $n = 9$; $p = 0.000$; $r = 0.966$).

Individual susceptibility

Micronucleus frequencies

By analysing individual values of 13 volunteers, we found a statistically increased number of MNBNCs at V2 in dose 75 μg/cm^2 ($p = 0.004$) and V11 and V12 in dose 15 μg/cm^2 ($p < 0.05$) compared with the negative control (Figure 10).

DNA SBs and oxidised bases

By analysing individual values of 13 volunteers, we found statistically increased numbers of SBs with V1 at 3 μg/cm^2 ($p = 0.027$), V2 at 15 μg/cm^2 ($p = 0.02$) and 75 μg/cm^2 ($p = 0.002$),V3 at 15 and 75 μg/cm^2 ($p < 0.001$), V4 at 3, 15 and 75 μg/cm^2 ($p < 0.001$), V5 at 3 μg/cm^2 ($p = 0.015$) and 75 μg/cm^2 ($p < 0.001$) and V7, V11 and V13 at 15 μg/cm^2 ($p < 0.001$), compared with the negative control. Also, we found statistically increased numbers of SBs FPG with V1 at 3, 15 and 75 μg/cm^2 ($p < 0.001$), V2 at 3 μg/cm^2 ($p = 0.008$) and V4 and V5 at 3 and 75 μg/cm^2 ($p < 0.001$) (Figures 11 and 12).

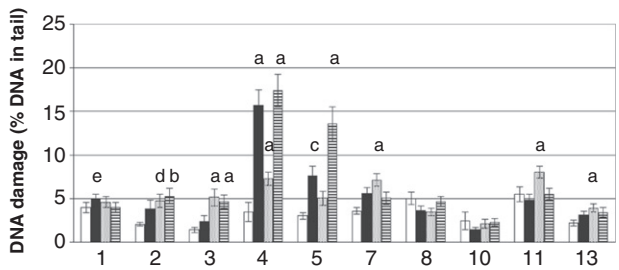

Figure 11. Individual DNA damage values (SBs) in PBMCs of volunteers measured by the comet assay after exposure to PLGA-PEO NPs for 24-h measure with the comet assay: Control (white), 3 μg/cm^2 (black); 15 μg/cm^2 (spotted), 75 μg/cm^2 (striped). DNA damage is expressed as % tail DNA. Data are presented as mean ± SEM. $^a p < 0.001$, $^b p = 0.002$, $^c p = 0.015$, $^d p = 0.02$, $^e p = 0.027$.

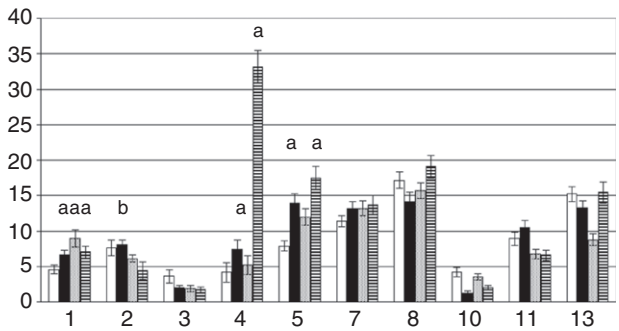

Figure 12. Individual DNA damage values (strand breaks plus oxidised purines – SBsFPG) in PBMCs of volunteers after 24-h exposure to PLGA-PEO NPs measured with the comet assay modified with lesion-specific enzyme FPG. Control (white), 3 μg/cm^2 (black); 15 μg/cm^2 (spotted), 75 μg/cm^2 (striped). DNA damage is expressed as % tail DNA. Data are presented as mean ± SEM. $^a p < 0.001$, $^b p = 0.008$. .

Discussion

A human blood cell model has been used to examine the effects of different NPs on immune cell viability and functions of cells, including proliferative activity of lymphocytes, cytotoxic activity of NK cells and phagocytic activity of neutrophils and macrophages. Also, the genotoxic effect of NPs was evaluated using the comet assay in PBMCs and micronucleus formation in fresh peripheral whole-blood cultures from human volunteers. Our findings indicate that PLGA-PEO NPs were not toxic up to dose 3 μg/cm^2; cytotoxic effect was displayed only at high dose 75 μg/cm^2 (106 μg/ml) when measured as [^{3}H]-thymidine incorporation into cells. Cytotoxicity of peripheral blood cells varies in different time intervals when decreased for 24 h NP treatment vs. 4 h exposure and similarly for 72 h treatment vs. 48 h. We hypothesise that significant decrease in [^{3}H]-thymidine incorporation into human blood cells in specific time intervals (4 and 48 h) after NP exposure may be related to more sensitive phases of cell cycle when NPs were added to cultures for last 4 and 48 h of whole 72 h cultivation period. Moreover, low proliferative response of non-stimulated peripheral blood cells measured as counts per minute/culture (200–470 cpm) may contribute to the variability in cytotoxicity. Taking into account a wide range of factors influencing data on cytotoxicity of NPs, starting with the method of synthesis and finishing at batch to batch variability, comparison with similar studies is difficult. A published study on *in vitro* toxicity of PLGA NPs (2.5–50 μg/well) evaluated by the MTT assay in HEK 293 cells after a 4 h exposure found that different NP formulations were non-toxic,

with only slight reduction in cell viability at higher concentrations studied (Basarkar et al. 2007). On the other hand, Eyles et al. (2003) reported some unspecific cytotoxicity if mice splenocytes were co-cultured with high concentrations of PLGA particles, although toxic effects were not seen at concentrations where maximum levels of cytokine secretion and cellular proliferation were recorded.

In the lymphocyte functional assay, three different mitogens and one antigen have been used to study the immune response of various lymphocyte subsets. Our results suggest different sensitivities of T- and B-cell responses to the PLGA-PEO NPs, the most significant effect being the suppression of T-cell response to CD3 antigen. In contrast with classical toxicological studies, no clear dose-dependent response was seen in *in vitro* cultures treated with NPs. The marked suppression of function at low dose, without a corresponding effect at high dose, may be caused by better dispersion of low concentrations of tested NPs in culture. In summary, although several significant differences in T-lymphocyte proliferative activity and T-dependent B-cell response of peripheral blood lymphocytes *in vitro* stimulated with mitogens and antigen were found, in fact statistically significant suppression in proliferative activity of exposed cultures reached 69–95% of values in unexposed controls. Even if the differences are statistically significant, it is less probable that they have important biological meaning.

In our experimental set-up, a significantly decreased killing activity of NK cells was recorded in cultures after 4 h exposure to PLGA-PEO NPs. However, after prolongation of exposure to 24 h we observed recovery to normal values comparable to corresponding controls. After longer (24 h) NP exposure, we expected similar or even more pronounced loss of killing activity after NP exposure. Possible explanation that unexpected restored killing activity after 24 h exposure to NPs could be related to the short-term stability (15 min) of PLGA-PEO NPs in cell culture medium brings more questions than answers. We suppose that results of killing activity of NK cells in 4 and 24 h treatment period (Figure 5) more less mimic the finding of cytotoxicity measured as incorporation of [^{3}H]-thymidine into the peripheral blood cells (Figure 3). Similar pattern of response implicates the explanation that cytotoxicity might be responsible for decrease of killing function of NK cells. Moreover, substantial decrease in killing activity of NK cells after 24 h vs. 4 h (21% vs. 37%) in control cultures may contribute to the loss of original killing function of these cells.

Published papers studying the effects of NPs on NK cell numbers/activity show different results. Andersson-Willman et al. (2012) found down-regulation of FcγRIII (CD16) expression on NK cells in the PBMC population *in vitro* treated with ZnO NPs. Similar decrease in NK cell count was observed *in vivo* in TiO_2 orally dosed mice (Duan et al. 2010); suppression of NK population by TiO_2 exposure led to an increase in tumour growth *in situ* (Moon et al. 2011).

On the other hand, in rats exposed to similar nanosized TiO_2 particles via inhalation, an increase of NK cells at day 8 after exposure was observed (Gustafsson et al. 2011). Increased killing activities of NK cells by chitosan NPs were found also in ovalbumin-immunised mice (Wen et al. 2011). Moreover, enhanced distribution of NK cells in spleen was found in mice i.p. treated with nanosilica (Park et al. 2009).

Significant intentional stimulation of NK cell population by glycodendrimers, as anticancer immune modulation through carbohydrate-mediated immune recognition, showed an enhanced NK cell activity proportioned to the percentage of activated NK cells in mice inoculated with melanoma (Vannucci et al. 2003). Another dendrimer (GN8P) was shown to delay the development of rat colorectal carcinoma as well as mouse melanoma, and to potentiate antigen-specific antibody formation in healthy mice via NK cell stimulation (Hulikova et al. 2009). Lipid NPs encapsulating G3139 promoted proliferation of NK cells and triggered a strong antitumour immune response in mice bearing L1210 subcutaneous tumours (Pan et al. 2009).

No impact of NPs on NK cells was also described in several studies. TiO_2 NPs found had no effect on expression of CD16 on NK cells in the *in vitro* exposed PBMC population (Andersson-Willman et al. 2012). No alterations in NK cell activity were also found in rats after 28-day oral exposure to non-coated or PVP-coated silver NPs (van der Zande et al. 2012).

Some published results support our finding of stimulatory effect of polymeric NPs on phagocytic cells showing that PLGA NPs sensitise polymorphonuclears and monocytes to secrete O(2) (−) upon stimulation (Segat et al. 2011). On the other hand, *in vivo* study of PLGA-PEO NPs engineered for oral applications found low concentration of the expression of pro-inflammatory cytokines IL-2, IL-6, IL-12p70 and TNF-α in plasma and peritoneal lavage in PLGA-PEO-treated mice (Semete et al. 2010). Several other NPs are known to activate phagocytic cells. After exposure to silver NPs, production of IL-1b, a critical cytokine involved in induction of innate immunity, significantly increased as particle size decreased (Yang et al. 2012). Nanodiamond and nanoplatinum liquid activated human monocyte-derived dendritic cells *in vitro* (Ghoneum et al. 2010), and gold nanorods with surface-coupled peptide sequences modulated cytokine release in macrophages and dendritic cells (Bartneck et al. 2012). Amorphous silica NPs promoted monocyte adhesion to human endothelial cells (Napierska et al. 2012). LPS-stimulated macrophages (human monocytic cell line THP-1) phagocytising titanium NPs <200 nm synergistically up-regulated 17 inflammation-related genes more than twofold (Taira et al. 2009). The macrophages labelled with ferucarbotran NPs (Fe_2O_3 and Fe_3O_4 coated with carboxydextran) showed an increase in the production of reactive oxygen species (ROS) (Yang et al. 2011). Production of ROS can be effectively scavengered by nanoceria NPs in U937 cell population (Lord et al. 2012). On the other hand, several studies demonstrated that some NPs do not have a classical proinflammatory effect, such as ZnO NPs on THP1 cells (Prach et al. 2013). CeO_2 NPs neither modulate pre-existing inflammation nor prime for subsequent exposure to LPS in human monocytes from healthy subjects (Hussain et al. 2012) but inducing autophagy acts as a prodeath mechanism and leads to increased cytotoxicity of human monocytes (Hussain & Garantziotis 2013).

Several technical limitations of the present *in vitro* experiments of the assessment of immunotoxicity need to be considered: short-term survival of primary cells in cultures and decreasing cell function in time. Short stability of NPs in cell-culture medium means that in few hours cells were exposed to PLGA-PEO microparticles and aggregates rather than NPs.

Setting up appropriate controls for immune assays is an important step to obtain valid results. CYF at 40 mg/ml demonstrated its ability to decrease the cytotoxic activity of NK cells. The response was significantly diminished after 4 or 24 h exposure. The phagocytic activity of granulocytes and monocytes and the respiratory burst of phagocytes were significantly suppressed at all time intervals, even after 4 h exposure to CYF. Selected doses of CYF failed to express cytotoxicity when measured as [^{3}H]-thymidine incorporation in non-stimulated peripheral blood cells. However, significant suppression was found in all mitogen (PHA, ConA, PWM) and antigen-stimulated (CD3) cell cultures and several exposure intervals. A 4 h exposure interval was too short to show altered T-cell function but T-dependent B-cell response was significantly decreased.

In addition to the assessment of immune effect, it is very useful to measure DNA stability using the same model. Both the

micronucleus and the comet assays are reliable tests for assessing the genotoxic potential of NPs. As these methods measure different endpoints downstream from genotoxicity, the combination of the two assays used in our study can give a reliable picture of potential genetic instability caused by exposure to NPs.

Our results, similar to the published data of He et al. (2009) as well as Kazimirova et al. (2012), show no significant differences in the frequency of MNBNCs between the negative control and PLGA-PEO NPs tested with the CBMN assay in human lymphocytes, while the classic clastogen, mitomycin C, induced a clear increase in MNBNC frequencies. This suggests that PLGA-PEO NPs do not have potential genotoxicity.

In general, MN are only counted in BNCs in the CBMN assay, because these cells have finished one nuclear division. Recent studies have indicated that aneugens, but not clastogens, also induce MN in MNCs in the CBMN assay (Elhajouji et al. 1998; Rosefort et al. 2004; Kirsch-Volders et al. 2011). These data suggest that MN in MNCs may be a useful marker to distinguish clastogens from aneugens and increase the sensitivity of the test. In our study, PLGA-PEO NPs did not increase the number of MNMNCs at any of the tested doses compared with the negative control.

The alkaline comet assay can capture with high sensitivity various kinds of DNA damage, e.g., single- and double-strand DNA breaks, alkali-labile sites, DNA–DNA and DNA–protein-cross links and single-SBs associated with incomplete excision-repair sites (Tice et al. 2000; Dusinska & Collins 2008). In addition, the use of lesion-specific repair enzymes, with the ability to introduce breaks at sites of damaged DNA, increases the sensitivity and specificity of the assay and can provide knowledge about the nature of DNA damage caused by NPs. FPG is a DNA-repair enzyme that recognises oxidised bases. The main substrate for FPG is 8-oxoG, the major product of purine oxidation, but as well as certain ring-opened purines (Fapy derivates, FaPyAde and FaPyGua) (Dusinska & Collins 1996; Collins et al. 1996). FPG can also detect alkylation damage (N7-methylguanine) (Angelis et al. 1999; Speit et al. 2004).

Our results are in agreement with Kazimirova et al. (2012) who found in TK6 cells no SBs or oxidised bases after the exposure to PLGA-PEO NPs. Here we show that PLGA-PEO NPs induce neither SBs nor oxidised DNA lesions in human PBMCs, suggesting that PLGA-PEO NPs are not potentially genotoxic.

Analysis of the correlations between number of MN, SBs, net FPG sites and the proliferative activity of T- and B-lymphocytes was performed in a small population ($n = 10$) with complete parameters measured and thus results can only indicate possible association between these biomarkers. Statistical analysis revealed a significant negative correlation of the number of MN with the proliferative activity of lymphocytes exposed to several mitogens. Findings are not surprising and can be attributable to a decreased ability of cells with damaged DNA to divide.

The association of oxidised bases with the SBs FPG found in non-treated as well as in cells treated with PLGA-PEO NPs is not surprising as oxidised bases represent a high proportion of this damage. Additionally, the standard alkaline comet assay measures single- and double-SBs and alkali-labile sites that might be the result of DNA oxidation.

The inverse correlation of oxidised bases with CD3 may be related to the response of blood cells to PLGA-PEO NPs as the CD3 was suppressed after the exposure. However, no significant increase in oxidised DNA lesions, only a trend, was found in exposed cells compared to controls. This may be due to the high individual variability in response to NP exposure and the low number of subjects.

We also compared individual results of genotoxic parameters with immunologic parameters of 11 volunteers. High inter-individual variability in immune parameters was observed as expected. Nevertheless, a similar individual trend of response to NPs despite low or high starting individual control values was recorded.

The highest number of SBs, seen in human subject V4 (15.6 and 17.3% tail DNA), was most likely related to the highest individual cytotoxicity measured as uptake of [^{3}H]-thymidine into proliferating peripheral blood cells (58.1%, 53.6% of control value).

Analysis of the data in model subject V2 showed a dose-dependent increase in DNA damage measured by the comet assay and the highest individual values of MN associated with the lowest values of proliferative response of T- and B-cells and phagocytic activity of monocytes within the group. However, the decreased proliferative response of lymphocytes seen in individual volunteers was not always accompanied with higher DNA damage detected by the comet and CBMN assays or vice versa. This finding may imply the presence of other confounding factors including different defence mechanisms (DNA repair, antioxidative defence) involved in the markedly increased DNA damage in individual subjects. Obviously, further studies with higher numbers of subjects are needed to understand the mechanisms of immune and genotoxic responses.

Conclusions

In conclusion, PLGA-PEO NPs were not toxic for human peripheral blood cell model up to dose 3 μg/cm^2; dose of 75 μg/cm^2 (106 μg/ml) displays significant decrease in [^{3}H]-thymidine incorporation into DNA of proliferating cells after 4 h (70% of control) and 48 h (84%) exposure to NPs.

In non-cytotoxic concentrations (0.12 and 3 μg/cm^2), moderate but significant suppression of proliferative activity of T-lymphocytes and T-dependent B-cell response was found in cultures stimulated with PWM > CON A, and no changes in PHA cultures. Decrease in proliferative function was the most significant in T-cells stimulated with CD3 antigen (up to 84%). Cytotoxicity of NK cells was suppressed moderately (92%) but significantly in middle-dosed cultures (4 h exposure). After 24 h, cytotoxic activity did not differ from controls. On the other hand, in low PLGA-PEO NPs dosed cultures, significant stimulation of phagocytic activity of granulocytes (119%) > monocytes (117%) and respiratory burst of phagocytes (122%) was recorded.

Our findings demonstrate differing sensitivities of various immune peripheral blood cells – lymphocytes, NK cells, granulocytes and monocytes to the effect of PLGA-PEO NPs. The same doses of PLGA-PEO NPs suppressed proliferative function of lymphocytes and killing activity of NK cells and in the meantime stimulated phagocytic activity of granulocytes, monocytes and respiratory burst of phagocytes.

Genotoxicity assessment revealed no increase in the number of MNBNCs and no induction of SBs or oxidised DNA bases in PLGA-PEO NPs-treated peripheral blood cultures. To conclude on immuno- and genotoxicity of PLGA-PEO NPs, more experiments with various particle size, charge and composition need to be performed.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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