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Engineered Nanomaterial Impact in the Liver following Exposure *via* an Intravenous Route–The Role of Polymorphonuclear Leukocytes and Gene Expression in the Organ

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Abstract

Background and methods: Following exposure *via* a number of routes (inhalation, ingestion or injection), some Nanomaterials (NMs) translocate to secondary tissues, prominently the liver. This study investigated the effects of an array of NMs, varying in their physicochemical characteristics, consisting of two types of Zinc Oxide (ZnO), two Multi-walled Carbon Nanotubes (MWCNT), one silver (Ag) and one titanium dioxide (TiO_2) on the liver, following Intravenous (IV) exposure of C57/BL6 mice. The animals were exposed to either a single dose of NM (128 μg/ml–100 μl) or three doses of (64 μg/ml–100 μl), every 24 hr. Animals were dissected 6, 24, 48 and 72 hr after the single IV injection, or 72 hr after the triple injection regime.

Results and conclusions: A Myeloperoxidase (MPO) assay was utilised to quantify neutrophil influx into the tissue. However, as MPO is also found in other granulocytes in smaller quantities, the neutrophils in the liver tissue were also labelled, using a specific neutrophil cell surface marker (Ly-6B.2). A wide array of NMs (including ZnO, Ag, TiO_2 and MWCNT) induced a neutrophil influx into the liver, as early as 6 hr post IV exposure. However, the neutrophils were only involved in the initial phases of the immune response against the NMs, as the leukocyte numbers had returned to control levels after 48 hr. Finally, analysis of mRNA expression in mice livers showed alterations in levels of C3, IL6, IL10, CXCL2 and ICAM-1.

Keywords: Liver; Nanomaterials; Neutrophils; Tissue migration; Gene expression

Introduction

Engineered Nanomaterials (NMs) (with at least one dimension between 1-100 nm) are being developed and exploited because of their unusual and interesting properties exhibited at the nano-scale, compared to larger materials [1,2]. These properties have led to their incorporation into over 1300 consumer products [3]. Their widespread utilisation in everyday products suggests that exposure to humans and the environment is ever increasing, and since air pollution particles of similar sizes are known to induce morbidity and mortality [4], concerns about the environmental and human health impacts of NMs have been raised [1].

The lungs and the gastrointestinal tract are in constant contact with the external environment-hence it is not surprising to find these systems being primary exposure sites for NMs [5,6]. Research indicates that NMs administered via ingestion, inhalation or intravenous injection rapidly reach secondary tissues, especially the liver [7-9]. As a secondary exposure site, the liver has huge importance, as it has been shown to accumulate NMs at high volumes (>90%), compared to other organs [9,10], and alongside the kidneys, may be responsible for the clearance of NMs from the blood [7-9]. The prominent advances in the field of nanomedicine have resulted in an increase in direct entry of NMs into the circulatory system. The presence of nanomaterials in the blood allows distribution to a wide range of target organs, including the liver.

The liver is the metabolic centre of the body [9]. It has a crucial role in metabolic homeostasis, as it is responsible for the storage, synthesis, metabolism and re-distribution of carbohydrates, fats and vitamins. It also produces large numbers of serum proteins and an array of enzymes, cytokines, and various important serum proteins such as complement components and acute phase proteins, crucial in the mammalian innate immune system [11].

Neutrophils also referred to as Polymorphonuclear leukocytes (PMNs) are the most abundant of all white blood cells, comprising of up to 70% of all leukocytes [12]. Neutrophils are the front line effectors of the innate immune system: after differentiation, these cells circulate in the bloodstream, before migrating into tissues [13]. These cells are solely responsible for surveillance and constituting the first line of defence against foreign antigens [14]. Hence, it is not surprising that in the event of an inflammatory response, there is usually an increase in PMN numbers, mobility, tissue influx and phagocytic ability [12]. Neutrophils are the most active phagocytic cells in the host's immune system arsenal. They are capable of ingestion of antigens into specialised phagosomes, and producing a wide array of cytotoxic agents to eliminate the foreign particle [15]. The role of PMNs in disposal of NMs is not yet fully understood, however, it has been shown that nanomaterial exposure in the lungs results in a large increase in PMN numbers in the tissue [4].

Here, we investigated the leukocyte influx into the liver, following IV exposure of mice to a panel of NMs including Ag, ZnO, TiO_2 and MWCNT. These NMs, as part of the FP7 ENPRA (Risk assessment of

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engineered nanoparticles) consortium, were chosen as they represent NMs in a variety of products, and therefore, very real prospect of the materials reaching the blood stream, hence translocating to the liver. In order to confirm whether these nanomaterials could induce a neutrophilic response in the liver, and to identify whether this response was short-lived or more prolonged, as this could potentially be associated with disease initiation. Furthermore, we investigated the changes in the expression of complement factor C (C3), interleukin 6 (IL6), chemokine C-X-C motif Ligand 2 (CXCL2), IL10, Tumour Necrosis Factor-α (TNF-α), Fas ligand, albumin and Intracellular Adhesion Molecule 1 (ICAM-1) in the liver, following nanomaterial exposure, to gain a better understanding how the organ responds, following a NM challenge.

Materials and Methods

Nanomaterials were purchased as stated: NM 110 (ZnO-BASF Z-Cote; zinkite, uncoated, 100 nm), NM 111 (ZnO-BASF Z-Cote; zinkite coated with triethoxycaprylylsilane, 130 nm), NM 300 (Ag-RAS GmbH; capped with polyoxylaurat Tween 20-<20 nm), NM 400 (entangled MWCNT-Nanocyl; diameter 30 nm), NM 402 (entangled MWCNT-Arkema Graphistrength C100; diameter 30 nm). The above mentioned nanomaterials were sub-sampled under Good Laboratory Practice conditions and preserved under argon in the dark, until use. The TiO_2 NRCWE samples were procured by the National Research Centre for the Working Environment. Sub-sampling was completed into 20 ml Scint-Burk glass pp-lock with Alu-Foil (WHEA986581; Wheaton Industries Inc.), after pooling. NRCWE 001, TiO_2 rutile 10 nm was purchased from NanoAmor (Houston, USA), and also used for production of NRCWE 002 (TiO_2 rutile 10 nm with positive charge) [16]. Investigated nanomaterials were characterised by a combination of analytical techniques, in order to infer primary physical and chemical properties useful to understand their toxicological behaviour. A list of the measured physical and chemical properties of the selected NMs was previously described (Supplementary table 1) [16]. Furthermore, the hydrodynamic size distributions of the NMs dispersed in water with 2% mouse serum was investigated at 1-128 µg/ml concentration range by Dynamic Light Scattering (DLS), using a Malvern Metasizer nano series–Nano ZS (USA) (Table 1).

Female C57/BL6 mice of 8 or 9 weeks were obtained from The University of Edinburgh (UK). After arrival, the animals were weighed and randomly housed in polypropylene cages, stored in rooms with a 12 hr light period, with a temperature and relative humidity of 21 ± 2°C and 50 ± 5%, respectively. All mice were given free access to tap water and standard mouse chow diet. The animals were kept under specific pathogen free conditions.

Prior to injections, the NMs were dispersed in sterile water with 2% mouse serum, according to the ENPRA protocol [17]. Mice were randomly assigned into cages in groups of 3, before being injected *via* the lateral tail vein, using a 27 gauge needle. All animals were kept under isoflurane anaesthesia, during the injection process (placed on a thermostat controlled heating device at 37°C). Animals were exposed to either a single dose of the different nanomaterials (128 µg/ml in 100 µl), or three doses of 100 µl of 64 µg/ml, every 24 hr. A positive control of Lipopolysaccharide (LPS) (50 µl of 100 µg/ml) was also employed. The mice were dissected 6, 24, 48 and 72 hr after the single IV injection or 72 hr, after the triple injection regime (anaesthetised before being killed by cervical dislocation). The liver was then divided into four pieces. The middle lobe was divided into two–one half (about 0.2 g of tissue) was stored in RNAlater* (Life Technologies, UK). The left lobe was removed for MPO analysis (about 0.3 g of tissue). The right lobe was preserved in 10% formaldehyde for immunohistochemistry (about 0.3 g of tissue).

A mouse MPO ELISA kit (Hycult Biotech, USA) was utilised to quantify neutrophil influx into the liver tissue. Briefly, the tissue was lysed in a buffer consisting of 5 mM EDTA, 10 mM Tris, 10% glycerine, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and 28 µg/ml aprotinin (pH 7.4). The samples were centrifuged twice at 1500 g for 15 mins at 4°C to reduce contamination with cell debris. All samples were diluted 4 fold before being used in the ELISA kit, according to the manufacturer's instructions.

The fixed tissue sections were transferred to embedding cassettes, before being dehydrated in 70% ethanol (2 changes, 1 hr each), 80% ethanol (2 changes, 1 hr each), 95% ethanol (2 changes, 1 hr each), 100% ethanol (3 changes, 1 hr each) and Histoclear II (3 changes, 1 hr each). The tissues were then embedded in paraffin wax (56-58°C- 2 changes, 1.5 hr each). The blocks were cut into thin slices (4 µm), before being transferred to a 45°C water bath. The paraffin sections were moved onto polysine glass slides, brushed into position, before being dried overnight at 37°C. The slides were rehydrated in Histoclear II (2 changes, 3 mins), Histoclear II 1:1 with 100% ethanol (3 mins), 100% ethanol (2 changes, 3 mins), 95% ethanol (3 mins), 70% ethanol (3 mins), and 50% ethanol (3 mins). The sections were then washed in Tris Buffered Saline (TBS) plus 0.025% Triton X-100 (2×5 mins), and blocked 10% FCS in TBS for 2 hr at room temperature. The primary antibody diluted in TBS with 1% BSA (rat anti-mouse Ly-6B.2 alloantigen-AbD Serotec, UK) was applied (100 µl per slide). The slides were incubated overnight at 4°C.

The slides were rinsed 2×5 mins in TBS plus 0.025% triton, and incubated in 0.3% H_2O_2 in TBS for 15 mins. The HRP conjugated secondary antibody was applied (human anti rat IgG2b: HRP–AbD Serotec, UK), before incubation at room temperature for 1 hr. The slides were rinsed 3×5 TBS and developed with 100 µl of DAB substrate kit (abcam, USA). The slides were washed and mounted with Mowiol 4-88 (Polysciences Inc., UK).

Liver samples were homogenised in liquid nitrogen, using a mortar and pestle. Homogenised tissues were stored at -80°C, before RNA was

NM code	NM type	Average size (suppliers information)	Size in water with 2% serum (DLS) Ψ
NM 110	ZnO	100 nm	142.7 nm
NM 111	ZnO	130 nm	165.31 nm
NM 300	Ag	<20 nm	42.94 nm
NM 400	MWCNT	30 nm 5 µm long	NA
NM 402	MWCNT	30 nm 5 µm long	NA
NRCWE 002	TiO_2	10 nm	116.54 nm

Ψ Intensity based size average in biological media after 15 mins

Table 1: Average size of tested NMs as supplied by suppliers and as measured by DLS.

extracted and isolated using the MagMAX™-96 Total RNA Isolation Kit (Ambion, USA). RNA concentration and purity were measured on a Nanodrop 2000c system (Thermo Scientific, UK). The High Capacity cDNA RT kit (Applied Biosystems, UK) was used according to the protocol, to transcribe RNA into cDNA. Equal quantities of RNA from 3 animals in the same treatment group were pooled and 300 ng of RNA were used in RT reactions. PCRs were conducted in triplicate on a 7900 RT fast PCR system and SDS 2.3 software in 384 well plates (Applied Biosystems, USA), using TaqMan kits, with FAM dye under standard TaqMan conditions for 50 cycles. The following Applied Biosystems kits were used: Mouse kits Mm00437762-m1–B2m (housekeeping gene); Mm00436450-m1–CXCL2; Mm00439614-m1–IL10; Mm00443258-31–TNF-α; Mm00437858-m1–C3; Mm00438864-m1–Fas ligand; Mm00516023-m1–ICAM-1; Mm00802090-m1–albumin and Mm00446190-m1–IL6.

All data are expressed as mean ± standard error of the mean. For statistical analysis, the experimental results were compared to their corresponding control values, using an ANOVA with Tukey's multiple comparison. All statistical analysis was carried out utilizing Minitab 15. A p value of <0.05 was considered to be significant. All experiments were repeated a minimum of three times.

Results and Discussion

This study was conducted as part of a large consortium (FP7 funded project–ENPRA), to investigate the potential hazard of a wide range of nanomaterials on a variety of targets, in order to use the data for generation of a structure activity relationship and for modelling risk assessment. Several studies have demonstrated that NMs entering the body via the lungs, ingestion or direct injection accumulate in the liver [7-9]. In previous studies, it has been shown that hepatocytes are capable of producing potent neutrophil chemoattractants (human–IL8 [15,16,18,19] rat-CINC-3) [20], following exposure to foreign matetrials, or in disease models. We therefore investigated neutrophil infiltration into the liver, following IV exposure of mice to a panel of engineered nanomaterials. Our previous studies have demonstrated that the panel of engineered nanomaterials investigated could be divided into a relatively high toxicity group and a lower toxicity group, according to their ability to induce cytotoxicity in the C3A cell line [16], primary human hepatocytes [19], primary rat co-cultures of hepatocytes and kupffer cells. Hence, six nanomaterials were chosen-three of which were shown to be of higher toxicity (Ag and two ZnO NMs), and three that were of relatively low toxicity (positively charged TiO_2 and two MWCNTs), in the *in vitro* systems.

Myeloperoxidase is a lysosomal protein stored in granules of all granulocytes, and is found in largest quantities in neutrophils. In order to quantify PMN infiltration into the liver, we measured this enzyme in tissue samples. A modest but statistically significant increase in MPO in the liver, as early as 6 hr, following intravenous injection of the NMs was detected (Figure 1) (p<0.05). MPO content of the liver tissue had decreased to control levels by 48 hr, following exposure to Ag and TiO_2 (Figure 2). Multiple doses of Ag and TiO_2 NMs did not enhance the MPO up-regulation further (Figure 3).

To confirm the increase in MPO in liver homogenates (Figure 1-3), and whether or not this was associated with the neutrophil influx, liver sections from exposed animals were stained using a specific neutrophil cell surface marker (Ly-6B.2). Staining demonstrated the presence of neutrophils in the tissues exposed to the positive control and the panel of nanomaterials, 6 hr post exposure, which were not evident in the negative control (Figure 4). Furthermore, the relative numbers
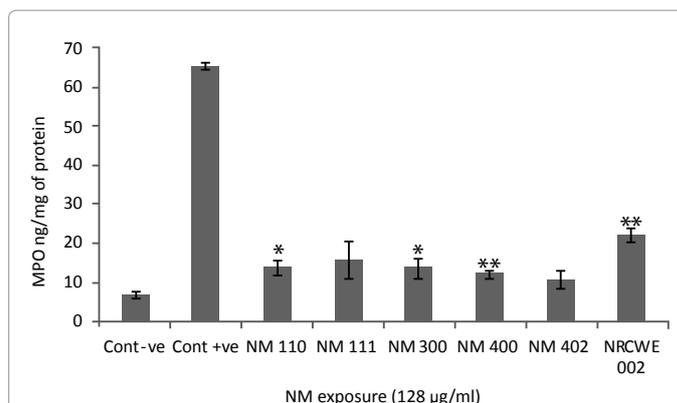

Figure 1: MPO measured in the liver tissue following 6 hr exposure to 100 μl in 128 μg/ml of the panel of NMs (NM 110–ZnO uncoated; NM 111–ZnO coated; NM 300–Ag; NM 400–MWCNT; NM 402-MWCNT; NRCWE 002–positively charged TiO_2. A positive control of LPS (50 μl-100 μg/ml) was also employed. The negative control is representative of animals injected with 100 μl of PBS. (n=3 ± SEM) *P <0.05, **P<0.005 compared to negative control.

Figure 2: MPO measured in the liver following 24, 48 and 72 hr exposure to 100 μl of 128 μg/ml the NM 300–Ag and NRCWE 002–positively charged TiO_2. A positive control of LPS (50 μl-100 μg/ml) was also employed. The negative control is representative of animals injected with 100 μl of PBS. (n=3 ± SEM) *P<0.05, **P<0.005 compared to negative control.
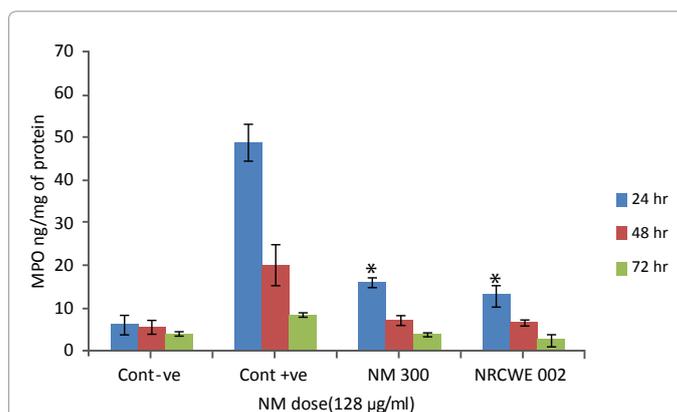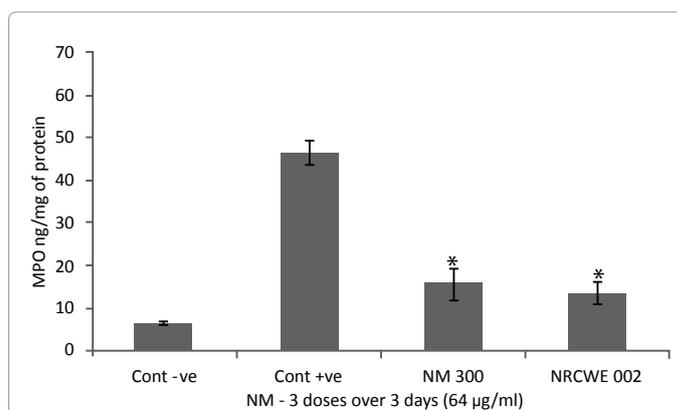

Figure 3: MPO measured in the liver following 3 repeated doses of NMs over three days (3 doses of 100 μl of 64 μg/ml every 24 hr). The animals were dissected 72 hr following the initial dose. The negative control is representative of animals injected with three doses of 100 μl of PBS, while the positive control animals were injected with three doses of LPS (50 μl - 50 μg/ml per injection). (n=3±SEM) *P<0.05, **P<0.005compared to negative control.

of neutrophils stained in the tissues were comparable to the levels of MPO in liver in the time course experiments, showing that there was a decrease in neutrophil staining (Ag and TiO_2) (Figure 5). Multiple dose of the silver and TiO_2 also reflected the MPO results, showing that repeated exposures did not further enhance the neutrophil influx into the tissue (Figure 6).

Very low doses (12.8 μg of NMs per animal for single exposure or 19.2 μg per animal for repeated exposure experiments) of NMs were injected into the mice in this study to represent a realistic exposure and to avoid toxicity to the animal. These doses are not dissimilar to other studies in which animals have been exposed to NMs *via* an IV route [21,22]. The findings in this study suggest that neutrophils are involved in the organs immunity to the foreign nanomaterials. To our knowledge, the role of neutrophils in the liver, following nanomaterial exposure has not been addressed previously, however, it has been shown that nautrophils can accumulate within hepatic microvasculature, following increase in inflammatory mediators such
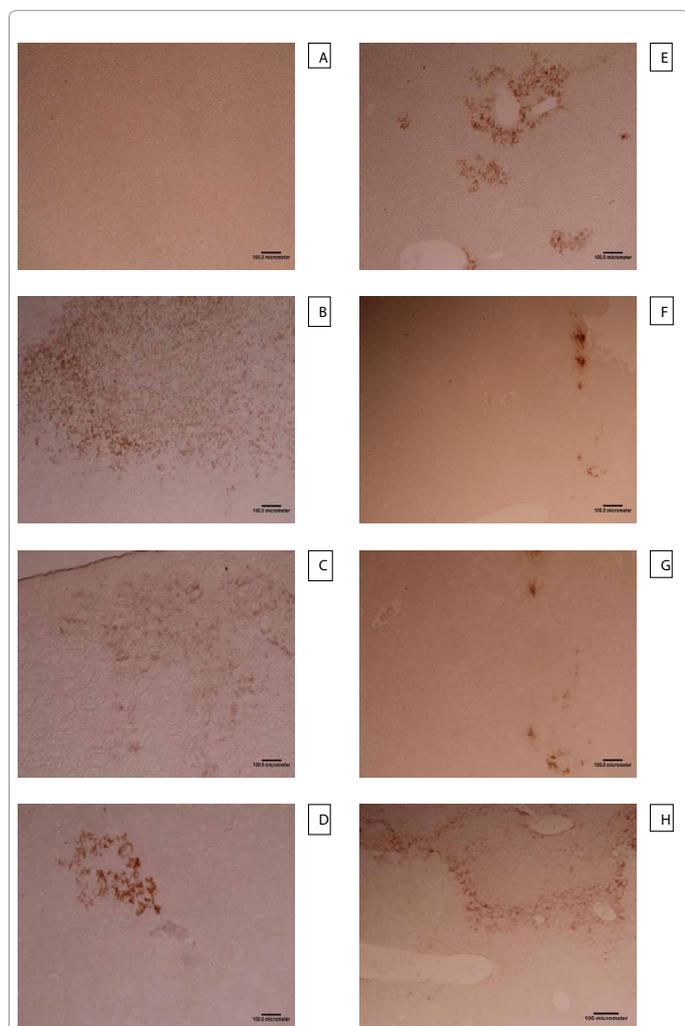

Figure 4: Ly6B.2 staining of mouse liver tissue by immunohistochemistry, 6 hr post intravenous exposure to ENPRA panel of engineered nanomaterials (100 μl-128 μg/ml of NMs). A positive control of LPS (50 μl-100 μg/ml) was also employed. The negative control is representative of animals injected with 100 μl of PBS.

A) PBS **B)** LPS **C)** NM 110 **D)** NM 111 **E)** NM 300 **F)** NM 400 **G)** NM 402 **H)** NRCWE 002.

Figure 5: Ly6B.2 staining of mouse liver tissue by immunohistochemistry representing samples from animals 24, 48 or 72 hr post intravenous exposure to ENPRA panel of engineered nanomaterials (100 μl-128 μg/ml of NMs).

A) NM 300–24 hr **B)** NRCWE 002–24 hr **C)** NM 300–48 hr **D)** NRCWE 002–48 hr **E)** NM 300–72 hr **F)** NRCWE 002–72 hr.

Figure 6: Ly6B.2 staining of mouse liver tissue by immunohistochemistry following three repeated doses over three days (3 doses of 100 μl of 64 μg/ml of NMs every 24 hr). The animals were dissected 72 hr, following the initial dose.

A) NM 300 **B)** NRCWE 002.

as TNF-α, IL1, MIP-2 and PAF [23,24]. Furthermore, in a recent study, an increase in numbers of neutrophils in the liver of BALB/c mice was observed during anaphylaxis [25].

In this study, it appears that the neutrophils are involved predominately in the initial phases of the immune response, on exposure to the NMs. The initial increase in neutrophil numbers in liver tissue was resolved by 48 hr post exposure. Neutrophils in other organs i.e. the lung are important part of the early innate immune response, as early as 4 hr post exposure to different antigen challenges [26,27], and can be involved in long term inflammation [28]. After the initial infiltration of the neutrophils into the tissue, the resident macrophages (Kupffer cells) may take over the immune response to the nanomaterials. It was also very interesting to note that repeated doses of the nanomaterials did not result in further accumulation of the polymorphonuclear leukocytes into the tissue, confirming our previous suggestion that neutrophils play no further part in the maintenance or the continuation of the inflammatory response.

It has been shown that the most common neutrophil mediated response in the liver is governed by the adhesion of the cells to hepatocytes (*via* ICAM-1 binding Mac-1) [24,29]. This adhesion triggers the formation of Reactive Oxygen Species (ROS) by NADPH oxidase, and release of proteases through the degranulation of the PMNs. This process could lead to neutrophilic hepatitis, characterised by self aggravating mechanisms, resulting in chronic disease [29]. This prolonged neutrophils response does not happen, following exposure to the nanomaterials utilised in this study. As already mentioned, the neutrophils decrease in number with time after the initial exposure. In addition, the up-regulation of ICAM-1 mRNA was only noted after 24 hr and had returned to normal levels 48 hr post IV NM exposure.

The expression of a range of genes related to inflammation, oxidative stress and apoptosis was analysed in the livers of mice exposed to the panel of engineered nanomaterials. Analysis of mRNA expression in mouse liver 6 hr after intravenous injection of 100 μl of 128 μg/ml of the panel of nanomaterials showed a decrease in C3 following exposure to ZnO (NM 110 and NM 111), MWCNT (NM 400 and NM 402) (Table 2). We noted a decrease in IL6 mRNA levels, following exposure to ZnO (NM 110, NM 111) and Ag NMs (NM 300) (Table 1). An increase in IL10 was observed, following exposure to ZnO (NM 110) and TiO$_2$ (NRCWE 002) (Table 1). Finally, a large increase in CXCL2 was demonstrated, following exposure to ZnO. No significant changes were detected in the expression of albumin, TNF-α, albumin, Fas ligand or ICAM-1, following treatment with any of the nanomaterials (Table 2).

Repeated exposure of the C57/BL6 mice to three doses of the Ag (NM 300) and positively charged TiO$_2$ (NRCWE 002) nanomaterials resulted in a large increase in IL10 levels, while smaller yet significant decreases in C3 and ICAM-1 mRNA were noted, following exposure to both NMs (Ag and TiO$_2$) (Table 3).

Finally, the animals were exposed to a single dose of 100 μl of 64 μg/ml of the Ag and positively charged TiO$_2$ NMs by injection *via* the lateral tail vein. The animals were dissected 24 hr, 48 hr and 72 hr after the initial injection. We found that in all instances, the increase or decrease in mRNA was most evident after the 24 hr exposure. For all the genes investigated in this study, resolution to control levels had occurred 72 hr post exposure (Tables 4 and 5).

Complement factor 3 is one of the most abundant and important

proteins in the complement cascade. C3 activation is essential for all the functions of the complement system. It is crucial in promoting phagocytosis, supporting the local immune response against foreign agents, and instructing the adaptive immune responses to select the appropriate antigens for any eventual antibody response [30]. Moreover, C3 might be capable of interaction with the coagulation system and contributing to pro-coagulation, and ultimately, a prothrombotic state [31]. The decrease in C3 expression might suggest that exposure to low doses of NMs in this study results in overall tolerance in the liver, rather than a fully developed immune response.

There was a decrease in IL6 mRNA levels, following exposure to the ZnO and Ag NMs, after 6 hr exposure. IL6 is a multifunctional cytokine that acts on a number of cell types, including B and T lymphocytes, hepatocytes, hematopoietic progenitor cells and neuronal cells [32]. Under different circumstances, the cytokine can be both pro- and anti-inflammatory. In the liver, IL6 is a potent inducer of acute phase proteins, which are in most part pro-inflammatory proteins (including C reactive protein, complement factors and serum amyloid A) [33]. Once again, the decrease in IL6 expression advocates that exposure to low doses of these NMs results in tolerance in the liver.

IL10 is a multifunctional cytokine with diverse effects on a wide range of hemopoietic cells. The cytokine's principle function is to terminate inflammatory responses [34]. IL10 also regulates growth or differentiation of B and T lymphocytes, NK cells, mast cells, most granulocytes, dendritic cells, keratinocytes, and endothelial cells [35]. IL10 also plays a key role in differentiation of regulatory T cells, which figure prominently and importantly in the control of immune responses, and developing a tolerant immune state [35]. Analysis of IL10 mRNA showed an increase, following exposure to exposure to

Gene Expression	NM 110	NM 111	NM 300	NM 400	NM 402	NRCWE 002
IL6	-200%	-50%	-200%	0	0	0
IL10	+75%	0	0	0	+50%	+200%
C3	-50%	-75%	-75%	-50%	-75%	0
CXCL2	+200%	0	0	0	0	0
TNF-α	0	0	0	0	0	0
Fas ligand	0	0	0	0	0	0
ICAM-1	0	0	0	0	0	0
Albumin	0	0	0	0	0	0

Table 2: mRNA expression of inflammatory cytokines and receptors, FasL and albumin in C57/BL6 mice liver. The animals were exposed to 100 μl of 128 μg/ml of panel of nanomaterials for 6 hr by injection *via* the lateral tail vein. mRNA expression was analysed by real time PCR with B2m utilised as an endogenous control.

Gene Expression	NM 300	NRCWE 002
IL6	0	0
IL10	+200%	+200%
C3	-100%	-100%
CXCL2	0	0
TNF-α	+50%	0
Fas Ligand	0	0
ICAM-1	-100%	-100%
Albumin	0	0

Table 3: mRNA expression of inflammatory cytokines and receptors, FasL and albumin in C57/BL6 mice liver. The animals were exposed three times (0 hr, 24 hr and 48 hr) to 100 μl of 64 μg/ml of the Ag (NM 300) and the positively charged TiO$_2$ NMs by injection *via* the lateral tail vein. The animals were dissected 72 hr after the first injection and the mRNA expression was analysed by real time PCR, with B2m utilised as an endogenous control.

Gene Expression (NM 300)	Day 1	Day 2	Day 3
C3	-75%	-50%	0
IL6	+100%	+100%	0
IL10	+200%	+75%	0
CXCL2	0	0	0
TNF-α	0	0	0
Fas Ligand	+75%	0	0
ICAM-1	+75%	0	0
Albumin	0	0	0

Table 4: mRNA expression of inflammatory cytokines and receptors, FasL and albumin in C57/BL6 mice liver. The animals were exposed to a single dose of 100 µl of 128 µg/ml of the Ag NM (NM 300), by injection *via* the lateral tail vein. The animals were dissected 24 hr, 48 hr and 72 hr after the initial injection and the mRNA expression was analysed by real time PCR with B2m utilised as an endogenous control.

Gene Expression (NRCWE 002)	Day 1	Day 2	Day 3
C3	-75%	0	0
IL6	0	0	0
IL10	+200%	+200%	0
CXCL2	0	0	0
TNF-α	0	0	0
Fas Ligand	+75%	0	0
ICAM-1	+75%	0	0
Albumin	0	0	0

Table 5: mRNA expression of inflammatory cytokines and receptors, FasL and albumin in C57/BL6 mice liver. The animals were exposed to a single dose of 100 µl of 128 µg/ml of the TiO_2 NM (NRCWE 002) by injection *via* the lateral tail vein. The animals were dissected 24 hr, 48 hr and 72 hr after the initial injection and the mRNA expression was analysed by real time PCR, with B2m utilised as an endogenous control.

NM 110 (uncoated ZnO), NM 300 (Ag), NM 402 (MWCNT) and NRCWE 002 (TiO_2). The increase in IL10 mRNA was the largest and the most significant of all the genes investigated in this study, once again suggesting tolerance as being the overall response in the organ, following acute low exposure to NMs.

CXCL2 also known as Macrophage Inflammatory Protein 2 alpha (MIP-2) is a chemokine secreted from a number of cell types, in particular, monocytes and macrophages [36]. CXCL2 is a potent chemoattractant for neutrophils and hematopoetic stem cells [36]. A large increase in CXCL2 was observed following exposure to NM 110 (uncoated ZnO), following 6 hr exposure. It is important to note that increases compared to base levels of CXCL2 were also demonstrated following the 6 hr exposure to other nanomaterials, however, due to large fluctuations in the control group of animals, these changes could not be regarded as significant. Up-regulation of CXCL2 could be associated with the short term acute recruitment of PMNs into the liver.

No significant changes were detected in the expression of albumin, TNF-α, Fas ligand or ICAM-1, following 6 hr exposure to any of the nanomaterials utilised in this study. The fact that there was no change in the levels of TNF-α would again suggest that there is no significant pro-inflammatory response, following exposure to the NMs. FasL (also referred to as CD95L) is a homotrimeric type II transmembrane protein signalling through trimerisation with CD95 on the target cells, leading to death by apoptosis [37]. The findings here would suggest that there is no apoptosis, following exposure to these particular nanomaterials at the time points used here, however, it is possible that FasL may be up-regulated at later time points, in relation to the clearance of PMN from the liver. There was no change in albumin gene expression suggesting that the NM challenge at these low doses does not affect liver function.

Repeated exposure of the mice to three doses of the Ag (NM 300) and positively charged (NRCWE 002) nanomaterials again resulted in large increases in IL10 levels, while smaller yet significant decreases in C3 and ICAM-1 mRNA were noted, following exposure to both NMs. ICAM-1 (Intracellular Adhesion Molecule 1) is a molecule involved in attraction and adhesion of inflammatory cells to endothelial and epithelial cells, and its soluble form, sICAM-1, is linked to activation of these cells in pathological responses [38]. It is up-regulated in liver associated diseases, such as in hepatitis C [38]. As already mentioned, binding of ICAM-1 to Mac-1 on hepatocytes is crucial to neutrophil mediated response in the liver. The overall decrease in ICAM-1 mRNA suggests that neutrophils are not involved at the latter stages of the liver immunity, despite fresh foreign nanomaterial challenges.

Finally, a group of animals were exposed to a single dose of 100 µl of 64 µg/ml of the Ag and positively charged TiO_2 NMs. The animals were dissected 24 hr, 48 hr and 72 hr after the initial injection. We found that in all instances, the greatest changes in mRNA occurred after the 24 hr exposure. These changes resolved and all the genes investigated returned to background levels, after 72 hr post exposure.

It is important to emphasize the limitations of this study. It may be important to examine earlier time points to gain a better understanding of the role of neutrophils in the liver, following a NM challenge. Future studies may include the utilisation of microarray analysis for a more comprehensive gene expression profile in the liver. Any potential changes in a large number of genes, including those of the cytochrome P_{450} family, heat shock proteins and IL1 family of proteins could be important indicators of NM impact on the liver. Furthermore, it might be important to examine presence of neutrophils in blood and compare with liver tissue. It is possible that these cells are also responsible for initiating the reparative process that is later managed by macrophages.

Overall, the findings in this study suggest that intravenous exposure of mice to the ENPRA panel of engineered nanomaterials results in neutrophil governed acute effects in the liver. The low doses used were not sufficient to cause any longer term inflammation in the liver tissue. Any changes that were observed after 24 hr post exposure, in terms of leukocyte infiltration and changes in gene expression related to inflammation, oxidative stress and apoptosis had resolved 72 hr post exposure.

Acknowledgements

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