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Fast and Robust Proteome Screening Platform Identifies Neutrophil Extracellular Trap Formation in the Lung in Response to Cobalt Ferrite Nanoparticles

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ABSTRACT

Despite broad application of magnetic nanoparticles in biomedicine and electronics, only a few in vivo studies on biocompatibility are available. In this study, toxicity of magnetic metal oxide nanoparticles on the respiratory system was examined in vivo by single intratracheal instillation in mice. Bronchoalveolar lavage fluid (BALF) samples were collected for proteome analyses by LC-MS/MS, testing Fe₃O₄ nanoparticles doped with increasing amount of cobalt (Fe₃O₄, CoFe₂O₄ with iron to cobalt ratio 5:1, 3:1, 1:3, Co₃O₄) at two doses (54µg, 162µg per animal) and two time points (day 1 and 3 post instillation). In a discovery phase, in-depth proteome profiling of few representative samples allowed for comprehensive pathway analyses. Clustering of the 681 differentially expressed proteins (FDR < 0.05) revealed general as well as metal oxide specific responses with an overall strong induction of innate immunity and activation of the complement system. Highest expression increase could be found for a cluster of 39 proteins, which displayed strong dose-dependency to iron oxide and can be attributed to neutrophil extracellular trap (NET) formation. In-depth proteome analysis expanded the knowledge of in vivo NET formation. During screening, all BALF samples of the study (n=166) were measured label-free as single-injections after short gradient (21 min) LC separation using the Evosep One system, validating the findings from the discovery and defining protein signatures which enable discrimination of lung inflammation. We demonstrate a proteomics-based toxicity screening with high sample throughput easily transferrable to other nanoparticle types. Data are available via ProteomeXchange with identifier PXD016148.

KEYWORDS

quantitative proteomics, LC-MS/MS, bronchoalveolar lavage fluid, magnetic metal oxide nanoparticles, iron cobalt oxide nanoparticles, neutrophil extracellular trap formation, NETosis
The field of nanotechnology is constantly expanding and fast, reliable screening techniques for biocompatibility of nanomaterials are needed to ensure human safety. Nanomaterials are particulate substances with at least one dimension less than 100 nm. Due to their properties arising from their composition, surface and size, their application spectrum is broad, ranging from biomedicine, electronics, health care, food, cosmetics, textiles and others. Single parameter in vitro assays e.g. for extracellular lactate dehydrogenase (LDH), reactive oxygen species (ROS), inflammation and DNA strand breaks are well established tests to assess cytotoxicity and genotoxicity of chemicals including nanomaterials. Although reliable they are lacking comprehensive depth to deal with the individual complexity of cellular responses to the immense amount of newly generated materials. In order to improve biocompatibility of engineered nanoparticles it is also imperative to better understand the mechanisms of action of adverse health effects in vivo. With recent advances in liquid chromatography and mass spectrometry we propose a robust analysis platform for biocompatibility testing of nanomaterials which allows sensitive and comprehensive measurements of cellular responses with high sample throughput. As a proof of principle, we tested simultaneously different types of magnetic nanoparticles at different concentrations and exposure times. Magnetic nanoparticles have a wide range of applications due to their superparamagnetic property and size, and are of great interest in biomedicine (e.g. magnetic resonance imaging (MRI), molecular imaging, magnetic particle imaging, targeted tumor therapy, drug delivery). Among them, iron oxide nanoparticles are the most used and studied magnetic nanoparticles due to their high biocompatibility and ease of synthesis. The iron oxide \( \text{Fe}_3\text{O}_4 \) (magnetite) is one of the most magnetic minerals, with \( \text{Fe}_3\text{O}_4 \) nanoparticles or crystals naturally occurring in bacteria or in the brain of migratory animals navigating in the earth’s magnetic field. Iron oxide nanoparticles change properties including band-gap and consequently redox activity when doped with other metals such as cobalt. It has been shown that cobalt ferrite (CoFe\(_2\)O\(_4\)) nanoparticles display increased magnetic properties and chemical stability, making them superior contrast agents in MRI. Cobalt nanoparticles are widely used in the technology sector for magnetic sensors, magnetic memory, and recently also for wireless communication. Although cobalt-based nanoparticles have been connected with increased cytotoxicity, genotoxicity, oxidative stress and tumor formation, parallel testing of Co, CoO and Co\(_2\)O\(_4\) nanoparticles showed lowest cytotoxicity and no DNA damage response for Co\(_2\)O\(_4\). Similarly, testing of Co\(_2\)O\(_4\) nanoparticles in vitro in cell lines representing lung, liver, nervous and gastrointestinal system, showed only mild cytotoxic or genotoxic effects only observable at high concentrations (> 100 µg/ml). Daily dietary cobalt intake has been estimated at 5-50 µg/day, with a very small fraction coming from vitamin B12 (Cobalamin). Danish occupational exposure limit for cobalt is 0.05 mg/m\(^3\). System-wide omics analyses are becoming more popular in nanosafety research investigating the effects of nanomaterials on cell lines (reviewed in), however,
only a few in vivo studies exist to date, in large part as a result of the time, cost and need for ethical approval. From these system-wide characterizations, the most commonly observed changes include oxidative stress, metabolism, cytoskeleton and cell cycle. Inhalation of nanoparticles is the most likely non-intentional occupational exposure type. From classical approaches it is well known, that inhalation of nanomaterials can induce immune and complement system activation and increases the risk to develop chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) or lung cancer, as well as cardiovascular disease. Although a few omics studies have been conducted on nanomaterial toxicity after inhalation,\textsuperscript{12-17} or pulmonary exposure,\textsuperscript{18-22} information on magnetic or iron oxide nanoparticles is missing, and a robust high throughput screening method with associated bioinformatics workflow allowing widespread utilization and broad generalization of the approaches has been lacking.

Here we studied the effects of magnetic iron oxide (Fe\textsubscript{3}O\textsubscript{4}) nanoparticles doped with cobalt oxide (Co\textsubscript{3}O\textsubscript{4}) at different ratios after intratracheal instillation in mice using label-free quantitative proteomics. Carbon nanoparticles (carbon black, Printex 90) were included as inflammation-inducing reference material,\textsuperscript{22-27} which is a standard in nanotoxicology and environmental research mimicking air pollution from combustion, diesel engines and tire wear emissions. Carbon black has an annual industrial production of 10 million tons, most of which is used in tires, the rest can be found in paints, rubber and plastics. The concentrations used for instillation correspond to exposure for 3-8 working days at the occupational exposure limit according to the Occupational Safety and Health Administration. Our analysis pipeline consists of high resolution mass spectrometry connected to the recently introduced Evosep One LC system, specifically developed for clinical proteomics, which enables robust and fast measurements by a combination of low-pressure sample flow with in-loop gradient storage and minimal overhead time.\textsuperscript{28} This setup enables the analysis of >60 samples per day and allows for large-scale comprehensive nano-bio and mechanistic nanotoxicology studies for the elucidation of molecular initiating events and resulting adverse outcome pathways. After performance evaluation of the Evosep One LC system, two proteomic strategies were followed, with a discovery phase including few samples (<10) for a classic in-depth label-based proteomic profiling and a screening phase including many samples (>100) for validation of the platform.
RESULTS AND DISCUSSION

Experimental Design

The goal of this study was to investigate magnetic iron cobalt oxide nanoparticles (cobalt doped Fe₃O₄ nanoparticles with increasing amounts of cobalt) after pulmonary exposure while in parallel presenting a proteomics platform that is easily transferable to large-scale nanotoxicology screening as part of an integrated assessment and testing approach for regulation of nanoparticles. BALF is a proximal biofluid that can be used to monitor airway inflammation and toxic responses in the lung. It is routinely sampled for differential lung diagnostics and has been discussed as a source for early detection of lung cancer. In order to assess effects of metal oxide nanoparticles upon inhalation, bronchoalveolar lavage fluid from mice dosed by single intratracheal instillation was collected and subjected to classical biocompatibility assays as well as proteome analysis (Figure 1A). Magnetic oxide nanoparticles with iron and cobalt oxide (Fe₃ₓCoₓO₄) at different ratios (1:0, 5:1, 3:1, 1:3, 0:1) were tested at two concentrations (54 µg, 162 µg per animal) and two time points after instillation (day 1, day 3). Timepoints for proteome analysis (day 1 and day 3 post instillation) were chosen based on previous studies which showed that exposure to various metal oxides induce pulmonary acute phase response, which peaks at day 1 or day 3.²⁹,³⁰ As a positive control carbon black (CB) nanomaterial known to induce lung inflammation³¹ was included for both time points, but only at the high dosage (162 µg per animal).

Proteomics experiments were divided into three parts – test of reproducibility, discovery and screening phase (Figure 1B, C). The reproducibility of the recently introduced Evosep One LC system was evaluated by measuring of technical replicates (n=16). During the discovery phase, selected representative samples with 3 biological replicates per group (total n=9) including pure iron oxide nanoparticles, pure cobalt oxide nanoparticles and vehicle controls were subjected to in-depth proteome profiling by extensive pre-fractionation and including isobaric tandem mass tag (TMT) labeling following a classical LC-MS/MS setup. This step allowed us to identify affected pathways and generate hypotheses regarding mechanisms of the effects of nanoparticles. During screening, all samples of the study were measured label-free as single-shot injections separated on short gradients of 21 min using the robust, high-throughput Evosep One LC system. This step allowed a fast screening of the 5 different types of magnetic metal oxide nanoparticles on BALF, at two concentrations and two time points together with their representative controls (total n=166). All screening measurements were completed in only 2.7 days. An overview of the LC-MS/MS setup and analytical pipeline for all three proteomic parts is depicted in Figure 1D.
Synthesis and Characterization of Nanoparticles

The nanoparticles used in this study provided by Promethan Particles were synthesized by continuous hydrothermal synthesis starting from metal salts as precursors with iron(III) nitrate nonahydrate (Fe(NO₃)₃ x 9H₂O) and cobalt(II)acetate tetrahydrate (Co(C₂H₃O₂)₂ x 4H₂O) for iron and cobalt oxide, respectively. The salts were used at different ratios to produce nanoparticles with iron to cobalt composition of 5:1, 3:1 and 1:3. Synthesis and characterization of the nanoparticles used have been described in detail. Nanoparticles were provided as liquid dispersions, safe for the end user to prevent accidental inhalation. All metal oxide nanoparticles used had a primary particle diameter below 40 nm based on transmission electron microscopy (TEM) imaging (Figure S1A) and a zeta potential between -4 and 24 mV in deionized water. Since the nanoparticles were uncoated, in deionized water they formed agglomerates with sizes of 103 nm for Co oxide and up to 1635 nm for Fe-Co 3:1 based on dynamic light scattering (DLS) analysis. Average surface area by Brunauer–Emmett–Teller (BET) analysis was 50 m²/g except for Fe-Co 5:1 (100 m²/g) and Fe-Co 3:1 (132 m²/g) (Figure S1B). Carbon black or Printex90 has been characterized previously by BET with an average size of 14 nm and a surface area of 300 m²/g.

Classic Nanoparticle Compatibility Tests: Pulmonary Inflammation, Cytotoxicity and Genotoxicity

To evaluate the extent of pulmonary inflammation induced by the nanoparticles the cellular content of BALF was assessed. Differential cell counting was performed for neutrophils, macrophages, lymphocytes, eosinophils and epithelial cells (Figure S2A, Table S1). Normal cell counts for BALF include >85% alveolar macrophages, 10–15% lymphocytes, ≤3% neutrophils, ≤1% eosinophils and ≤5% epithelial cells. For all treatment groups, there was a strong neutrophil influx, except for low dose pure iron oxide nanoparticles which reached base level 3 days after exposure. On contrary, macrophages showed pulmonary evasion upon nanoparticle treatment, with similar iron dose dependency as neutrophils. Lymphocyte influx was visible after 3 days of instillation with mild increase for all treatment groups at high dose. Eosinophils showed a prominent late response with a clear correlation with increasing cobalt content in the nanoparticles and were also increased after carbon black instillation. Epithelial cells were slightly affected in a cobalt responsive manner, with normalization back to baseline levels after 3 days of instillation. Similar to the neutrophil influx profile, the acute phase response gene Saa3 was strongly increased at day 1 after instillation, displaying dose dependency and iron inducibility (Figure S2A). Cytotoxicity was evaluated by measuring extracellular lactate dehydrogenase (LDH) and total protein content (Figure S2B). Elevated LDH
was observed 3 days after instillation for high dose nanoparticle exposure with cobalt-dose dependency as for carbon black. Total protein content in BAL fluid was elevated with dose dependency for both time points with highest values for pure cobalt oxide nanoparticles. As a measure for genotoxicity DNA strand brakes were evaluated by the comet assay\textsuperscript{35–37} in both BALF and lung as well as liver, the main target organ for nanoparticle accumulation after translocation (Figure S2C). Increased levels of DNA strand breaks were observed for BAL cells from mice exposed to pure iron oxide nanoparticles, and for mice exposed to Fe\textsubscript{2}CoO\textsubscript{4} nanoparticles with 1:3 and 3:1 iron to cobalt ratio. The cytotoxicity observed for the cobalt-group could have interfered with an inflammatory response.

To summarize, according to classical toxicity testing all the tested nanoparticles induced dose dependent acute inflammatory and acute phase responses whereas particles with high iron content induced DNA strand breaks in BAL cells. There was a clear iron dose response for the extent of pulmonary neutrophil influx and macrophage evasion. This observation has to the best of our knowledge not been reported before. However, there is a link of siderophore binding proteins in neutrophils for bacterial iron starvation and pathogen secreted iron-binding siderophores.\textsuperscript{38} In a recent study it was shown that bacterial siderophores not just bind iron to support their growth but also to steal iron from neutrophils to hijack their anti-inflammatory response by preventing reactive oxygen species (ROS) generation.\textsuperscript{39} Macrophages, known to play a crucial role in iron uptake, storage and recycling with specific regard to erythrocyte phagocytosis\textsuperscript{40} contain a repertoire of iron-binding or transporting proteins. Similar as for neutrophils, iron content has been suggested to be important for ROS generation in macrophages.\textsuperscript{41} Cobalt dose dependency could be observed for eosinophil influx and an increase in epithelial cells, the latter as a sign for increased cytotoxicity with higher cobalt content. Cobalt has been linked to the induction of occupational hard metal-induced asthma with strong pulmonary eosinophilic inflammation.\textsuperscript{42} Further, it has been shown that after instillation with cobalt nanoparticles the amount of solubilized cobalt ions correlate with eosinophil numbers,\textsuperscript{43} which supports our observation of cobalt oxide dose dependent pulmonary eosinophil influx. Best tolerated were pure iron oxide nanoparticles at lower concentration, consistent with their known biocompatibility. On the other hand, pure iron oxide nanoparticles induced DNA strand breaks, indicating genotoxicity.
Proteomics

Reproducibility of Evosep One

Requirements for clinical proteomics include high sample throughput, accuracy, robustness, simple sample preparation, and deep proteome coverage while dealing with the high dynamic range in biological matrices spanning up to 11 orders of magnitude for serum and plasma or 10 orders for bronchoalveolar lavage fluid. Deep proteome coverage is traditionally achieved by reducing sample complexity by peptide separation into multiple fractions and/or using long liquid chromatography gradients which increases measurement time and reduces sample throughput. While measurement time can be reduced by incorporating labeling strategies (e.g. isobaric chemical labeling TMT) which allows multiplexing currently of up to 16 samples, this adds additional steps to the sample preparation. The dynamic range issue inherent to biological samples can be addressed by depletion of a few highly abundant proteins such as albumin but again increases sample processing and therefore variability. Recent advances in liquid chromatography and high-resolution mass spectrometry rekindled the interest in clinical proteomics. Robustness and reproducibility of the Evosep One LC system was evaluated by repeated measurements (n=16) of one representative BALF sample using the 21 min gradient method (Figure 2), including intra-assay variability. The representative BALF sample was distributed in 16 wells to perform in parallel individual protein extraction, digestion and peptide loading on EvoSep tips. On average 450 proteins were quantified with protein measurements covering 6 orders of magnitude (Figure S3A). Label free quantitation (LFQ) intensities of six selected proteins spanning the whole dynamic range are shown, demonstrating high reproducibility. High correlation between technical replicates was observed with an average Pearson correlation coefficient of 0.98, ranging from 0.958 to 0.988 for pairwise comparisons of protein intensities. Likewise, the median coefficient of variation (CV) was 19% based on 203 quantified proteins (no missing values, n=16). Allowing increasing numbers of missing values, the CVs increase to a maximum of 25.3% based on 474 proteins (n>2) (Figure 2, Figure S3B). In clinical diagnostic assays a CV < 20 is considered acceptable. Applying this filter 53% of the proteins with no missing values passed. The analytical performance of the EvoSep One system presented here is in the expected range and similar to what has been previously reported.28 Thus, the validation step has demonstrated that the presented workflow has acceptable inter- and intra-assay reproducibility.
Discovery phase

The effects of iron and cobalt oxide nanoparticles on the proteome content of BALF were profiled in depth achieved by extensive pre-fractionation. In combination with chemical isobaric TMT labeling which allowed multiplexing of the 9 samples in one experiment accurate protein quantification was ensured. In-depth profiling of BALF led to the complete quantification of 1766 proteins (Figure S4A). Principal component analysis (PCA) displays the expected clustering of replicates and separation between experimental groups (control (CTRL), iron oxide nanoparticles (Fe3O4 NP; abbrev. as Fe), cobalt oxide nanoparticles (Co3O4 NP; abbrev. as Co)) (Figure 3A). Differential expression analysis was performed, and 681 proteins were found to be significant (FDR < 0.05) (Table S2). Protein expression distribution between experimental conditions is shown (Figure 3B) with 431 proteins differentially expressed for Fe vs CTRL, 480 proteins for Co vs CTRL and 220 for Fe vs Co. Differentially expressed proteins were clustered by applying unsupervised fuzzy-c means algorithm (Table S3) with the number of protein members per cluster annotated (Figure 3C). Protein content changes in BALF can be described by a general increase (cluster 5) or decrease (cluster 6) in response to metal oxide nanoparticles. Metal oxide nanoparticles specific changes are represented by clusters 1-4, with increase to Fe3O4 nanoparticles (clusters 1 and 3) or Co3O4 nanoparticles (cluster 4) and mild decrease to Co3O4 nanoparticles (cluster 2). The most prominent expression differences can be observed for the iron oxide responsive proteins of cluster 3. Gene set enrichment analyses for all deregulated proteins (Figure S4B) shows a high content of extracellular secreted proteins, highly associated with extracellular vesicles, exosomes and blood microparticles, as well as focal adhesion, cytoskeleton, extracellular matrix and nucleosome. Functionally, proteins with e.g. endopeptidase inhibitor activity and oxidoreductases were highly enriched. In accordance with sample treatment iron ion binding has been found significant only for iron oxide nanoparticles. Among the top GO biological process terms or KEGG pathways enriched were complement and coagulation cascades and metabolic pathways with a considerable response to cobalt oxide, whereas immune system and response to metal ion or bacterium appeared to be more prominent in response to iron oxide. Similarly enriched were proteins associated with phagosome, lysosome, bacterial invasion of epithelial cells and systemic lupus erythematosus. Those results are in accordance with findings in previous proteomics or transcriptomics studies, however the iron oxide and cobalt oxide dependent shift for enriched functions has not been reported. Performing enrichment separately for the six clusters (Figure 3D) confirms the stronger immune response for iron oxide nanoparticles, whereas cobalt oxide is more associated with wound healing and complement activation. General downregulation is associated with metabolic enzymes with high content of proteins involved in oxidation-reduction. Based on MetaCore enrichment (Figure S5-10, lower panels) both iron oxide response clusters (1 and 3) are associated strongly with neutrophil
mediated immunity (Figure S5, S7), which is in accordance with the iron dose dependency observed for the pulmonary neutrophil influx (Figure S2A). Proteins from cluster 2 showing moderate downregulation with cobalt oxide response are enriched for iron import and epithelial progenitor cell differentiation as well as metabolism of xenobiotics by cytochrome 450 (Cyp2f2, Gstm1, Gsto1, Adh5). Proteins of cobalt oxide response cluster 4 (upregulation) are associated with wound healing including cytoskeleton remodeling, complement activation and fibrosis development. More differential enrichment analysis of proteins from cluster 6 (general downregulation) revealed enzymes connected to glutathione metabolism, NRF2 regulated oxidative stress, protein folding, as well as carboxylic and oxoacid metabolic process (Figure S10). Downregulation of proteins of clusters 2 and 6 might be related to the observed pulmonary macrophage evasion, however, the iron dose response expected from differential cell counting was not observed. Among the most strongly enriched proteins in BALF samples upon single instillation with metal oxide nanoparticles were histone proteins, identified with 14 isoforms covering core histones (H2A, H2B, H3, H4) and linker histones (H1) distributed in clusters 3 (intense iron oxide dose-dependent upregulation, 10 histone isoforms), 4 (cobalt oxide dose-dependent upregulation, 1 histone isoform) and 5 (general upregulation, 3 histone isoforms).

Cluster 3 is of particular interest as it displays the strongest changes in expression with enhanced specificity for iron oxide nanoparticles and shows in addition pronounced enrichment for nucleosome assembly among clusters (Figure 3D). A functional protein-protein interaction network (Figure 3E, left panel) of the 38 members of cluster 3 consists of histones, immune response and neutrophil-associated proteins, with myeloperoxidase (Mpo), neutrophil elastase (Elane, NE), and proteinase 3 (Prtn3) among them indicative of neutrophil extracellular trap (NET) formation. NET formation or NETosis is a defense mechanism where neutrophils externalize a DNA mesh decorated with anti-inflammatory proteins such as endopeptidases to capture and defend against pathogens. "NETosis in systemic lupus erythematosus (SLE)" as the most enriched pathway with MetaCore analysis (FDR 10^{-17}) further confirms this observation (Figure S7). Graphic representation of this pathway with overlaid expression shows a 30% coverage with 10 out of 31 proteins identified (Figure 3E, right panel). Cluster 3 “NETosis in SLE” proteins are histone H1, histone H1.2, histone H2A, histone H2, histone H4, Mpo (abbreviated as PERM on MetaCore pathway map), Camp, and Ncf1 with Elane (abbreviated as Leukocyte elastase on MetaCore pathway map) and histone H3 only significant for exposure to iron oxide nanoparticles. NETosis proteins Mapk14, PKC and Ncf2 clustered together with intermediate iron oxide response cluster 1. This indicates that additional potential candidates for NETosis can be found in clusters 1 and 3. Two types of NETosis can be distinguished, phagocyte NADPH oxidase (Nox2) dependent and independent, which are studied in vitro using the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) and the
calcium ionophore A23187 as classical stimulants, respectively. The PKC activator PMA leads to cytosolic ROS generation by Mpo perforating the nuclear membrane while Elane translocates to the nucleus and initiates chromatin de-condensation and extortion. The calcium ionophore A23187 induces the production of mitochondrial ROS and the activation of the SK3 channel which leads to PAD4 mediated hypercitrullination of mostly histones. NETosis is an important defense mechanism of neutrophils against pathogens, chronic NET formation, however, has been associated with the induction of autoimmune diseases (rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)) based on production of PAD4-mediated citrullinated autoantigens (mostly histone H3), as well as chronic lung diseases and pulmonary cancer metastasis. Persistent inhalation of irritants like in tobacco lead to chronic NET formation, while NETs have been associated with awakening dormant cancer cells linking it to lung cancer induction. Inducers of NETosis are bacteria, viruses, but also crystals, immune complexes and chemical changes. The association of nanoparticles and NETosis induction is a very young field and has been observed for polystyrene, nanodiamonds, graphene oxide, Au and Ag nanoparticles (reviewed in) and is not induced by particles larger than 1000nm. Depending on the stimulating agent different mechanisms inducing NETosis have been discussed, which might influence the nature of the NETs. In an effort to elucidate NET associated proteins several groups applied LC-MS/MS. Comparing our data (clustered 681 DEGs, Figure S11A) to the comprehensive studies on proteins associated with NETs (Figure S11B), we further validated our clustering results. Compared to Lim and colleagues (164 NET proteins), 64 proteins were found in common, with a large proportion of these being cluster 3 proteins. Comparing to the most comprehensive NETproteome study (791 NET proteins), 136 proteins were common with a high percentage of proteins for clusters 1 and 3. Similar observations were made in two other studies, with one study distinguishing between the NET proteome of healthy donors and autoimmune patients (RA, SLE). The distribution of the overlapping proteins indicates the importance of clusters 1 and 3. Interestingly, there was an increased overlap among NETs of autoimmune patients and the cobalt responsive cluster 4. Besides neutrophils other immune cells can also form extracellular traps (ETs) as has been observed for macrophages (METs) and eosinophils (EETs), explaining the distribution of NET proteins over the clusters. In the case of cobalt dependent cluster 4 together with differential BALF cellular content this could indicate EET formation. NETs are also known to induce alternative complement pathways which goes together with the strong enrichment observed for cluster 5 proteins. Considering all NET proteome studies 213 out of 681 significant deregulated proteins were found to be in common with a similar overrepresentation of proteins of clusters 1 (49%) and 3 (63%) as already seen for single study comparisons (Figure S11C, Table S4). Comparing our data to the 4 comprehensive NET proteome studies, then 25 proteins are in common and can be regarded as core proteins among NET associated proteins (Figure S11D,
Table S5). 24 of 38 proteins of cluster 3 (63%) (Figure S7, Figure S11C) are validated NET proteins with lactotransferrin (Ltf) and lipocalin 2 (Lcn2) as iron binding proteins. Among the 14 additional potential NET associated proteins were the iron-binding proteins hemoglobin alpha and beta. Other iron-binding proteins with significant changes include ferritin (cluster 1). Among the histones, 7 out of 10 are known to be citrullinated, indicating NOX2-independent NETosis. In the referenced NET proteome studies non-physiological inducing agents were used in vitro, while our study adds knowledge from in vivo NET formation from physiological “real” agents. In the study by Chapman53 NET associated proteins were quantified for both NETosis types (PMA, A23187) side by side. Signature proteins based on relative expression intensity for each type are H3 histones, annexins and azurocidin for NOX2-dependent and LCN2, H1 histones, CRISP3, MMP8 and MMP9 among others for NOX2-independent NETosis. Based on H1 histones, which are members of cluster 3 in our study and are absent or poorly expressed in PMA-induced NETosis, we can conclude that pulmonary metal oxide nanoparticle exposure leads to predominantly NOX2-independent NETosis.

Screening phase

From the discovery phase in-depth profiling both general and specific changes in the BALF proteome to specific metal oxide nanoparticles were observed. For validation purposes as well as supporting the transfer of nanomaterial testing to a broader clinical/environmental set-up we integrated into our proteomics workflow the Evosep One LC system, which allows robust and accurate LC-MS/MS measurements with high sample throughput (60 samples per day with 21 min gradients). Different types of metal oxide nanoparticles (Fe3O4, CoFe2O4 (Fe Co 5:1), CoFe2O4 (Fe Co 3:1), CoFe2O4 (Fe Co 1:3), Co3O4) were tested at two doses and two time points (4 conditions: day1 low dose – D1LOW, day 1 high dose – D1HIGH, day 3 low dose – D3LOW, day 3 high dose – D3 - HIGH) after a single pulmonary instillation. In total, BALF proteomes of 166 mice were measured by LC-MS/MS in only 2.7 days. Robust measurements were achieved for all samples (Figure S12A) with an average of 400 quantified proteins per sample (Figure S12B). PCA (Figure 4A) clearly sets apart vehicle controls (2% serum instillation) from nanoparticle-treated animals. While the nanoparticle treatment groups are not completely separated, the color gradient of the samples suggests dose-dependency of the response to the metal oxide used with BALF samples from iron oxide nanoparticle instillation furthest away from cobalt oxide and cobalt iron oxide in between following decreasing iron cobalt composition. The inflammation control (carbon black, Printex90) clusters with cobalt oxide nanoparticles, which highlights the increased cytotoxicity of cobalt. Performing PCA on sample subsets splitting for time point and dose, iron oxide nanoparticle response is temporary with BALF
samples from the low dose and later time point cluster together with the controls (Figure S12C). This observation shows that pure iron oxide nanoparticles are, as expected, the most biocompatible of the nanoparticles tested. Differential expression analysis was performed for each treatment group and condition with comparisons made against pooled controls for each time point (FDR < 0.05) (Figure 4B, Figure S12D, Table S6) In total, 330 proteins were differentially expressed, with 159 already found during the discovery phase. Most changes were observed for the high dose at day 1 post instillation with 44 proteins commonly significant different for all iron and cobalt oxide nanoparticle treatments (Figure 4C). Among those were histones (6 isoforms), Lcn2, Ngp and S100a9, already identified as strong responders to iron oxide nanoparticles (cluster 3). Lipocalin 2 (Lcn2) regulates iron homeostasis and is released by e.g. neutrophils to bind and neutralize bacterial siderophores as part of an antimicrobial defense mechanism by preventing iron uptake by bacteria. Lipocalins can be found on the surface of mucosal surfaces such as lung but also intracellularly and in blood circulation. Lipocalin 2 has been found to be elevated in the serum of patients with COPD and asthma. The strongest expression changes were found for neutrophil extracellular trap (NET) associated proteins from the discovery cluster 3 which displayed high iron dose dependency, confirming our findings from the discovery phase. Histone coverage included 5 H1 linker histone isoforms (Figure S13A) and 4 core histone isoforms covering H2B, H3 and H4 (Figure S13B). Extracellular histones as part of NETs are bactericidal but have been shown also to be highly cytotoxic and contribute largely to tissue injury. Among the linker histones, most pronounced was Hist1h1c (H1C), able to regulate IRF3 mediated IFNβ signaling. Common proteins from other conditions (low dose day 1, high dose day 3) are shown in Figure S12E. The most unique proteins were found following exposure to pure cobalt oxide nanoparticles for all conditions (Figure S12D). Comparing all samples, 45 proteins were found to be unique to cobalt and were enriched in Nrf2-mediated antioxidant stress response and detoxification including e.g. Sod2, Sod3, Gpx1 and Txn1. As already observed by PCA, the response to pure iron oxide nanoparticles according to the BALF proteome changes is transient, especially at low dose. Enrichment analysis (Figure 4D, Figures S14) gave similar results as for the discovery in-depth profiling. Protein changes were associated with immune system, inflammation, complement and blood coagulation, response to wounding and actin cytoskeleton remodeling. In summary, it becomes apparent that cobalt oxide containing nanoparticles induce a delayed response while pure iron oxide nanoparticles are cleared most efficiently.
Clustering of significantly different proteins from the day 1 high dose condition (Figure 4E) confirmed observations made during the discovery phase. Similar clusters were found and by including nanoparticles with incremental increase of cobalt oxide composition (Fe 1:0, Fe Co 5:1, Fe Co 3:1, Fe Co 1:3, Co 0:1), the dose-dependency of the metal oxide nanoparticle type could be followed. Comparing the screening cluster assignment to the discovery clusters and visualizing the overlap between the two strategies (discovery, screening) shows that both results are in accordance (Figure 4F, left panel). By selecting proteins which were assigned to the same cluster type (black arrow, Figure 4F, left panel), we defined a protein signature of 91 from the screening data and called them NP response. Of those, selecting the best protein of each cluster, defined by the highest combined membership score, a six-protein signature was generated (Gpi, Scgb1a1, S100a9, Gsn, Cfh, Sod1). A NETosis signature consisting of 31 proteins based on overlapping proteins with positive iron oxide nanoparticle response (discovery clusters 1 and 3, screening clusters 1 and 2) was also defined (white arrow, Figure 4F, left panel). All three protein signatures allowed the discrimination of controls versus nanoparticle treated samples (Figure 4F, right panel) similarly as profiling around 400 proteins from the whole dataset (Figure 4A) with treatment dependency visible. In order to test the discriminatory power of these signature proteins (Table S7) they were applied to a comprehensive BALF dataset covering the time course of bleomycin induced reversible lung fibrosis, which can be seen after 3 to 21 days of treatment, while from 28 days onwards, the healing process was initiated and the samples cluster with the untreated samples. The 6-protein signature was not sufficient to separate meaningful the samples. Applying the NETosis signature, two clear groups emerged, separating controls with very early stage from medium time point, whereas other stages were distributed in both groups. Applying our protein signature of 91 proteins on this dataset and filtering for proteins with no missing values we further reduced our signature to 24 proteins and were able to separate the experimental groups in the same way as for the whole dataset based on 400 quantified proteins (Figure S15). With this example it was demonstrated that the proposed nanotoxicology platform produces not only meaningful results but is also a powerful tool for signature or biomarker discovery.

CONCLUSIONS

A nanotoxicology proteomics screening platform transferable to all kinds of nanomaterials is presented here. As proof of principle cobalt ferrite (CoFe₂O₄) magnetic nanoparticles were selected with iron and cobalt content at different ratios, which were intratracheal instilled in mice and bronchoalveolar lavage collected. The Evosep One LC system combined with high resolution mass
spectrometry and short gradient peptide separation ensured robust and fast measurements with high samples throughput desirable in diagnostics. The 21 min/60 samples a day protocol allowed average quantification of 400 proteins per BALF sample. The performance of the Evosep One LC system was good allowing fast and robust measurements with median CV of 19%. In line with previous studies, immune system and complement activation were dominating responses from pulmonary nanoparticle exposure. Based on differential expression analysis iron oxide nanoparticles were better tolerated with faster clearance than any of the Co doped variants. Most prominent changes in the BALF proteome with strong iron dose response could be attributed to NETosis or NET formation. NET associated proteins quantified during discovery phase by deep proteome profiling were validated by comparison to other NET proteome studies and potential NET candidates were added. While most studies on NETosis are limited to the cell culture environment using non-physiological inducing agents, knowledge to NETosis formation in vivo in response to real life stimuli was added. Combining discovery and screening data protein signatures were generated which can be applied to detect lung injury or NETosis.

METHODS

Animal experiments

Female C57BL/6J BomTac mice were obtained from Taconic Europe (Ejby, Denmark). Mice were allocated arbitrarily to experimental groups and were acclimatized for 1 week before the start of experiments. All mice were housed with up to 6 animals per cage in controlled environmental conditions; temperature (21±1 °C), humidity (50% ± 10%) and 12 hours light/dark period. Mice had access to food and water ad libitum. All mice were dosed by a single intratracheal instillation at 8-9 weeks of age and average weight of 20 g as previously described. Metal oxide nanoparticles (iron oxide doped with cobalt oxide at different ratios; Fe₂O₄ (magnetite), CoFe₂O₄ (cobalt ferrite, Fe Co 5:1), CoFe₂O₄ (Fe Co 3:1), CoFe₂O₄ (Fe Co 1:3), Co₃O₄), and reference material (carbon black, Printex 90) were instilled one material at a time at doses of 54 µg and 162 µg/mouse in 2% murine serum. Three mice were included for each material as the vehicle control group (instillation with 0 µg/mouse in 2% murine serum), which were combined for statistical analysis for each exposure time point. Six mice per dose group were included for each time point (day 1 and day 3). Mice were euthanized at 1 day and 3 days post-exposure. All procedures complied with the EC Directive 86/609/EEC and Danish law regulating experiments with animals (The Danish Ministry of Justice, Animal Experiments Inspectorate, permission 2006/561-1123).
Intratracheal Instillation

For animal instillations the nanomaterial dispersions were diluted in 2% murine serum in 0.45 µm MilliQ filtered Nanopure water and sonicated in for 16 min using a 400 W Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) mounted with a disruptor horn and operated at 10% amplitude as described. The dispersions were continuously cooled by ice/water. Dilutions were prepared directly after sonication and were further sonicated for 2 minutes after re-suspending. Size distribution was immediately measured using DLS. Nanoparticle dispersions were administered in 50 µl volume.

Nanoparticle characterization

Synthesis

Magnetic iron oxide, cobalt oxide and cobalt-doped iron oxide nanoparticles were produced at Promethean Particles Ltd (Nottingham, UK) by continuous hydrothermal synthesis starting from metal salts as precursors, with iron(III) nitrate nonahydrate (Fe(NO$_3$)$_3$ x 9H$_2$O, Sigma Aldrich) and cobalt(II)acetate tetrahydrate (Co(C$_2$H$_3$O$_2$)$_2$ x 4H$_2$O, Sigma Aldrich) for iron and cobalt oxide, respectively. For Co doping the salts were used at different ratios to produce nanoparticles with 5:1, 3:1, and 1:3 Fe Co compositions. Synthesis and characterization of the particles were described in detail. Nanoparticles were distributed as aqueous solutions.

Determination of particle elemental composition

Samples were analyzed on a Perkin Elmer Optima 8000 (Perkin Elmer, Shelton, CT, USA) by inductively coupled plasma optical emission spectrometry (ICP-OES) in radial mode to determine the relative abundance of each element in the nanoparticle solutions. Triplicates of each solution were digested in 24% ICP grade hydrochloric acid for 24 h. These samples were subsequently diluted 10000-fold in 2% nitric acid prior to ICP-OES analysis. The OES torch was aligned using 1 ppm manganese at wavelength 257.610 nm and the iron and cobalt measurements were taken at 238.204 and 228.616 nm respectively.

DLS and Zeta potential characterization

Samples were diluted 1 in 5000 using deionized water (pH 7.2, 18.2 mΩ·cm) prior to measurement at 25 °C on a Malvern Zetasizer (nanoZS) (Malvern Panalytical, Malvern, U.K). Samples were
allowed to equilibrate for 2 min prior to analysis with each sample analyzed in triplicates. These data were collected and averaged to determine the hydrodynamic diameter, polydispersity index (PDI), zeta potential and electrophoretic mobility.

**Transmission electron microscopy**

TEM analysis was performed using a JEOL 1400EX 80 kV system. TEM grids were prepared by a dropcasting method, whereby a 10 μl drop of the nanoparticle suspension was deposited on a 300 mesh carbon-coated copper TEM grid (Agar Scientific, UK). The drop was left for approximately 2 hours to allow the nanoparticles to adhere to the carbon membrane, and grids were rinsed with ultrapure water to remove excess water to avoid agglomeration. Particle diameter measurements were conducted using the Gatan Digital Micrograph software by measuring at least 100 particles for each composition.

**Bronchoalveolar lavage fluid preparation**

Lungs were flushed twice with 1 ml saline using a BAL cannula (BD InsyteTM, 20 GA, 1.1 x 48 mm, 5 ml/min) placed in the trachea. BALF recovery was 1.5 ml per animal. Cellular content was removed by centrifugation (400 x g, 10 min, 4 °C) and aliquots of supernatants were stored at -80 °C until analysis. Samples were only frozen and thawed once and were never re-frozen.

**Cellular content of Bronchoalveolar lavage fluid**

BAL cell numbers were determined as previously described.31 The total number of BAL cells was determined in a NUCLEOCOUNTER (NC-200.) to obtain total BAL cell counts. Differential cell counts were determined by differential cell counting. Forty μl cell suspension (differential count) was collected on CYTOSPIN slides (1000 rpm, 4 min). After centrifugation, the cells were stained using the May Grünwald and Giemsa. Cell differentiation was performed counting 200 cells per slide.

**Cytotoxicity testing: total protein content and LDH assay**

Total protein content in BAL fluid was measured by Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufactures protocol. Samples and standards were assayed in duplicates in 96-well plates and incubated for 30 min at 37°C. Absorbance was measured at 550 nm on a Victor2
1420 Multi label counter (Wallac, PerkinElmer). Protein concentrations of all acellular BAL fluid samples were calculated based on the standard curve of known albumin concentrations.

Cytotoxicity was determined using the LDH Cytotoxicity Detection Kit (Roche). 100 µl of BAL fluid were assayed in 96-well plates in duplicates. 100 µl of LDH reaction mixture were added to each well and plates were incubated at room temperature for 30 min, protected from light. Absorbance was measured at 492 nm with a reference wavelength of 630 nm.

**Saa3 mRNA levels**

Total RNA was isolated from the left lung and lateral lobe of liver (6-23 mg) using Maxwell® 16 LEV simply RNA Tissue Kit (Promega, USA) according to the manufacturer’s instruction. Isolated RNA was stored at -80ºC until further analysis. Saa3 mRNA levels in lungs and Saa1 in liver was assessed as makers of pulmonary and hepatic acute phase response. cDNA synthesis was prepared with Taqman Reverse Transcription Reagent Kit (Applied Biosystems) according to manufacturer’s protocol as previously described.²⁵ The relative gene expression of target genes Saa1 and Saa3 was determined by RT-qPCR on Viia™ 7 (Applied Biosystems) and calculated by comparative method $2^{-\Delta \Delta CT}$ using 18S levels for normalization. The nucleotide sequence of Saa3 primers and probe were forward: 5’ GCC TGG GCT GCT AAA GTC AT 3’, reverse: 5’ TGC TCC ATG TCC CGT GAA C 3’ and probe: 50 FAM- TCT GAA CAG CCT TGG CAT CGC T-TAMRA 3’ and Saa1 (Mm00656927_g1). Target and reference genes were all run in triplicates in 384-well reaction plates (Thermo Fisher) including RT controls and negative controls without synthesized cDNA. Day to day variation of the plate control was less than 25%.

**Genotoxicity testing: Comet assay**

The comet assay was performed as previously described.³⁵ DNA strand break levels were determined on frozen BAL cell suspensions, lung and liver tissue (3 x 3 mm piece of median lobe). Organ samples were snap frozen in NUNC cryo-tubes directly after dissection and kept at -80 ºC until analysis. Frozen BAL cells preserved in DMSO were thawed quickly at 37 ºC while frozen tissues were homogenized in Merchant’s medium by a steel mesh 0.2 mm. Cells were suspended in 0.7% agarose final concentration at 37 ºC. Cells were embedded on 20-well Trevigen CometSlides™ (30 µL per well). Cooled slides were placed in lysis buffer overnight at 4 ºC. Next day, slides were rinsed in electrophoresis buffer, and alkaline treated for 40 min. Electrophoresis was run with 5% circulation (70 ml/min) for 25 min at applied voltage 38 V (1.15 V/cm electrophoresis chamber) and measured
current 700 mA. Slides were neutralized (2 x 5 min), fixed in ethanol for 5 min and on warm plate at 45°C for 15 min. Cells were stained in 20 ml/slide bath with TE buffered SYBR®Green fluorescent stain for 15 minutes, dried at 37°C for 10 min, UV-filter and cover slip were applied and DNA damage was analyzed by IMSTAR Pathfinder™ system. The results are presented as average %TDNA value and tail length for all cells scored on each Trevigen CometSlide well. The day-to-day variation and electrophoresis efficiency was validated by including on each slide A549 epithelial lung cells exposed to PBS or 60 µM H₂O₂ used as negative and positive controls as described.³⁵

**Proteomics sample preparation**

BALF protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer’s protocol. All samples were brought to the same protein concentration (0.1 µg/µl) by dilution with saline. Protein extraction was performed using 4 M urea/thiourea. After reduction with dithiothreitol and alkylation with iodoacetamide, proteins were digested at room temperature with Lys-C for 4 h and trypsin overnight at a 1:50 enzyme to protein ratio. For in-depth BALF profiling (discovery phase) samples from three experimental groups (day 1 high dose; CTRL, Fe₃O₄, and Co₃O₄ nanoparticles) were selected. A total of 9 samples with 3 biological replicates per group were TMT-labeled (TMT10plex minus TMT10-131, Thermo Scientific) according to the manufacturer’s protocol and were run as 9plex. Combined TMT-labeled peptides with 5 µg per channel were desalted on R2/R3 resin in StageTip format. Unlabeled peptides for reproducibility evaluation and screening were loaded on Evosep tips in 0.5% formic acid in water after C18 activation with 100% acetonitrile and equilibration.

**High pH reversed phase pre-fractionation**

For in-depth BALF profiling 45 µg of 9plex TMT labeled peptides were separated into 16 fractions by high pH reversed phase pre-fractionation using an UltiMate 3000 LC system (Thermo Scientific) equipped with an Acquity UPLC CSH C18 column with 300 µm × 100 mm, 1.7 µm (Waters). Peptides were eluted over a gradient from 4% to 48% solvent B over 60 min (solvent A: 20 mM ammonium formate, adjusted to pH 9.6 by NH₄OH; solvent B: 80% acetonitrile 20% 20 mM ammonium formate, adjusted to pH 9.6 by NH₄OH) and pooled according to the UV spectrum. A reference BALF library composed of representative samples of the study (pooled mix of 40 µg) was fractionated label-free.
into 33 fractions and run together with the BALF screening samples to increase protein identification from the match between run functionality in MaxQuant analysis.

Liquid chromatography and mass spectrometry

LC-MS/MS analysis for TMT-labeled BALF samples (discovery, in-depth profiling) were carried out using an EASY-nLC 1000 system connected to a Q Exactive HF mass spectrometer (ThermoFisher Scientific). Peptides were separated using a 136 min gradient on a 15 cm column with 3 µm Reprosil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany). For LC separation the aqueous solvent A contained 0.1% (v/v) formic acid in water and the organic mobile phase 0.1% (v/v) formic acid in 95% (v/v) acetonitrile. Flowrate of the LC gradient was 250 nl/min for all steps, starting from 1% solvent B, increasing to 7% over 3 min, to 25% over 110 min, to 45% over 10 min, to 100% over 5 min and a column wash with 100% solvent B for 8 min. MS	extsuperscript{1} spectra were acquired in positive ionization mode data dependently using a top10 method. MS	extsuperscript{1} spectra were recorded at a resolution of 60000 in a scan range from m/z 300-1650, with an automated gain control (AGC) target ions of 1x10	extsuperscript{6}, maximum injection time of 25 ms in profile mode. MS	extsuperscript{2} spectra were recorded at a resolution of 60000 in a scan range of m/z 200-2000 in profile mode, with an AGC target of 5x10	extsuperscript{4}, maximum injection time of 22 ms, isolation window m/z 1.2, normalized collision energy of 34, including charge states 2-4, dynamic exclusion of 15 s and first fixed mass at m/z 110.

LC-MS/MS analysis for label-free BALF samples (reproducibility evaluation, screening including a reference library) was carried out using an Evosep One LC system connected to a Tribrid Fusion Lumos mass spectrometer (ThermoFisher Scientific). Peptides were separated using a 21 min gradient on a 7 cm column with 3 µm Reprosil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany) using the default parameters defined by Evosep One. MS full spectra were acquired in positive ionization mode data dependently using a top5 method. MS spectra were recorded at a resolution of 60000 in a scan range from m/z 300-1650, with an AGC target of 1x10	extsuperscript{6} ions and a maximum injection time of 25 ms in profile mode. MS	extsuperscript{n} spectra were recorded at a resolution of 15000 in the scan range of m/z 200-2000 in centroid mode, with an AGC target of 5x10	extsuperscript{4}, a maximum injection time of 22 ms, an isolation window of m/z 1.5 and normalized collision energy of 27.
Mass spectrometry data analysis

MS raw files were analyzed with MaxQuant software (versions 1.5.7 and 1.6.1,66,67) and generated peak lists were searched against the murine UniProt FASTA reference proteome database downloaded in May 2018 (52528 entries). The FDR was set to 1% for both protein and peptide identification. A maximum of two missed cleavages were allowed with a minimal peptide length of seven amino acids. Cysteine carbamidomethylation was set as fixed modification, methionine oxidation and N-terminal acetylation were set as variable modifications. Label-free BALF samples for reproducibility evaluation and screening were analyzed allowing for match between runs with matching time window of 0.7 min and alignment time window of 3 min. For TMT-labeled BALF samples (discovery) the TMT10plex was selected as isobaric label, excluding TMT10-131. For protein quantification a minimal ratio count of one was allowed.

Statistics and bioinformatics analysis

The animal study was carried out on several different calendar dates. Vehicle controls were included on all calendar dates. Vehicle controls were subsequently pooled for each post-exposure day (day 1 and day 3). BAL data, Saa3 mRNA levels and genotoxicity were analyzed using two-way ANOVA with individual particle and dose level set as fixed category variables. Log-transformation was used in some cases. In case of statistically significant interaction effects across the fixed variables, one-way ANOVA was performed with dose as fixed categorical variable. If statistical significance main effects were observed, pairwise comparison was performed using Dunnett’s test or Tukey’s post hoc test.

Statistics and bioinformatic analysis for proteomics were performed in the R environment. Differential expression analysis was performed with the limma package68 using Bayes moderate T test. Vehicle controls included for each nanoparticle exposure were pooled per exposure time point (day 1, day 3). Proteins with FDR < 0.05 were considered significant and were clustered with fuzzy c-means algorithm using the Mfuzz package.69 Plotting of expression patterns was based on a minimal membership value of 0.7. Pearson correlation coefficients were calculated for proteins with minimal 3 out of 16 quantitative measurements. Gene set enrichment analysis was carried out with the STRINGdb package70 for the categories gene ontology biological process, gene ontology molecular function, gene ontology cellular compartment, and KEGG pathways. Protein protein interaction networks were generated with the stringApp v1.4.071 within Cytoscape v3.7.1.72
Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016148. Submitted were MS raw files, search parameter files (mqpar.xml), MaxQuant software versions, UniProt data base FASTA file and MaxQuant search output (proteinGroups.txt) for both Discovery and Screening.

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.xxx. Supporting Information provides nanoparticle characterization, classic nanoparticle compatibility testing, protein-protein interaction networks of clustered significant proteins, GO term and KEGG enrichment analysis, histone expression and protein signature testing.
Figure legends

Figure 1 Experimental design and proteome analysis workflow. (A) Mice were treated by single intratracheal instillation with either 0 µg (vehicle control – CTRL), 54 µg (low dose – LOW) or 162 µg (high dose – HIGH) of nanoparticles per animal. Bronchoalveolar lavage fluid (BALF) was obtained by two washes with saline one day or three days post instillation. (B) Reproducibility: To evaluate reproducibility of the Evosep One LC system technical replicates (n=16) of a single BALF sample were measured as 21 min gradients. Discovery: In-depth BALF proteome profiling was performed on selected samples from day 1 post instillation with high dose nanoparticle treatment (162 µg/animal). Samples of iron oxide (Fe$_3$O$_4$) and cobalt oxide (Co$_2$O$_3$) nanoparticle treatment were compared to controls with three biological replicates per group. Screening: Within the whole study the effects of six different types of nanoparticles (Fe$_3$O$_4$, CoFe$_2$O$_4$ (Fe Co 5:1), CoFe$_2$O$_4$ (Fe Co 3:1), CoFe$_2$O$_4$ (Fe Co 1:3), Co$_3$O$_4$, and carbon black) were tested, at two doses (LOW, HIGH; except for carbon black (HIGH only)) and two time points (one day and three days post instillation). In total, BALF samples of 166 animals were measured. (C) Schematic of proteome analysis workflow. To obtain maximal proteome coverage, BALF samples of the discovery phase were pre-fractionated (high pH reverse phase, 15 fractions) and run as 120 min gradients with classic nanoLC system setup. Samples were TMT labeled and run together as 9plex. BALF samples of the whole study (n=166, Screening) as well as technical replicates (Reproducibility) were run label-free as single-shot injections on 21 min gradients using the Evosep One LC system. MS raw files were analyzed by MaxQuant. Downstream bioinformatic analyses were performed within the R environment as indicated with protein-protein interaction network visualization in Cytoscape. (D) Summary of analytical depth and measurement time for Discovery and Screening phase.

Figure 2 High reproducibility of the Evosep One LC system. (A) Reproducibility of LFQ intensities for selected proteins covering 6 orders of magnitude. (B) Matrix of Pearson Correlation coefficients for 16 technical replicates. (C) Distribution of coefficients of variation (CVs) based on proteins with n=16, n>7 and n>2 quantitative measurements within 16 technical replicates. Median CVs (annotated with black dot) were 19%, 24.1% and 25.3% corresponding to 203, 350 and 474 quantified proteins, respectively.

Figure 3 Deep BALF proteome profiling – Discovery phase. (A) Principal component analysis. (B) Venn diagram of differentially expressed proteins (FDR < 0.05) for three comparisons: Fe vs CTRL, Co vs CTRL, Fe vs Co. (C) Fuzzy c-means clustering of differentially expressed proteins (FDR <
Number of protein members per cluster are indicated. Clusters of proteins with specific response for iron oxide or cobalt oxide are color coded. Iron oxide – red. Cobalt oxide – blue. (D) Tile plots depict enrichment analysis for proteins of each cluster for selected terms of Gene Ontology Biological Processes (GOBP) and KEGG pathways (KEGG). (E) Left panel: protein-protein interaction network of cluster 3 with strong response to iron oxide. Members of this cluster are classical NETosis signature proteins (Elane, MPO, Prtn3, histones). Proteins represented as turquoise nodes were also identified in LC-MS/MS studies as NETosis associated proteins.52–57 Significant enriched terms were annotated and sorted from top (most significant) to bottom (less significant) including nucleosome (red), immune system process (blue), neutrophil aggregation (yellow), neutrophil mediated killing (green) and respiratory burst (turquoise). Histone isoforms are annotated with their associated histone family (H1, H2A, H2B, H3.2, H4). Right panel: “NETosis in SLE” pathway (highly enriched for cluster 3, FDR 10^{-17}) map generated by MetaCore analysis with overlaid protein expression of Fe vs CTRL (1) and Co vs CTRL (2) comparisons. Red expression bars indicate up-regulation of significant proteins (FDR < 0.05). Detailed pathway legend can be found on the MetaCore website: https://portal.genego.com/legends/MetaCoreQuickReferenceGuide.pdf

Figure 4 Fast screening of BALF proteome by highly reproducible Evosep LC system – Screening phase. (A) Principal component analysis of BALF samples of all animals (n=166) (B) Numbers of differentially expressed proteins (FDR < 0.05) per treatment group. All comparisons were made against pooled controls per time point. (C) Common proteins which show differential expression upon metal oxide nanoparticles treatment (high concentration) at day one post instillation. (D) Normalized enrichment for 10 selected GOBP terms for comparisons among the 4 experimental conditions (D1 LOW, D1 HIGH, D3 LOW, D3 HIGH). Maximal -log10(FDR) value per enrichment term over all conditions was set to 1. Abbreviations of enriched terms: defense (defense response), imm. sys. (immune system process), inflamm. (inflammatory response), coagul. (blood coagulation), wounding (response to wounding), cytoskeleton (actin cytoskeleton organization), endopet. act. (regulation of endopeptidase activity), iron (iron ion homeostasis), metal (response to metal ion), oxid. stress (response to oxidative stress). (E) Fuzzy c-means clustering of differentially expressed proteins (FDR < 0.05) from day 1 post instillation with 162 µg nanoparticles/animal (DAY 1 HIGH). Clusters of proteins with specific response for iron oxide or cobalt oxide are annotated (red – iron oxide, blue – cobalt oxide). (F) Overlapping significant differentially expressed proteins from discovery and screening phase are shown. Distribution of cluster assignment is depicted. Protein members of clusters with best matching pattern were
selected (black arrow), representing a signature of 91 proteins (NP response). Based on highest combined membership values one protein per cluster was selected (annotated gene name), representing a signature of 6 proteins (NP response best member). Based on iron oxide response pattern (white arrow), 31 proteins were selected, representing a NETosis signature. (G) Principal component analyses of the whole dataset filtered for the 3 protein signatures: NP response, NETosis and NP response best member with 91, 31, and 6 proteins, respectively, resemble grouping of total BALF screening.
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**Lung**

- Cobalt iron oxide NPs
- Bronchoalveolar lavage fluid (BALF)

**BALF proteomics**

| Strategy       | #samples | #quant. proteins |
|----------------|----------|------------------|
| Discovery      | 9        | 1700             |
| Screening      | 166      | 400              |

**Functional analysis**

- Neutrophil extracellular traps
Fig 2

A) LPA Intensity vs Technical Replicate

B) Heatmap showing Pearson Correlation between proteins and technical replicates

C) Coefficient of Variation per protein

Quantitative values per protein
Fig 3

A

B
differentially expressed proteins: 681

Fe-CTRL

Co-CTRL

431

107

195

60

66

220

66

25

Fe-Co

C

Cluster 1 128

Cluster 2 69

Cluster 3 39

Cluster 4 148

Cluster 5 101

Cluster 6 196

D

viral process
response to wounding
oxidation-reduction process
nucleosome assembly
immune system process
defense response
actin cytoskeleton organization

KEGG

Systemic lupus erythematosus
Ribosome
Phagosome
Pertussis
Metabolic pathways
Lysosome
Focal adhesion
Complement and coagulation
Bacterial invasion of epithelial cells

E

Cluster 3

selected significant enriched terms
nucleosome
immune system process
neutrophil aggregation
neutrophil mediated killing
respiratory burst

NETosis in SLE

proteins present in NETome studies
