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Susceptibility to quantum dot induced lung inflammation differs widely among the Collaborative Cross founder mouse strains

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Abstract

Quantum dots (QDs) are engineered semiconductor nanoparticles with unique physicochemical properties that make them potentially useful in clinical, research and industrial settings. However, a growing body of evidence indicates that like other engineered nanomaterials, QDs have the potential to be respiratory hazards, especially in the context of the manufacture of QDs and products containing them, as well as exposures to consumers using these products. The overall goal of this study was to investigate the role of mouse strain in determining susceptibility to QD-induced pulmonary inflammation and toxicity. Male mice from 8 genetically diverse inbred strains (the Collaborative Cross founder strains) were exposed to CdSe–ZnS core–shell QDs stabilized with an amphiphilic polymer. QD treatment resulted in significant increases in the percentage of neutrophils and levels of cytokines present in bronchoalveolar lavage fluid (BALF) obtained from NOD/ShiLtJ and NZO/HILtJ mice relative to their saline (Sal) treated controls. Cadmium measurements in lung tissue indicated strain-dependent differences in disposition of QDs in the lung. Total glutathione levels in lung tissue were significantly correlated with percent neutrophils in BALF as well as with lung tissue Cd levels. Our findings indicate that QD-induced acute lung inflammation is mouse strain dependent, that it is heritable, and that the choice of mouse strain is an important consideration in planning QD toxicity studies. These data also suggest that formal

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genetic analyses using additional strains or recombinant inbred strains from these mice could be useful for discovering potential QD-induced inflammation susceptibility loci.

Keywords

Quantum dots; Lung inflammation; Collaborative Cross mouse strains; Heritability; Genetics

1. Introduction

The market for nanotechnology-enabled products continues to accelerate as the number of novel applications for engineered nanomaterials (ENMs) increases. This has raised concerns regarding environmental and occupational exposures to ENMs. Recent epidemiological studies of workers in ENM manufacturing facilities suggest that occupational ENM exposure may be associated with increased risk of respiratory and cardiovascular toxicity (Cui, 2013; Liao et al., 2013; J.S. Lee et al., 2015). Thus, better understanding of ENM toxicity is important for human health and the continued success of the nanotechnology industry.

Quantum dots (QDs) are a class of ENM commonly composed of a cadmium/selenium (CdSe), cadmium/tellurium (CdTe), or cadmium/selenium/tellurium (CdSeTe) semiconductor crystalline core surrounded by a multilayer shell of higher band gap material such as zinc/sulfide (ZnS) (Dabbousi et al., 1997; Kim et al., 2004; Medintz et al., 2005; Azzazy et al., 2007; Ho et al., 2013). The superior optical properties such as brightness and photostability of QDs compared to traditional organic dyes make them useful imaging tools. Bioconjugated QDs have been used as excellent direct and indirect immunofluorescent probes for intra- and extracellular targets (Alivisatos, 2004; Michalet et al., 2005; Zrazhevskiy et al., 2010; Probst et al., 2013); to label and deliver drugs to tumors (Savla et al., 2011); and to map sentinel lymph nodes in vivo (Gao et al., 2004; Kim et al., 2004). QDs have also been utilized as biosensors and in the electronics and energy industries (Hu et al., 2010; Jung et al., 2012; Bae et al., 2013).

The diverse uses of QDs highlight the need to fully understand their toxicity. QDs are known to be cytotoxic in a cell-type, dose, particle size, and surface coating-dependent manner, and release of Cd²⁺ ions plays a role in their cytotoxicity (Kirchner et al., 2005; Lovric et al., 2005; Clift et al., 2008; Hoshino et al., 2011; McConnachie et al., 2012; Smith et al., 2012). Previous studies have confirmed that Cd²⁺ ions can generate reactive oxygen species (ROS), that QDs can release Cd²⁺ ions, and that QDs have been observed to elicit ROS production and to impact glutathione (GSH) levels (Derfus et al., 2004; Wang et al., 2004; Kirchner et al., 2005; Lovri et al., 2005; Clift et al., 2010). Thus, QD-induced cytotoxicity is at least partially due to antioxidant depletion and oxidative stress. This is supported by studies in which GSH precursors and inducers have reduced QD cytotoxicity (Lovri et al., 2005; Neibert and Maysinger, 2011).

To our knowledge, no published epidemiological studies exist regarding environmental or occupational exposures to QDs. However, several in vivo animal studies have found little toxicity following intravenous administration QDs (Hauck et al., 2010; Su et al., 2011; Ye et

al., 2012), which is important if QDs are to be used clinically. However, thrombi harboring QDs have been found in the lungs of mice injected with high doses of CdSe QDs, although thrombi formation was preventable with heparin pretreatment (Geys et al., 2008). In terms of dermal exposures, QDs can be taken by human keratinocyte cells and showed little toxicity up to 20 nM in concentration (Zhang et al., 2011). QDs have also been shown to penetrate into the outer layers of stratum corneum and epidermis, but not all the way through, porcine skin (Zhang et al., 2008). In contrast, pulmonary exposure to QDs (a potential route of exposure in occupational settings) has been shown to result in neutrophilic and granulomatous inflammation, compromised alveolar-capillary barrier function, DNA damage, and cytotoxicity (Oberdorster et al., 2005; Jacobsen et al., 2009; Ma-Hock et al., 2012, 2013; Ho et al., 2013; Roberts et al., 2013). Furthermore, as observed for in vitro studies, surface coatings (i.e. —COOH vs. —NH₂ vs. polyethylene glycol) are known to influence the toxicity and disposition of QDs in mouse and rat lungs (Ho et al., 2013; Roberts et al., 2013). We chose to use trioctylphosphine oxide/poly (maleic anhydride-alt-1-tetradecene) (TOPO-PMAT) coated QDs in this study because of the reproducibility, stability, and solubility that is achieved through incorporating the hydrophobic surface ligand TOPO during synthesis and subsequently adding the amphiphilic polymer PMAT. TOPO-PMAT coating of CdSe/ZnS QDs also results in the retention of excellent optical properties (McConnachie et al., 2012).

Mice are often used as models for mammalian in vivo toxicity testing. Inbred strains of mice have been extensively used to investigate the role of genetic background in determining susceptibility to a number of agents. Moreover, through formal genetic analyses, differential responses of inbred mouse strains can increase our understanding of the mechanisms underlying toxicity by revealing genes that influence the response to toxic exposures (Festing et al., 2001; Harrill et al., 2009; Bradford et al., 2011).

The overarching goal of this study was to establish the range of sensitivity to QD-induced lung inflammation and toxicity among the 8 founder strains of the Collaborative Cross (CC), which is an international project to develop new recombinant inbred (RI) mouse strains having high inter-strain genetic variability (Churchill et al., 2004). The CC founder strains have the following designations: 129S1/SvImJ, A/J, C57BL/6J, NOD/LtJ and NZO/HILtJ (which are traditional laboratory strains), and CAST/EiJ, PWK/PhJ and WSB/EiJ (which are strains recently derived from wild mice). Including 8 strains of mice in our experimental design allows for a robust examination of inter-strain variability, which is important since most in vivo QD toxicity studies have been limited to a single mouse strain and conclusions reached in one strain may not hold true for others. Indeed, we found that mouse strain has a major effect on QD-induced lung inflammation, suggesting that in future studies formal genetic analyses using additional strains or recombinant inbred strains from these mice could be useful for discovering loci that are associated with susceptibility to QD-induced inflammation.

2. Materials and methods

2.1. Ethics statement

The University of Washington Institutional Animal Care and Use Committee (IACUC) approved all animal procedures carried out in this study (UW Protocol Number 2384-08), and no adverse behavioral effects or apparent suffering were observed.

2.2. Animals

Specific pathogen free (SPF) male mice from the following strains were purchased from Jackson Laboratories (Bar Harbor, ME): 129S1/SvImJ (n = 28; 12 QD treated, 12 saline, 4 naïve), C57BL/6J (n = 49; 28 QD treated, 17 saline, 4 naïve), A/J (n=37; 22 QD treated, 11 saline, 4 naïve), CAST/EiJ, NOD/LtJ, NZO/HILtJ, PWK/PhJ and WSB/EiJ (n=16; 6 QD, 6 saline, 4 naïve). Hereafter, mouse strain names are abbreviated as indicated in the parentheses following each strain: 129S1/SvImJ (129), C57BL/6J (B6), A/J (AJ), CAST/EiJ (CAST), NOD/LtJ (NOD), NZO/HILtJ (NZO), PWK/PhJ (PWK) and WSB/EiJ (WSB). Mice were received at approximately 6 weeks of age and all experiments were done within 4 weeks of arrival at the University of Washington. Baseline measures were made in naïve mice. Animals were housed under modified SPF conditions in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility at the University of Washington.

2.3. Quantum dots

The amphiphilic polymer coated QDs used in this study were synthesized and characterized in the laboratory of Dr. Xiaohu Gao in the Department of Bioengineering at the University of Washington as previously described (Pellegrino et al., 2004; Bagalkot and Gao, 2011; McConnachie et al., 2012, 2013). They consisted of a CdSe core, a ZnS shell, and an outer coating of tri-n-octylphosphine oxide, poly(maleic anhydride-alt-1-tetradecene) (TOPO-PMAT).

2.4. Oropharyngeal aspiration of QDs

Animals were anesthetized prior to treatment using 4% Isoflurane. Mice received a dose of 6 µg Cd equivalents/kg body weight (BW) of 10 nM TOPO-PMAT CdSe/ZnS QDs in 0.9% sterile saline, which is comparable to a hypothetical 40 hour work week cumulative exposure of 4.6 mg/kg calculated using the current Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL) of 5 µg/m³ and average human breathing rates from the EPA Exposure Factors Handbook for ages 16–71 (1.62 m³/h), and 70 kg as a worker body weight. Oropharyngeal aspiration (OPA) was used to deliver either QDs or 1.53 µl/g BW 0.9% sterile saline as a vehicle control. As noted above, 4 naïve control animals from each strain received no treatment. Mice were euthanized using CO₂ narcosis and cervical dislocation 8 h after dosing. This time point was selected in order to detect both cytokines and neutrophils in the same BAL sample. Prior in vitro studies with alveolar macrophages had shown that although 4 h after exposure to QDs was optimal for detection of cytokines, there was still a detectable level of cytokines at 8 h (V. Lee et al., 2015). Moreover, sufficient neutrophil influx was detected in mice 8 h after QD exposure

via nasal instillation (McConnachie et al., 2013). Tissues were removed and flash frozen in liquid nitrogen and bronchoalveolar lavage was performed as described below. Additional AJ and B6 mice were euthanized immediately, 30 min, 1 h and 3 h following OPA of the same dose of QDs (n = 4 per time point) or saline (n = 2 at 3 h, and 1 at the other time points) for the purpose of analyzing cadmium levels in lung tissue.

2.5. Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed 3 times with 3 lung inflations using 1 ml phosphate buffered saline (PBS) per lavage, as previously described (Weldy et al., 2011). CAST, WSB and PWK mice received 700 μ l PBS per lavage due to the reduced lung volume in these smaller strains.

2.6. Flow cytometry

BAL samples were centrifuged at ~500 g to pellet cells. The supernatant from the 1st lavage was stored at -80 °C for later analyses and is referred to hereafter as bronchoalveolar lavage fluid (BALF). Supernatants from the 2nd and 3rd lavages were discarded. The cells from all 3 lavages were then pooled and processed for immunophenotyping and flow cytometry to identify the relative number of macrophages and neutrophils present in BALF using a slightly modified method of that performed previously (Weldy et al., 2011). Cells were stained for CD11b, Ly-6G/Ly-6C (Gr1) and F4/80 with a phycoerythrin conjugated monoclonal antibody against Cd11b (AbCam, Cambridge, MA), biotinylated anti-mouse Gr1 conjugated to streptavidin/AlexaFluor350 (BioLegend, San Diego, CA) and anti-F4/80 conjugated to Alexafluor 488 (eBioscience, San Diego, CA). Gating of flow cytometric bivariate plots was used to determine the percentage of neutrophils and macrophages in BALF. Neutrophils were classified as cells high in Cd11b and low in F4/80 fluorescence. Macrophages were identified as being high in F4/80 fluorescence and either high or low in Gr1 fluorescence. F4/80⁺/Gr1^{hi} cells have been described in a previous study as inflammatory monocytes (Mosa et al., 2009).

2.7. BALF cytokine/inflammatory protein measurements using ELISA microarray

Slides were printed using a piezoelectric GeSiM NanoPlotter 2 and processed as described previously (Servoss et al., 2009). All ELISA reagents for the 18 cytokine assays (amphiregulin (AMR), eotaxin, granulocyte-colony stimulating factor (GCSF), interleukins 1 α , 6, and 12 (IL-1 α , 6, and 12), GRO1 oncogene (KC; CXCL1), matrix metalloproteinase 2 (MMP2), small inducible cytokine A5 (RANTES; CCL5), thymus and activation-regulated chemokine (TARC; CCL17), tumor necrosis factor (TNF α), vascular endothelial growth factor A (VEGF), monocyte chemotactic protein 1 (MCP1), macrophage-derived cytokine (MDC), macrophage inflammatory proteins 1 α (MIP-1 α , CCL3), 1 β (MIP-1 β , CCL4), 1 γ (MIP-1 γ , CCL9) and 2 (MIP-2, CXCL2)) were purchased from R&D Systems. In brief, for every chip, each of the 18 capture antibodies for the individual ELISAs were printed onto 4 spots. Samples were thawed on ice, centrifuged to remove any particulates, and diluted with 0.1 volume of 10 $\times$ assay buffer (final assay concentration: 0.1 mg casein/ml and 1000 pg green fluorescent protein/ml) in PBS. Samples were then processed in triplicate on the ELISA chips as described (Servoss et al., 2009). After overnight incubation with the

samples, chips were washed and incubated with a mixture of biotinylated detection antibodies. The biotinylation signal was amplified using the biotinyltyramide amplification system and the fluorescent signal added using tagged streptavidin (Woodbury et al., 2002). The fluorescent intensity of the spots was imaged using a Tecan Reloaded laser scanner and quantitated using ScanArray Express software. Standard data were generated using serial dilutions of expressed antigens. The fluorescent data were normalized based on results from an internal calibrant assay (Zangar et al., 2009). Standard curves were then fit with the Protein Microarray Analysis Tool (ProMAT) using the four-parameter logistic curve-fitting option (White et al., 2006) and used by ProMAT to generate antigen concentration values for the samples based on the adjusted spot fluorescent intensities.

2.8. BALF total protein

Total protein in BALF samples was measured using the Bio-Rad Protein Assay (BioRad, Hercules, CA) according to the manufacturer's instructions and absorbance was read at 590 nm using a SpectraMax 190 spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA). All samples and standards were analyzed in triplicate.

2.9. Cadmium analysis

Samples of frozen right lung tissue (~20–40 mg) from mice sacrificed 8 h after OPA from each strain were randomly selected for analysis (6 QD treated and 3 saline treated). In addition, samples were also taken from an additional 4 QD treated AJ and B6 mice immediately, 30 min, 1 h and 3 h after OPA. For saline treated controls in both strains, samples were taken from 1 mouse immediately, 30 min and 1 h after OPA, and from 2 mice 3 h after OPA. Following nitric acid/microwave digestion, Cd levels were measured using inductively-coupled plasma mass spectrometry (ICP-MS) following a modified EPA Method 6020A with an Agilent 7500ce ICP-MS (US-EPA, 2007). The cadmium content in QD dosing solution was confirmed by the same method. The limit of quantification (LOQ) was 0.1 ng Cd per sample. The amount of Cd per tissue was normalized by tissue weight. Samples that had Cd levels below the LOQ were assigned the value of the LOQ/square root of 2 before being divided by lung tissue weight. The kinetics of Cd clearance from the lungs was assessed using an exponential decay model.

2.10. Lung total glutathione

Total glutathione levels in lung homogenates were measured after reducing all disulfide forms using previously described procedures (Weldy et al., 2011).

2.11. Statistical analyses

All data were processed and analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA), GraphPad Prism (GraphPad Software, La Jolla, CA), and R (R Core Team, 2014). Data from all endpoints were log transformed to improve normality prior to analysis. Two-way analysis of variance (ANOVA) was performed to examine QD treatment and mouse strain effects. If a significant treatment effect was detected, post-hoc t-tests were performed between treatment groups and resulting p-values were adjusted using the False Discovery Rate (FDR) method in the p.adjust function in R. Post hoc t-tests were performed

separately between QD and saline groups and between saline and naïve groups. In a secondary analysis, we performed t-tests on absolute BALF cell numbers between QD and saline treated mice in each strain. The strain order in all data plots, which were created using the ggplot2 package in R, is determined by the ratio of the percent neutrophils in BALF in QD treated mice compared to strain matched saline controls in ascending order (Fig. S1) (Wickham, 2009). Colors used for mouse strains in the plots are those designated by the CC organizers for the founder strains. In order to reveal potential relationships among outcome measures across strains, a hierarchical clustering analysis was performed. Prior to clustering, nonlog-transformed data were converted into z-scores within each assay, essentially normalizing each assay to a mean of 0 and a standard deviation of 1. Individual animal z-scores in each assay were averaged for each strain and treatment group. Clustering was performed using the heatmap.2 function in the gplots package in R, with defaults of Euclidean distance and complete linkage as methods for dendrogram formation (Warnes et al., 2013). This is a modified method from those used in previous nanomaterial studies where multiple groups and assays are compared (Shaw et al., 2008; McConnachie et al., 2012). Simple linear regression was used to further examine potential relationships seen in the cluster analysis and to analyze relationships between lung glutathione and BALF neutrophils, cytokines, and lung Cd. Broad-sense heritability was calculated using one-way ANOVA for select phenotypes to estimate the genetic contribution to the overall phenotypic variability. Inter-class correlation, r_1 , was calculated as $(MSB - MSW) / (MSB + (n - 1)MSW)$, where MSB is the mean square between strains and MSW is the mean square within strains obtained from ANOVA analysis, and n is the number of mice. The coefficient of genetic determination, g^2 was calculated as $(MSB - MSW) / (MSB + (2n - 1)MSW)$ (Billat et al., 2005; Rutledge et al., 2014). The sensitivity analysis performed on percent neutrophils in BALF entailed removing potential outliers using Tukey's interquartile range (IQR) method which defines outliers as either less than Q1 minus 1.5 times, or Q3 plus 1.5 times the IQR within each strain and treatment group, and then repeating the two-way ANOVA and t-tests (Tukey, 1977).

3. Results

3.1. Cd content in lung tissue

Cd was measured as a marker of QD presence in the lung 8 h after exposure. Cd content was highly variable across the strains and a significant interaction between mouse strain and QD treatment was observed (Fig. 1A) indicating strain dependent differences in disposition. B6, PWK, and WSB mice all had average levels of <0.1 ng Cd/mg tissue, with B6 being the lowest (Fig. 1A). AJ mice had an average of 0.16 ng Cd/mg tissue. The 129, CAST, NOD, and NZO mice had average Cd levels between 2.5 and 3 ng Cd/mg tissue (Fig. 1A). In order to further investigate the notable variability present at 8 h post-OPA, we also measured Cd levels in AJ and B6 mice immediately, 30 min, 1 h and 3 h after OPA (Fig. 1B). Interestingly, QDs appeared to have a longer half-life in AJ mice (4.5 h, 95% CI: 2.8 to 10.9 h) compared to B6 mice (1.2 h, 95% CI: 1.0 to 1.6 h). In keeping with their slower clearance, QD treated AJ mice had significantly higher lung Cd levels at 8 h than B6 mice. While there is a suggestion that AJ mice might have higher levels than B6 even immediately following QD exposure, this potential difference was not statistically significant.

3.2. Neutrophils in BALF

We found high variability across strains for BALF neutrophils and observed a significant interaction between mouse strain and QD treatment. In particular, NOD and NZO mice showed a significant increase in the percentage and absolute numbers of neutrophils in BALF following QD treatment compared to strain-matched saline-treated controls (Fig. 2A and Table S1). Notably, with the exception of one mouse, all treatment groups of B6 had very few neutrophils. The presence of neutrophils in BALF from the remaining strains (129, AJ, CAST, PWK and WSB) was too variable to detect any statistically significant treatment related differences.

3.3. Macrophages in BALF

We found significant elevations in percent Gr1⁺ macrophages in QD treated animals compared to saline controls in NZO and B6 mice (Fig. 2B). Interestingly, 129, AJ, NOD, and PWK mice showed comparable levels of Gr1⁺ macrophages in QD and saline treated mice, yet in these strains saline treated mice were different from their respective naïve controls (Figs. 2B and S4). No differences were observed in absolute numbers of Gr1⁺, total macrophages, or total BALF cells between QD and saline treated mice in any strain (Table S1).

3.4. Cytokines in BALF

Of the 18 cytokines and inflammation-associated proteins assayed with the ELISA microarray, the concentration of 6 (IL-10, IL-1 α , IL-12, IL-6, MIP-1 β , and MIP-2) was below the limit of detection. Of the remaining 12 cytokines analyzed all were affected by strain; Eotaxin, MDC, and RANTES were also affected by QD treatment, and KC, MIP-1 α , MIP-1 γ , and G-CSF, were affected both by strain and QD treatment and had significant elevations in QD vs. saline treated mice in at least one strain (Figs. 3 and S3). KC was elevated in QD treated AJ and WSB mice relative to their respective saline controls (Fig. 3A). MIP-1 α was elevated in BALF from QD treated NOD mice relative to saline controls (Fig. 3B). In B6 and NZO mice, the levels of MIP-1 α were similar in QD and saline treated mice but saline treated mice were different from their respective naïve controls (Figs. 3B and S4). Levels of MIP-1 γ were significantly higher in QD-treated AJ and 129 mice compared to their respective saline controls (Fig. 3C). In CAST, NOD, and NZO mice, levels of MIP-1 γ were similar in QD and saline treated mice but saline treated mice were different from their respective naïve controls (Figs. 3C and S4). BALF G-CSF levels were elevated in QD treated AJ mice compared to saline controls (Fig. 3D).

3.5. Total protein in BALF

We measured the total protein present in BALF supernatants as a marker of compromised capillary/alveolar barrier function. While we found both strain and treatment were significant factors contributing to absolute levels of protein, we were unable to detect within strain differences in post-hoc analyses between QD-treated and saline-treated controls (Fig. 4A). In addition, in 129 mice, the levels of total protein in BALF were similar in QD and saline treated mice but saline treated mice were different from their respective naïve controls (Figs. 4A and S4).

3.6. Total glutathione in lung tissue

To investigate whether total lung glutathione levels were affected by QD exposure, total glutathione (consisting of GSH + GSSG) was measured in lung homogenates (Fig. 4B). While QD treatment and mouse strain significantly influenced glutathione levels we were unable to detect within strain differences in post-hoc analyses between QD-treated and saline-treated controls (Fig. 4B). In addition, in CAST, NZO, PWK and WSB levels of total glutathione were similar in QD and saline treated mice but saline treated mice were different from their respective naïve controls (Figs. 4B and S4). Modest but significant inverse associations were observed between the levels of lung tissue glutathione and BALF neutrophils, cytokines, and lung Cd (Fig. S2).

3.7. Clustering analysis

In order to objectively examine potential relationships among outcomes measures across all assay results among the different mouse strains and treatment groups, a cluster analysis of z-scores for each assay was performed (Fig. 5). Interestingly, at the highest level of clustering, the algorithm separated all strains according to treatment. In addition, there is a cluster of high z-scores for almost all endpoints in QD treated NZO and AJ mice. Within this cluster, a block of particularly high scores can be seen for percent neutrophils in BALF, MIP-1 α , KC, G-CSF, MIP-1 γ , MDC, and Gr1+ macrophages. QD treated WSB and NOD mice had similar z-scores for many of the same outcomes as AJ and NZO mice but were not adjacently clustered. Overall, the heat map echoed the results from each assay analyzed separately, such that mouse strains showing significant QD treatment in a specific endpoint tended to have the highest z-scores and be clustered with that assay in the heat map.

3.8. Correlation of neutrophils in BALF with Cd levels in lung tissue and BALF cytokines

To examine the relationship between lung Cd levels and the percentage of neutrophils in BALF, linear regression analysis was performed on the log-transformed values for neutrophils in BALF, versus the log-transformed lung tissue Cd levels. Relationships between lung Cd levels, BALF neutrophils, MIP-1 α , KC, and G-CSF were also investigated in the same manner since MIP-1 α , KC, and G-CSF were the cytokines most closely clustered with BALF neutrophils and that were affected by QD treatment in at least one mouse strain. Significant positive associations were found between Cd levels and MIP-1 α (Fig. 6A), KC (Fig. 6B), G-CSF (Fig. 6C), and between Cd levels and BALF neutrophils (Fig. 6D). BALF neutrophils were also positively associated with the levels of MIP-1 α (Fig. 7A), KC (Fig. 7B), and G-CSF (Fig. 7C).

3.9. Heritability estimates

Overall heritability for selected phenotypes was calculated separately for naïve, saline treated, and QD treated mice to estimate how much of the total variation was genetically determined (Table 1). Among naïve mice, the most heritable phenotypes were the levels of neutrophils in BALF, the levels of lung tissue glutathione, and the levels of BALF MIP-1 γ . In saline and QD treated mice, BALF MIP-1 γ was the trait with the highest heritability. Negative heritability estimates seen in saline treated mice indicate more phenotypic variability within rather than between strains. As seen in Fig. S4, some animals (potentially

outliers) were highly reactive to saline compared to other mice in the same strain, which can increase intra-strain variability and decrease heritability estimates. The heritability of BALF neutrophil response and glutathione levels was lower for saline and QD treated mice relative to that observed for naïve mice. However, heritability of these phenotypes was greater for QD-treated mice than saline treated mice.

4. Discussion

QDs have the potential to benefit society through many industrial and biomedical applications. However, their small size and heavy metal composition raises concerns about their safety. In this study we aimed to establish the range of sensitivity to QD-induced lung inflammation and toxicity among the 8 founder strains of the Collaborative Cross. We observed that exposure to TOPO PMAT CdSe/ZnS QDs via oropharyngeal aspiration induced an acute pulmonary inflammatory response in some of the mouse strains as defined by neutrophil influx and pro-inflammatory cytokine levels in BALF. NOD and NZO mouse strains exhibited significant increases in BALF neutrophils after QD exposure compared to saline treated controls. In addition, NOD and AJ mice exhibited significant increases in multiple pro-inflammatory cytokines relative to saline treated mice. Across all strains, we observed significant positive correlations between lung tissue Cd and BALF neutrophils in QD treated animals. In a secondary analysis, we reported absolute numbers of neutrophils, Gr1⁺ and total macrophages, and total BALF cells and found that QD-treated NOD and NZO mice had significantly higher levels of neutrophils compared to strain matched saline controls (Table S1).

To examine the robustness of the results for percent neutrophils in BALF, our primary indicator of inflammation, we performed a sensitivity analysis. We found two differences between the primary and sensitivity analyses in terms of statistical significance. First, the interaction between strain and treatment in the two-way ANOVA became marginally significant ($p = 0.068$) in the sensitivity analysis. The second difference we observed was that in the sensitivity analysis, QD-treated AJ mice showed significant increases in BALF neutrophils compared to their strain-matched saline control mice.

In the clustering analysis, data for the different outcome measures were normalized using z-score conversions in order to examine potential relationships across strains and treatments. Overall, we found that AJ mice clustered with NZO mice and had z-scores similar to those of NOD mice for neutrophils and many of the inflammatory cytokines. These observations complement the results from the sensitivity analysis suggesting that AJ mice are likely as susceptible to QDs as these other strains.

In addition, MIP-1 α and KC were the two cytokines clustered nearest to neutrophils in AJ and NZO QD treated mice, indicating that these two strains had some of the highest standardized values for neutrophil influx as well as these neutrophil chemotactic proteins. MIP-1 α (CCL3) is a C-C motif chemokine known to recruit neutrophils through receptors CCR1 and CCR5 (Reichel et al., 2012). Up-regulation of MIP-1 α has been observed after bleomycin or single wall carbon nanotube exposures in mice (Chou et al., 2008; Saito et al., 2008). In the case of bleomycin, which induces oxidative stress and fibrosis, IL-6 was

shown to mediate levels of MIP-1 α (Saito et al., 2008). KC is a C–X–C motif chemokine that binds CXCR2 and is the mouse homolog of human IL-8. Increases in lung KC levels have been observed in previous nanomaterial studies in rodents (Rossi et al., 2010; Ma-Hock et al., 2012; Ho et al., 2013). In a recent study of work place exposure to calcium carbonate nanoparticles, increases in the level of IL-8 were associated with increased exposure to these particles, indicating that ENM exposed workers may have increased lung neutrophil influx and inflammation (Cui, 2013). Other ENM human exposure studies in the workplace have shown decrease in pulmonary function, and increased signs of oxidative stress in the lung including increased malondialdehyde and 4-hydroxy hexenal in exhaled breath (J.S. Lee et al., 2015). Increased levels of vascular cell adhesion protein 1 (VCAM), a proposed biomarker for cardiovascular disease, were also observed in one of the studies (Vasan, 2006; Liao et al., 2013).

In our studies, NOD mice had KC and MIP-1 α z-score values similar to those in AJ mice, and NZO mice were not clustered together with these strains due to their responses in the other assays. Even though WSB mice did not exhibit statistically significant increases in BALF neutrophils, they had levels of KC similar to those of NOD mice, possibly indicating that they are also susceptible to QD-induced lung inflammation. Interestingly, across all strains, MIP-1 α and KC levels were the 1st and 2nd most strongly correlated with BALF neutrophils and lung Cd (Fig. 6). However, the strength and significance of the relationship appears to be different among the individual strains. While the exact explanation for these differences in response to these cytokines is obviously complicated, it is likely related to their genetics. For example, when we examined the correlation between KC cytokine levels and percent neutrophils within the individual strains, 5 strains (129, B6, AJ, NOD and NZO) showed a significant correlation (Table S2). The same 5 strains also showed significant correlations between percent neutrophils and G-CSF which regulates the development and release of bone marrow derived neutrophils, and potentially influences chemotaxis (Table S2) (Noursadeghi et al., 2005; Hamilton and Achuthan, 2013). Seven strains (all strains except PWK) showed a significant correlation between MIP-1 α and percent neutrophils in BALF (Table S2). Thus, it may be that certain strains respond more vigorously to one cytokine as the primary driver of neutrophilia (e.g. MIP-1 α but not KC for WSB mice), whereas other strains respond to all 3 factors (129, B6, AJ, NOD and NZO). Still others (PWK mice) seem not to respond to KC, MIP-1 α , or G-CSF. Furthermore, there are likely other cytokines, alarmins or other chemotactic factors that we have not measured that also drive the neutrophil response in a strain dependent manner. In addition, the significant increases in BALF of KC, MIP-1 α , and G-CSF and their correlation with BALF neutrophils and lung Cd suggests the potential involvement of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway in the observed QD-induced inflammation since the transcription of all three of these genes can be induced by NF- κ B (Grove and Plumb, 1993; Ohmori et al., 1995; Abraham, 2003). In addition, AJ, 129 and NZO mice had the highest z-scores for MIP-1 γ which has been associated with dendritic cell recruitment (Zhao et al., 2003; Rose et al., 2010).

Overall, the results of our study are in agreement with other studies investigating QD-induced pulmonary toxicity and injury in that all studies including our own have shown neutrophilic inflammation and pro-inflammatory cytokine induction (Jacobsen et al., 2009;

Ma-Hock et al., 2012, 2013; Ho et al., 2013; Roberts et al., 2013). Increased total protein levels in BALF are a valuable measure of alveolar/capillary barrier integrity (Matute-Bello et al., 2011). In contrast to other studies that found increased total BALF protein, with QD treatment, we did not observe increased total BALF protein in QD treated mice compared to genetically matched controls (Jacobsen et al., 2009; Roberts et al., 2013). We interpreted these findings as evidence that the alveolar/capillary barrier was not severely compromised after QD exposure. Moreover, preliminary examinations of representative lung tissue sections prepared from all eight strains found no consistent treatment-related histopathological changes (not shown). To our knowledge, our study is the first to evaluate QD-induced inflammatory responses in the lungs of different mouse strains.

We evaluated the levels of glutathione, which is an important thiol antioxidant and is known to form complexes with Cd and other metals, as a marker of oxidative stress and to determine its potential role in the observed inflammatory response (Ballatori, 1994). Depletion of GSH has been used previously as a marker of oxidative stress associated with QD exposure in an in vitro study where no GSSG was detected (Clift et al., 2010). In our study we observed that total glutathione levels were affected significantly by both strain and treatment and to varying degrees were correlated with levels of Cd, BALF neutrophils and cytokines (Fig. S2). However, we were unable to detect within strain differences in glutathione between QD and saline treated mice. Also, while not statistically significant, B6 mice, the strain most resistant to neutrophil influx, appear to have increased total glutathione in QD treated animals relative to saline controls, which may indicate that they are synthesizing more glutathione de novo after QD treatment. In an in vitro study investigating the role of glutathione in cellular response to QD exposure, uncapped CdTe QDs increased the levels of total and oxidized (GSSG) glutathione in rat pheochromocytoma (PC12) cells when measured 24 h after exposure (Neibert and Maysinger, 2011). In our previous work we exposed both cells and mice to the same relatively stable TOPO PMAT QDs used in this current study. We found them to be not very cytotoxic. However, they can increase transcription of the glutathione synthesis gene *Gclm* both in vitro and in vivo (McConnachie et al., 2012, 2013). We interpret our findings and those of other studies investigating QD exposure, glutathione, oxidative stress, and inflammation as evidence that pulmonary exposure to the QDs used in this study may result in mild oxidative stress and that GSH may potentially influence the inflammatory response. However this may depend on the mouse strain being used, as well as the dose, coating and stability of the QDs (Derfus et al., 2004; Wang et al., 2004; Kirchner et al., 2005; Lovri et al., 2005; Clift et al., 2010; Su et al., 2010).

Measuring tissue levels of Cd as a marker for QD exposure is a common method in in vivo QD toxicity studies. When we assessed Cd levels 8 h after OPA, we uncovered a surprising amount of variability across the different strains. Some strains had remarkably higher levels of Cd (129, AJ, CAST, NOD, NZO) than others (B6, PWK, and WSB). To further investigate the observed variability, we also measured lung tissue Cd levels in AJ and B6 mice immediately following OPA, and at 30 min, 1 h, and 3 h after dosing. Interestingly, the half life of QDs in the lung appeared to be longer in AJ than B6 mice and at 8 h after QD exposure, AJ mice had significantly higher lung Cd levels than B6 mice. While not

statistically significant, there is a suggestion that QD treated AJ mice might have higher levels of Cd than B6 mice immediately following OPA. This suggested immediate difference could be due to inherent strain differences in alveolar size and airway branching architecture (Soutiere et al., 2004; Metzger et al., 2008). Differences in lung physiology could also potentially explain part of the difference in QD clearance between B6 and AJ mice. A previous study using intratracheal administration of QDs in rats found that almost 100% of administered Cd remained in the lung at 1 and 3 days compared with the lung-associated lymph nodes (LALN), and kidneys (Roberts et al., 2013). However, starting at 7 days and continuing through the last measurement at 28 days, lung levels decreased and LALN and kidney levels increased. It should be noted that the kidney had ~3 times as much Cd as the LALN at all detectable points (Roberts et al., 2013). A similar pattern was observed when QDs were constantly inhaled such that over time Cd levels decreased in the lung, but increased in the liver and kidney between days 5 and 26 of exposure (Ma-Hock et al., 2012). Again, the lung held the majority of the Cd compared to other measured tissues (Ma-Hock et al., 2012). Even though half life in the lung was not calculated in these studies, it would have certainly been longer than those calculated in our study, which could possibly be explained by differences in species, dosing methods (OPA vs. intra-tracheal instillation and inhalation), and in QD coatings (—COOH and —NH₂ coated vs. TOPO-PMAT). In addition, we previously measured lung Cd in mice following intra-nasal administration of QDs and showed that the kinetics of QD clearance up to 24 h after nasal-instillation from the lungs were dependent on glutathione synthesis capacity (McConnachie et al., 2013). In this study, lung Cd was mildly but significantly associated with lung total glutathione levels. Results from our study and others suggest that Cd distribution following pulmonary exposure is likely species and strain dependent and that lung glutathione is a modulating factor in mice.

Interestingly, we also observed that in some outcome measures, saline and QD treated mice responded similarly but were different than naïve mice of the same strain, suggesting that strains exhibiting this pattern across multiple outcome measures may be highly reactive regardless of the type of treatment, and thus may not be particularly useful for isolating QD specific effects. Nonetheless, our positive estimates of heritability for a number of outcome measures (MIP-1 α , MIP-1 γ , KC, neutrophils and GSH) infer a genetic component for the QD induced inflammatory response.

In conclusion, TOPO-PMAT coated QDs induce mouse strain-dependent acute lung inflammation when delivered via oropharyngeal aspiration. Specifically, NOD and NZO mice showed significant increases in BALF neutrophils. NOD mice also showed significant increases in some pro-inflammatory cytokines in QD-treated mice over saline controls. Both NOD and NZO mice should be considered sensitive strains. AJ mice could also possibly be considered a sensitive strain given significant increases relative to saline controls in all pro-inflammatory cytokines where strain differences were detectable and the fact that when a potential outlier was removed, they showed highly significant increases in BALF neutrophils between these groups as well. In contrast, QD treatment did not elicit inflammation in B6 mice and we consider this strain to be the most resistant of the CC founder strains. Other strains showed high variability in BALF neutrophils and it is difficult to draw conclusions regarding their susceptibility. In addition, our data provide evidence

that MIP-1 α and to a lesser degree KC and G-CSF appear to be important components of the QD-induced neutrophil response in the lung. The significant associations observed in our data between Cd, glutathione, and BALF neutrophils suggest that TOPO-PMAT QDs induce mild oxidative stress. Collectively, the data from this study confirm that the ability of QDs to cause pulmonary inflammation depends on which mouse strain is examined and suggests that formal genetic analyses using additional strains or recombinant inbred strains derived from these mice could be useful for discovering potential QD-induced inflammation susceptibility loci.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Total Cd in frozen lung tissue by strain at 8 h post OPA (A) and at earlier time points in AJ and B6 mice (B). Cd levels at 8 h are shown using box plots to indicate the median, 25th and 75th percentiles of the data, and potential outliers (A). Individual animal data for each treatment group and strain is plotted on top of the boxplots. At earlier time points, the mean Cd values and standard errors are shown for both treatment groups of B6 and AJ mice.

Fig. 2.

Flow cytometric detection of (A) neutrophils and (B) Gr1⁺ macrophages as percentages of total cells in BALF. Levels of neutrophils and Gr1⁺ macrophages are shown using box plots to indicate the median, 25th and 75th percentiles of the data, and potential outliers.

Individual animal data for each treatment group and strain is plotted on top of the boxplots.

*=significant difference between treatment groups within a strain using an FDR of 10%.

Fig. 3.

Cytokines in bronchoalveolar lavage fluid. Results are shown for those cytokines where at least one strain showed a significant elevation for QD treated vs. saline treated mice. Data are shown using box plots to indicate the median, 25th and 75th percentiles of the data, and potential outliers. Individual animal data for each treatment group and strain is plotted on top of the boxplots. * = significant difference between treatment groups within a strain using an FDR of 10%.

Fig. 4.

Levels of (A) total protein in BALF and (B) total glutathione levels in lung tissue. For both measures, data are shown using box plots to indicate the median, 25th and 75th percentiles of the data, and potential outliers. Individual animal data for each treatment group and strain is plotted on top of the boxplots. *=significant difference between treatment groups within a strain using an FDR of 10%.

Fig. 5. Unsupervised hierarchical clustering analysis. Results from all treatment groups from all assays were converted to z-scores across each assay. Bright red indicates a high positive z-score and bright green indicates a high negative z-score. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Regression analyses of MIP-1 α , KC, BALF percent neutrophils compared to lung tissue Cd. All analyses only contain animals where measurements exist for both parameters so only the QD treated animals are included since saline treated animals have virtually no detectable Cd and are therefore not informative. Size and shape of the symbols are used to distinguish among the different mouse strains.

Fig. 7.

Regression analyses of MIP-1 α and KC compared to BALF neutrophils. All analyses only contain animals where measurements exist for both parameters and in the cases where Cd is one of the parameters. Size, shape and fill are used to distinguish among the different mouse strains and treatment groups.

Table 1

Broad sense heritability of qd response phenotypes for CC founder strains.

Phenotype	Naïve		Saline		QD	
	r^1	g^2	r^1	g^2	r^1	g^2
BALF neutrophils	0.62	0.49	-0.02 ^a	-0.01 ^a	0.15	0.09
BALF protein	0.44	0.31	0.28	0.18	0.33	0.22
Lung GSH	0.56	0.42	0.27	0.25	0.41	0.28
BALF G-CSF	0.30	0.20	-0.15 ^a	-0.08 ^a	0.39	0.27
BALF KC	0.38	0.26	0.24	0.15	0.64	0.50
BALF MIP-1 α	0.29	0.19	0.29	0.19	0.60	0.46
BALF MIP-1 γ	0.51	0.38	0.59	0.45	0.77	0.66
BALF Gr1 ⁺ Macs	0.05	0.03	0.27	0.17	0.41	0.28

^a Negative heritability estimate seen in saline treated mice indicate more phenotypic variability within rather than between strains. As seen in Fig. S4, some animals (potentially outliers) were highly reactive to saline compared to other mice in the same strain, which can increase intra-strain variability and decrease heritability estimates.