Macrophage Solubilization and Cytotoxicity of Indium-Containing Particles *In Vitro*

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Indium-containing particles (ICPs) are used extensively in the microelectronics industry. Pulmonary toxicity is observed after inhalation exposure to ICPs; however, the mechanism(s) of pathogenesis is unclear. ICPs are insoluble at physiological pH and are initially engulfed by alveolar macrophages (and likely airway epithelial cells). We hypothesized that uptake of ICPs by macrophages followed by phagolysosomal acidification results in the solubilization of ICPs into cytotoxic indium ions. To address this, we characterized the *in vitro* cytotoxicity of indium phosphide (InP) or indium tin oxide (ITO) particles with macrophages (RAW cells) and lung-derived epithelial (LA-4) cells at 24h using metabolic (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) and membrane integrity (lactate dehydrogenase) assays. InP and ITO were readily phagocytosed by RAW and LA-4 cells; however, the particles were much more cytotoxic to RAW cells and cytotoxicity was dose dependent. Treatment of RAW cells with cytochalasin D (CytoD) blocked particle phagocytosis and reduced cytotoxicity. Treatment of RAW cells with bafilomycin A1, a specific inhibitor of phagolysosomal acidification, also reduced cytotoxicity but did not block particle uptake. Based on direct indium measurements, the concentration of ionic indium was increased in culture medium from RAW but not LA-4 cells following 24-h treatment with particles. Ionic indium derived from RAW cells was significantly reduced by treatment with CytoD. These data implicate macrophage uptake and solubilization of InP and ITO via phagolysosomal acidification as requisite for particle-induced cytotoxicity and the release of indium ions. This may apply to other ICPs and strongly supports the notion that ICPs require solubilization in order to be toxic.

Key Words: indium; InP; ITO; solubilization; macrophage cytotoxicity.

phosphide (InP) and indium tin oxide (ITO). InP is used in the manufacture of semiconductors, injector lasers, solar cells, photodiodes, and light-emitting diodes due to its unique properties of superior band gap qualities, high electron mobility, and high breakdown voltage (Kirby *et al.*, 2009). ITO serves as a thin film surface coating in the manufacture of flat panel displays (e.g., liquid crystal displays) and mobile phone displays due to its characteristics of high electrical conductivity, transparency, and mechanical resistance (Guo *et al.*, 2011). In 2007, ITO accounted for 90% of the total indium demand in Japan (Tanaka *et al.*, 2010). ITO typically consists of approximately 90% indium oxide (In₂O₃) and 10% tin oxide (SnO₂) by weight and can be sintered or nonsintered.

Elevated production and use of ICPs in microelectronics have increased concerns over the safety of industry workers exposed to particulate aerosols when cutting, grinding, and polishing materials containing these compounds. Occupational inhalation exposure to ICPs, mostly ITO, has been associated with increased levels of indium detected in the blood, serum, and urine of workers and in the development of interstitial lung disease (Chonan *et al.*, 2007; Hamaguchi *et al.*, 2008; Homma *et al.*, 2005; Liu *et al.*, 2012; Miyaki *et al.*, 2003; Nakano *et al.*, 2009) as well as pulmonary alveolar proteinosis (Cummings *et al.*, 2010; Xiao *et al.*, 2010). There have been two fatal cases from ITO exposure (Cummings *et al.*, 2010; Homma *et al.*, 2003). In an effort to protect workers from inhalation exposure to ICPs, the American Conference of Governmental Industrial Hygienists (ACGIH, 2007) has recommended a time-weighted average threshold limit value of 0.1 mg/m³ based on pulmonary edema for all indium compounds. A critical issue to be addressed is whether the pulmonary toxicity of indium-containing compounds can be predicted based on the total amount of indium present in the compound and/or the solubility of the compound. Indium ions released from the parent compound by solubilization may well represent the toxic constituents of ICPs.

The worldwide demand for indium metal has increased dramatically in recent years primarily due to the growth of the microelectronics industry. Indium is often used in industrial applications as a binary compound in the form of indium-containing particles (ICPs) with examples being indium

In animal studies, pulmonary toxicity and carcinogenicity were observed after chronic inhalation exposure to InP and ITO particles in rodents (Gottschling *et al.*, 2001; Nagano *et al.*, 2011; NTP, 2001). Acute pulmonary toxicity was observed after oropharyngeal aspiration of InP and ITO particles in rodents (Kirby *et al.*, 2009; Lison *et al.*, 2009) or after intratracheal instillation in hamsters (Tanaka *et al.*, 2002, 2010). InP and ITO are insoluble at physiological pH (Hamaguchi *et al.*, 2008) and are initially engulfed by alveolar macrophages (and likely airway epithelial cells) following pulmonary deposition of particles. However, the mechanism(s) responsible for pulmonary toxicity is unclear. We reported earlier that in mice, the number of viable alveolar macrophages present in bronchoalveolar lavage (BAL) fluid decreased following aspiration of InP particles, whereas lactate dehydrogenase (LDH) activity increased, suggesting an increase in macrophage cell death (Kirby *et al.*, 2009). In the chronic inhalation carcinogenicity study with InP (NTP, 2001), indium was readily detected in blood and serum from particle-treated animals. Because ICPs are soluble at low pH, we hypothesized that the uptake of ICPs by macrophages followed by phagolysosomal acidification results in the solubilization of ICPs into cytotoxic indium ions, which can then interact with critical biomolecules.

Using InP and ITO particles as representative examples of ICPs, the objective of this study was to determine if InP and ITO were cytotoxic to macrophages or lung-derived epithelial cells *in vitro* and, if so, whether particle-induced cytotoxicity could be blocked by treatment with (1) cytochalasin D (CytoD), an inhibitor of phagocytosis (Paredes-Sabja and Sarker, 2012; Saxena *et al.*, 2008) and (2) bafilomycin A1 (BafA1), an inhibitor of vacuolar ATPase required for the lysosome-dependent acidification of phagosomes (Frankenberg *et al.*, 2008; Haggie and Verkman, 2009; Lahat *et al.*, 2008; Lukacs *et al.*, 1990; Xia *et al.*, 2008). In addition to determining particle-induced cytotoxicity, particle solubilization into ionic indium was also measured. We proposed that macrophage death caused by the solubilization of ICPs would result in the release of ionic indium extracellularly.

MATERIALS AND METHODS

Particles and reagents. InP particles were obtained from Johnson-Matthey (Ward Hill, MA) and had a hydrodynamic particle diameter of $\sim 1.5 \mu\text{m}$ based on dynamic light scattering (DLS) measurements. This was the same InP used in the NTP (2001) chronic study. Nonsintered ITO particles were obtained from Indium Corporation (Clinton, NY) and sonicated for 90 min in cell culture medium using a Branson 2210 ultrasonic bath (Branson Ultrasonics, Danbury, CT) to reduce particle agglomeration prior to use (final hydrodynamic particle diameter of $\sim 0.5 \mu\text{m}$ based on DLS measurements). Based on calculations using the particle diameters and densities (InP: 4.8 g/cm^3 ; ITO: 7.2 g/cm^3) as well as the total surface area of a culture well in a 24-well plate (1.9 cm^2), we estimated particle numbers per unit surface area (data not shown). A $200 \mu\text{g/ml}$ solution of InP or ITO contains approximately 1.3×10^7 or 2.2×10^8 particles/ cm^2 , respectively. Tin oxide (TO) particles with a particle diameter of $< 1 \mu\text{m}$ were obtained from Materion Corporation (formerly Cerac, Inc.; Mayfield Heights, OH) and sonicated for 90 min prior to use. CytoD (in dimethyl sulfoxide [DMSO]) was

purchased from Sigma-Aldrich (St Louis, MO). BafA1 was purchased from Sigma-Aldrich or LC Labs (Woburn, MA) and dissolved in 95% ethanol.

Cell lines. The mouse adherent macrophage cell line, RAW 264.7 (RAW), and the mouse adherent lung-derived epithelial cell line, LA-4, were obtained from American Type Culture Collection (ATCC, Manassas, VA). RAW cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, cat no. 11965, Life Sciences, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) + penicillin/streptomycin at 37°C (in 5% CO_2). LA-4 cells were cultured in Ham's F12K medium (ATCC, cat no. 30-2004) supplemented with 15% FBS + penicillin/streptomycin at 37°C (in 5% CO_2).

Cell culture. RAW cells were cultured for 24 h in 24-well plates (2×10^5 cells per well) and then washed once to remove nonadherent cells. Cells were treated in triplicate with InP or ITO particles (25, 50, 100, 200, or $400 \mu\text{g/ml}$ in 1 ml volume) in medium (+ 3% FBS) for 2 h to allow particle uptake. In some experiments, cells were similarly treated with TO particles (50, 100, 200, or $400 \mu\text{g/ml}$). Control cells were treated with medium without particles. After 2 h of treatment, cells were washed once and then cultured in medium (+ 3% FBS) for 24 h in order to measure particle-induced cytotoxicity. In some experiments, CytoD was used to specifically block phagocytic uptake by RAW cells as has been previously described (Paredes-Sabja and Sarker, 2012). RAW cells were pretreated with CytoD ($5 \mu\text{g/ml}$) or medium (+ 3% FBS) alone for 30 min followed by treatment for 2 h with medium alone, CytoD alone, InP ($200 \mu\text{g/ml}$), ITO ($400 \mu\text{g/ml}$), InP + CytoD, or ITO + CytoD. After 2 h of treatment, the extracellular particles were removed and the cells were cultured in the presence of CytoD or medium (+ 3% FBS) alone for 24 h. In separate experiments, BafA1 was used to specifically inhibit phagolysosomal acidification in RAW cells as has been previously described (Frankenberg *et al.*, 2008). RAW cells were pretreated with BafA1 (25 nM) or medium (+ 3% FBS) alone for 30 min followed by treatment for 2 h with medium alone, BafA1 alone, InP ($200 \mu\text{g/ml}$), ITO ($300 \mu\text{g/ml}$), InP + BafA1, or ITO + BafA1. After 2 h of treatment, cells were washed once to remove the extracellular particles and the BafA1 (because longer incubation times with BafA1 [$\sim 18 \text{ h}$] can inhibit particle uptake; Xu *et al.*, 2003) and then cultured in medium (+ 3% FBS) for 24 h.

LA-4 cells, which are mouse adenoma (and alveolar type II epithelial cell)-derived, were cultured for 24 h in 24-well plates (1×10^5 cells per well) and then washed once to remove nonadherent cells. Cells were treated in triplicate with InP or ITO particles (25, 50, 100, 200, or $400 \mu\text{g/ml}$ in 1 ml volume) in medium + 15% FBS for 2 h to allow particle uptake. Control cells were treated with medium without particles. After 2 h of treatment, cells were washed once and then cultured in medium for 24 h in order to measure particle-induced cytotoxicity. In some experiments, CytoD was used to block phagocytic uptake by LA-4 cells as has been previously described (Saxena *et al.*, 2008). LA-4 cells were pretreated with CytoD ($2.5 \mu\text{g/ml}$) for 30 min followed by treatment for 2–4 h with InP ($200 \mu\text{g/ml}$) or ITO ($400 \mu\text{g/ml}$) $\pm$ CytoD. Due to their amorphous morphology, LA-4 cells were trypsinized with 0.25% trypsin-EDTA (Gibco) for 3–5 min to promote cell rounding, which allows for better visualization of particle uptake by light microscopy.

Cytotoxicity assays. Cell culture medium was collected after 24 h of treatment, transferred to 1.5-ml eppendorf tubes, and briefly centrifuged. LDH enzymatic activity was measured in the supernatants following centrifugation as a marker of cell death using a microplate adaptation of a commercially available liquid LDH Reagent kit (cat no. L7572; Pointe Scientific, Canton, MI). The rate of reduction of NAD was measured as an increase in absorbance at 340 nm and was directly related to LDH activity. Because LDH is a cytosolic enzyme, detection of extracellular LDH activity is used as a biomarker of cellular injury (membrane damage/cell permeability). Cell viability was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. MTT reagent (Invitrogen, Eugene, OR) was diluted 1:10 in culture medium and then added at 0.5 ml per well for 2 h at 37°C (in 5% CO_2). Next, the MTT reagent was removed and DMSO was added at 0.5 ml per well for 10 min at room temperature. Samples were then transferred to 96-well plates and absorbance was measured at 550 nm. Viable and metabolically active cells reduce MTT to formazan dye. MTT and LDH absorbance values from

treated cells were divided by the mean corresponding value from untreated (medium only) control cells. Data are shown as fraction control (for MTT assay) or fold over control (for LDH assay).

Atomic absorption spectroscopy. RAW or LA-4 cells were cultured for 24 h in 6-well plates (5×10^5 cells per well) and then washed once to remove nonadherent cells. Cells were then treated for 24 h with medium alone, InP (200 $\mu\text{g/ml}$), or ITO (400 $\mu\text{g/ml}$) particles. Cell culture medium (2 ml) was collected after 24 h into PYREX glass centrifuge tubes (Corning, Tewksbury, MA) and centrifuged at $3000 \times g$ (room temperature) to pellet any residual cells and particles. After centrifugation, 0.5-ml aliquots of (cell and particle-free) supernatants were transferred to 1.5-ml eppendorf tubes and acid-digested overnight at 70°C in 50% perchloric acid:nitric acid (2:1 [vol/vol]). This acid digestion step serves to remove any organic material contaminants, to release any free indium that may be bound to protein(s) in the culture medium, and to concentrate the sample prior to analysis. The concentration ($\mu\text{g/l}$) of total ionic indium was measured by atomic absorption spectroscopy (AAS) using an AAnalyst 600 (PerkinElmer, Shelton, CT). LDH activity was also measured in the cell culture supernatants after centrifugation as previously described. In some experiments, RAW cells were pretreated with CytoD (5 $\mu\text{g/ml}$) or medium alone for 30 min followed by treatment for 24 h with InP (200 $\mu\text{g/ml}$) or ITO (400 $\mu\text{g/ml}$) $\pm$ CytoD. Controls for AAS experiments included supernatants from wells without cells treated with the same amount of particles in medium (background). Background indium concentration values were subtracted from the concentration values generated from particle-treated cells.

Statistics. One-way ANOVA with Tukey's test or unpaired Student's *t*-test, as appropriate, was performed using GraphPad Prism 5 (La Jolla, CA). All data are presented as mean $\pm$ SD with a *p* value $< .05$ considered statistically significant.

RESULTS

ICPs Are Cytotoxic to Macrophages

Alveolar macrophages ingest ICPs following aspiration of particles *in vivo* (Kirby *et al.*, 2009). Airway and lung tissue-resident epithelial cells are also capable of ingesting particles (Saxena *et al.*, 2008). Therefore, using an *in vitro* cell culture system, we initially characterized the phagocytic uptake of InP and ITO particles by both macrophages and lung-derived epithelial cells as well as particle-induced cytotoxicity.

RAW cells were treated with InP or ITO for 2 h to allow particle uptake. The phagocytic uptake of particles by RAW cells is apparent by light microscopy in the photomicrographs shown in Figures 1A (InP) and 2A (ITO). Treatment with CytoD blocked the uptake of both InP (Fig. 1B) and ITO (Fig. 2B) particles by

FIG. 1. InP particles are phagocytosed by macrophages and lung-derived epithelial cells. RAW cells treated with InP (200 $\mu\text{g/ml}$) (A). RAW cells cotreated with InP + CytoD (5 $\mu\text{g/ml}$) (B). LA-4 cells treated with InP (200 $\mu\text{g/ml}$) (C). LA-4 cells cotreated with InP + CytoD (2.5 $\mu\text{g/ml}$) (D). Photomicrographs ($\times 200$ magnification) in (A and B) are RAW cells after 24 h of culture and those in (C and D) are trypsinized LA-4 cells after 4 h of culture.

FIG. 2. ITO particles are phagocytosed by macrophages and lung-derived epithelial cells. RAW cells treated with ITO (400 $\mu\text{g/ml}$) (A). RAW cells cotreated with ITO + CytoD (5 $\mu\text{g/ml}$) (B). LA-4 cells treated with ITO (400 $\mu\text{g/ml}$) (C). LA-4 cells cotreated with ITO + CytoD (2.5 $\mu\text{g/ml}$) (D). Photomicrographs ($\times 200$ magnification) in (A and B) are RAW cells after 24 h of culture and those in (C and D) are trypsinized LA-4 cells after 4 h of culture.

the RAW cells. Following treatment for 2 h with InP or ITO, particle-induced cytotoxicity was measured after 24 h of culture. Cell viability (MTT values) decreased with increasing concentrations of InP (Fig. 3A) and was lowest at InP concentrations of 200 $\mu\text{g/ml}$ (0.43 ± 0.05 ; fraction control) and 400 $\mu\text{g/ml}$ (0.39 ± 0.04). Cell viability also decreased with increasing concentrations of ITO (Fig. 3C) and was lowest at the ITO concentration of 400 $\mu\text{g/ml}$ (0.49 ± 0.05). LDH levels in medium from InP-treated cell cultures increased with increasing concentrations of InP (Fig. 3B) and peaked at InP concentrations of 100 $\mu\text{g/ml}$ (6.71 ± 1.72 ; fold over control) and 200 $\mu\text{g/ml}$ (6.88 ± 1.68). LDH levels in medium from ITO-treated cell cultures also increased with increasing concentrations of ITO (Fig. 3D) and peaked at the ITO concentration of 400 $\mu\text{g/ml}$ (7.43 ± 0.49). After 24 h of culture, particle-loaded RAW cells were observed by light microscopy to be a mixed population of both adherent and floating cells, which still appeared to be structurally intact (data not shown). To confirm that the InP- and ITO-induced cytotoxicity observed was not due to particle overload in macrophages at higher doses, RAW cells were

treated with TO particles using the same methods and similar exposure levels (50–400 $\mu\text{g/ml}$). Despite being phagocytosed, TO particles were not cytotoxic to the RAW cells even at the highest concentration tested (data not shown) suggesting that the cytotoxicity of InP and ITO was not a nonspecific effect due to particle overload.

LA-4 epithelial cells were treated with InP or ITO using similar methods. The phagocytic uptake of particles by LA-4 cells is apparent by light microscopy in the photomicrographs shown in Figures 1C (InP) and 2C (ITO) following trypsinization to promote cell rounding (see Materials and Methods section). Treatment with CytoD blocked the uptake of both InP (Fig. 1D) and ITO (Fig. 2D) particles by the LA-4 cells. Following treatment for 2 h with InP or ITO, particle-induced cytotoxicity was measured after 24 h of culture. InP and ITO particles, despite being phagocytosed by the LA-4 cells (Figs. 1C and 2C, respectively), were much less cytotoxic even at the highest particle concentration of 400 $\mu\text{g/ml}$ (0.82 ± 0.04 for InP, Fig. 3A and 0.85 ± 0.04 for ITO, Fig. 3C) in contrast to the cytotoxicity observed with the RAW cells. Likewise, LDH

FIG. 3. Macrophages are more susceptible than lung-derived epithelial cells to the cytotoxic effects of ICPs. RAW or LA-4 cells were treated with InP or ITO particles (25, 50, 100, 200, or 400 μg/ml) or medium alone for 2 h, washed once to remove extracellular particles, and then cultured for 24 h in medium prior to measuring cell death by MTT (A and C) and LDH (B and D) assays. Raw absorbance values from treated cells were divided by the mean corresponding value from untreated (medium alone) control cells and expressed as fraction control (MTT) or fold over control (LDH). Data on graphs represent mean ± SD (for two to three combined experiments). RAW and LA-4 values at each dose were compared by unpaired Student's *t*-test (***p* < .0001, ***p* < .001, **p* < .01, ^*p* < .05). Abbreviation: ns = not significant.

levels in supernatants from particle-treated LA-4 cell cultures were unaffected by InP and ITO even at the highest particle concentration of 400 μg/ml (1.04 ± 0.06 for InP, Fig. 3B and 1.03 ± 0.12 for ITO, Fig. 3D). Particle-induced cytotoxicity was significantly increased ($p < .0001$) for the RAW cells compared with the LA-4 cells in both assays for InP at concentrations of 50, 100, 200, and 400 μg/ml and for ITO at concentrations of 200 and 400 μg/ml. For both InP and ITO, particle load may have stressed the LA-4 cells, which slightly decreased metabolic activity (reduced MTT values) but did not kill the cells (based on low LDH release). These data indicate that macrophages, compared with lung-derived epithelial cells, are more susceptible to the cytotoxic effects of phagocytosed InP and ITO particles.

Cytotoxicity of ICPs for Macrophages Is Dependent Upon the Phagocytic Uptake of Particles and Phagolysosomal Acidification

In order to determine if the phagocytic uptake and solubilization of InP and ITO particles within macrophages was required for particle-induced cytotoxicity, RAW cells were treated either with (1) CytoD to inhibit particle phagocytosis or (2) BafA1 to inhibit phagolysosomal acidification prior to measuring particle-induced cytotoxicity.

Treatment of RAW cells with CytoD, which blocked the phagocytic uptake of InP and ITO particles (as previously shown in Figs. 1B and 2B), also protected the cells from particle-induced cytotoxicity (Fig. 4). The viability of cells treated with InP + CytoD (0.74 ± 0.06) or ITO + CytoD

FIG. 4. Cytotoxicity of ICPs for macrophages is dependent upon the phagocytic uptake of particles. RAW cells were pretreated with CytoD (5 μ g/ml) or medium alone for 30 min followed by treatment for 2 h with medium alone, CytoD alone, InP (200 μ g/ml), ITO (400 μ g/ml), InP + CytoD, or ITO + CytoD. Cells were then cultured in the presence of CytoD or medium alone for 24 h prior to measuring cell death by MTT (A and C) and LDH (B and D) assays. Raw absorbance values from treated cells were divided by the mean corresponding value from untreated (medium alone) control cells and expressed as fraction control (MTT) or fold over control (LDH). Data on graphs represent mean $\pm$ SD (for one representative experiment); $p < .0001$ is for the InP/ITO treatment group compared with the InP/ITO + CytoD and CytoD groups (one-way ANOVA with Tukey's test).

(0.75 ± 0.04) after 24 h of culture was significantly increased ($p < .0001$) compared with the viability of cells treated with InP (0.43 ± 0.04) or ITO (0.55 ± 0.02) alone (Figs. 4A and 4C). The release of LDH by cells treated with InP + CytoD (1.04 ± 0.02) or ITO + CytoD (0.80 ± 0.07) was significantly decreased ($p < .0001$) compared with cells treated with InP (6.43 ± 0.42) or ITO (7.46 ± 0.37) alone (Figs. 4B and 4D). Treatment with CytoD alone also exhibited some cytotoxicity to RAW cells in terms of decreased cell viability (MTT values) compared with treatment with medium alone but not LDH release; however, cell viability was still significantly greater ($p < .0001$) compared with treatment with InP or ITO particles alone. In order to block phagocytosis, CytoD inhibits actin polymerization-dependent cytoskeletal changes; therefore, the reduced MTT values observed for macrophages treated with CytoD alone may reflect some degree of impaired

metabolism/cell proliferation as opposed to CytoD-mediated cell killing.

Treatment of RAW cells with BafA1 protected the cells from particle-induced cytotoxicity (Fig. 5). The viability of cells treated with InP + BafA1 (0.85 ± 0.002) or ITO + BafA1 (0.80 ± 0.05) after 24 h of culture was significantly increased ($p < .0001$) compared with cells treated with InP (0.50 ± 0.03) or ITO (0.41 ± 0.02) alone (Figs. 5A and 5C). The release of LDH by cells treated with InP + BafA1 (2.71 ± 0.24) or ITO + BafA1 (5.15 ± 0.37) was significantly decreased ($p < .0001$) compared with cells treated with InP (5.22 ± 0.23) or ITO (9.09 ± 0.38) alone (Figs. 5B and 5D). BafA1, unlike CytoD, did not block InP or ITO particle uptake, yet particle-induced cytotoxicity was inhibited at 24 h despite the fact that the RAW cells were loaded with particles (Supplementary fig. 2). Treatment with BafA1 alone also exhibited some cytotoxicity

FIG. 5. Cytotoxicity of ICPs for macrophages is dependent upon phagolysosomal acidification. RAW cells were pretreated with BafA1 (25nM) or medium alone for 30 min followed by treatment for 2 h with medium alone, BafA1 alone, InP (200 μ g/ml), ITO (300 μ g/ml), InP + BafA1, or ITO + BafA1. Cells were then washed once to remove the BafA1 and cultured for 24 h in medium prior to measuring cell death by MTT (A and C) and LDH (B and D) assays. Raw absorbance values from treated cells were divided by the mean corresponding value from untreated (medium alone) control cells and expressed as fraction control (MTT) or fold over control (LDH). Data on graphs represent mean $\pm$ SD (for one representative experiment); $p < .0001$ is for the InP/ITO treatment group compared with the InP/ITO + BafA1 and BafA1 groups (one-way ANOVA with Tukey's test).

to RAW 264.7 cells in terms of both cell viability (MTT values) as well as LDH release; however, overall cytotoxicity was significantly less ($p < .0001$) compared with treatment with InP or ITO alone. These data show that the phagocytic uptake of InP and ITO particles by macrophages and processing within the phagolysosomal acidification pathway is required for particle-induced cytotoxicity.

Macrophages Solubilize ICPs

AAS was used in order to determine if macrophages, which have phagocytosed ICPs, breakdown and solubilize the particles. As shown in Figure 6, the concentration of total (solubilized) ionic indium in medium from RAW cells treated with InP (264 ± 49.6 μ g/l) or ITO (312 ± 47.2 μ g/l) was greatly increased compared with LA-4 cells treated with InP (0 ± 0 μ g/l) or ITO (21.0 ± 23.3 μ g/l) after 24 h of culture (Figs. 6A and 6C).

As expected, LDH activity (i.e., cytotoxicity) was greater in medium from RAW cells treated for 24 h with InP (4.85 ± 0.28) or ITO (4.44 ± 0.17) compared with LA-4 cells treated with InP (1.08 ± 0.05) or ITO (1.38 ± 0.05) (Figs. 6B and 6D). These data indicate that the macrophages, but not the epithelial cells, rapidly (within 24 h) solubilize the InP and ITO particles into indium ions, which are then released into the surrounding medium by the dying cells. The concentration of total ionic indium in medium from RAW cells treated with InP alone (94.4 ± 16.7 μ g/l) was much higher (2.9-fold) compared with cells treated with InP + CytoD (33.0 ± 11.4 μ g/l) after 24 h of culture (Fig. 7A). Also, the concentration of total ionic indium in medium from cells treated with ITO alone (2089 ± 259 μ g/l) was much higher (3.6-fold) compared with cells treated with ITO + CytoD (582 ± 4.53 μ g/l). These data show that the phagocytic uptake of InP and ITO by macrophages is required

FIG. 6. ICPs are solubilized within macrophages but not lung-derived epithelial cells, which results in the extracellular release of ionic indium. RAW or LA-4 cells were treated for 24 h with InP (200 µg/ml) (A) or ITO (400 µg/ml) (C). The concentration (µg/l) of total ionic indium in cell culture medium was then measured by AAS. Control groups included supernatants from wells without cells treated with the same amount of particles in medium (background). Background indium concentration values were subtracted from the concentration values generated from particle-treated cells. LDH activity was also measured in the RAW and LA-4 cell culture medium after treatment with InP (B) or ITO (D). Data on graphs represent mean $\pm$ SD (for one representative experiment); $p < .0001$ is for the RAW cells + InP/ITO treatment group compared with the LA-4 cells + InP/ITO group (unpaired Student's *t*-test).

for particle solubilization and the extracellular release of indium ions.

DISCUSSION

The results of the present study strongly support the notion that following phagocytic uptake of InP and ITO by macrophages, the particles are solubilized within the cell via the phagolysosomal acidification pathway, which results in cell death and the subsequent release of indium ions extracellularly by the dying cells. This is based on data that used chemical inhibition of particle phagocytosis (CytoD) or phagolysosomal acidification (BafA1). Specifically, blockade of phagocytic

uptake of both InP and ITO particles by macrophages reduced particle-induced cytotoxicity. Likewise, inhibition of phagolysosomal acidification within macrophages reduced cytotoxicity but did not block particle uptake, a key difference. BafA1 treatment has also been previously shown to protect RAW cells from death induced by NH_2 -labeled polystyrene nanospheres by inhibiting lysosomal permeabilization (Xia *et al.*, 2008), which depends upon acidification (Cho *et al.*, 2011). Furthermore, CytoD treatment of RAW cells reduced the generation and extracellular release of indium ions presumably by blocking the cellular uptake and subsequent solubilization of InP and ITO particles within macrophages. Ideally, we would have preferred to use primary alveolar macrophages

FIG. 7. ICPs are solubilized within macrophages, which is dependent upon the phagocytic uptake of particles. RAW cells were pretreated with CytoD (5 µg/ml) or medium alone for 30 min followed by treatment for 24 h with InP (200 µg/ml) ± CytoD (A) or ITO (400 µg/ml) ± CytoD (B). The concentration (µg/l) of total ionic indium in cell culture medium was then measured by AAS. Control groups included supernatants from wells without cells treated with the same amount of particles in medium (background). Background indium concentration values were subtracted from the concentration values generated from particle-treated cells. Data on graphs represent mean ± SD (for one representative experiment); $p < .0001$ in (A) is for the InP + CytoD treatment group compared with the InP group, $p < .001$ in (B) is for the ITO + CytoD treatment group compared with the ITO group (one-way ANOVA with Tukey's test).

from BAL throughout this study; however, they were not used due to the high number of untreated mice that would need to be sacrificed in order to obtain enough naïve cells for these assays. In a small-scale experiment, InP and ITO were both found to be cytotoxic to BAL-derived mouse alveolar macrophages cultured *ex vivo* (Supplementary fig. 1), which confirms that

the cytotoxic effects of InP and ITO particles observed with a macrophage cell line were also applicable to primary cells.

InP and ITO were found to be much less cytotoxic to lung-derived epithelial cells compared with macrophages after 24 h of culture despite the uptake of particles. Airway and lung-derived epithelial cells (including alveolar type II cells) have been shown to readily ingest particles via an actin polymerization-dependent phagocytic mechanism (Saxena *et al.*, 2008) and do possess the necessary phagolysosomal machinery (Haggie and Verkman, 2009). However, it would appear that LA-4 cells are not as efficient as macrophages (which are professional phagocytes) in solubilizing the particles into ionic indium, which is likely the cytotoxic form. Indeed, our data showed that treatment of LA-4 cells with InP or ITO induced minimal particle solubilization (indium ion release) as well as cytotoxicity compared with RAW cells. In a previously published report (Lison *et al.*, 2009), ITO particles were similarly shown to be cytotoxic to rat macrophages but not epithelial cells *in vitro* after 24 h of culture. Future studies should address if prolonged treatment of LA-4 cells with ICPs results in increased solubilization and cytotoxicity. It is also possible that acute exposure of alveolar macrophages to ICPs at cytotoxic concentrations or prolonged chronic exposure at noncytotoxic levels results in the release of ionic indium, which may adversely impact the lung epithelium.

These findings with InP and ITO particles may also be applicable to other ICPs and, perhaps, metal particulate compounds in general. Future comparative studies will attempt to address if the solubilization and cytotoxicity of different ICPs of similar particle size, with regard to the quantity of ionic indium released by macrophages *in vitro*, correlates with *in vivo* pulmonary toxicity. If so, the relative solubilization of different ICPs within macrophages could be predictive of their *in vivo* toxicity, which would also suggest that the indium ions released by macrophages play a role in driving particle-induced pulmonary toxicity. The InP and ITO particles used in this study could not be compared with each other in regard to macrophage cytotoxicity and released indium ions due to differences in particle size.

In conclusion, based on *in vitro* data, our proposed model is that both alveolar macrophages and lung-derived epithelial cells have the capacity to phagocytose ICPs following pulmonary deposition; however, macrophages are much more efficient in the solubilization of the particles (via phagolysosomal acidification) into ionic indium, which is the cytotoxic entity of ICPs. Indium-induced necrosis and/or apoptosis of macrophages following exposure to ICPs results in the further release of ionic indium (along with other factors) extracellularly by the dying cells, which may then drive pulmonary toxicity downstream via, e.g., the induction of free radicals/reactive oxygen species (ROS) causing oxidative stress (Chibli *et al.*, 2011; Gottschling *et al.*, 2001; Lison *et al.*, 2009; Zurita *et al.*, 2007). InP particles can induce oxidative stress in macrophages *in vivo* (Gottschling *et al.*, 2001), which may

be the result of particle solubilization and the generation of ionic indium within macrophages. Therefore, indium-induced production of free radicals/ROS resulting in oxidative stress in macrophages as well as other airway/lung cells may play a role in lung pathogenesis following exposure to InP and ITO (Lison *et al.*, 2009). Zinc oxide (ZnO) nanowires have also been shown *in vitro* to be cytotoxic to human macrophages. Cell death, which involved hallmarks of both apoptosis and necrosis, was preceded by a lysosomal-dependent increase in intracellular ionic zinc, which was cytotoxic to the cell (Muller *et al.*, 2010). Furthermore, a recent study (Cho *et al.*, 2011) has suggested that ZnO nanoparticles are solubilized under acidic conditions within the phagolysosomal compartment of macrophages, which leads to cell death. Nanoparticle solubilization results in a local spike in ionic zinc, which damages and permeabilizes the lysosome causing the contents (along with the ionic zinc) to leak out into the cytoplasm, which kills the cell. The dying cell then releases the cytotoxic zinc ions extracellularly. Thus, based on our data, a similar mechanism of action to that of ZnO nanoparticles/wires may also apply to ICPs of various particle sizes.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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