Rosette Nanotubes Alter IgE-Mediated Degranulation in the Rat Basophilic Leukemia (RBL)-2H3 Cell Line

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ABSTRACT

In this study, the effects of rosette nanotube (RNT) exposure on immune cell viability and function were investigated in vitro using the rat basophilic leukemia (RBL)-2H3 cell line. RBL-2H3 viability was decreased in a dose- and time-dependent manner after lysine-functionalized RNT (K-RNT) exposure. In addition, K-RNTs had a significant effect on RBL-2H3 degranulation. When K-RNT exposure was concurrent with IgE sensitization, 50 and 100 mg l⁻¹ K-RNTs elicited a heightened degranulatory response compared with IgE alone. Exposure to 50 and 100 mg l⁻¹ K-RNTs also caused degranulation in RBL-2H3 cells not sensitized with IgE (0 ng ml⁻¹ IgE). Furthermore, in cells preexposed to K-RNTs for 2 h and subsequently washed, sensitized, and stimulated with IgE, a potentiated degranulatory response was observed. Using confocal laser scanning microscopy and a fluorescein isothiocyanate (FITC)-functionalized RNT construct (termed FITC¹/TBL19-RNT), we demonstrated a strong and direct affiliation between RNTs and RBL-2H3 cell membranes. We also demonstrated cellular internalization of RNTs after 2 h of exposure. Together, these data demonstrate that RNTs may affiliate with the cellular membrane of RBL-2H3 cells and can be internalized. These interactions can affect viability and alter the ability of these cells to elicit IgE-FcμR mediated degranulation.

Key words: degranulation; nanotoxicology; inflammation; self-assembly; rosette nanotubes

Biologically inspired rosette nanotubes (RNTs) are a self-assembling nanomaterial (NM) formed in solution from a guanine-cytosine (G-C) hybrid motif through a hierarchical, entropically driven process (Fenniri et al., 2001, 2002b; Morales et al., 2005). The fundamental self-assembling building block of RNTs can be synthesized to feature either 1 (Fig. 1A) or 2 G-C units (Fig. 1D-E). This leads to the formation of hexameric rosettes, which are maintained by either 18- or 36-hydrogen bonds, respectively. The resulting large and substantially hydrophilic supermacrocycles (Fig. 1B) subsequently stack upon one another to form a stable tubular nanostructure (Fig. 1C and 1F–H), which can be up to several 100 μm long.

RNTs hold potential in a variety of biomedical applications including targeted drug delivery, improved vascular implants, tissue engineering, and enhanced orthopedics (Fine et al., 2009; Song et al., 2011; Sun et al., 2012). RNTs are also metal-free, hydrophilic and can be dispersed in polar media, and thus offer advantages for these applications compared with many other engineered NMs (Fenniri et al., 2001, 2002b). Moreover, RNTs have tunable dimensions and can be engineered to express a diverse range of functional groups on their outer surface through covalent functionalization of the G-C molecule (Borzsonyi et al., 2010; Chhabra et al., 2009; Fenniri et al., 2002a; Tikhomirov et al., 2008). This allows for the manipulation of both their physical...
and biological properties (Fenniri et al., 2002a; Fine et al., 2009; Sun et al., 2012). Given the potential utility of RNTs, a thorough assessment of their potential interaction with cells, tissues, and systems is necessary.

A critical factor to consider when assessing the biocompatibility and toxicity of new materials are their interactions with various in vivo processes such as those facilitated by the immune system (Remes and Williams, 1992). Recent data has demonstrated that NMs may adversely influence immune cell responses in vivo during inadvertent exposure and following deliberate pulmonary, subcutaneous, intraperitoneal, and intravenous introduction (Chen et al., 2010; Kolosnjaj-Tabi et al., 2010;
Meng et al., 2011; Song et al., 2009; Gustafsson et al., 2011). For example, fullerenes have been demonstrated to act as potent anti-gens, promoting the production of fullerene-specific IgG antibodies, but have also been shown to be immunosuppressive, suppressing the inflammatory response of peripheral blood basophils and mast cells by inhibiting the activation of signaling intermediates required for exocytosis of immunogenic mediators (Braden et al., 2000; Chen et al., 1998; Ryan et al., 2007). In addition, even subtle changes in NM structure can alter their biological effects; for example, changing the density of functionalized polymers on NMs can switch the complement pathways that are activated upon NM exposure in human serum (Hamad et al., 2010; Sim and Wallis, 2011).

It is important to understand the response of immune cells occupying “sentinel locations” within tissues and at interfaces between the body and environment. Myeloid cells (i.e., macrophages and granulocytes) are found throughout body tissues including respiratory, intestinal, and mucosal epithelia and are one of the first activators of the inflammatory response (Passante and Frankish, 2009). Granulocytes (i.e., neutrophils and mast cells) contain granules in their cytoplasm, which when stimulated are released via a process called degranulation and include various mediators of immunity. In this study, we investigate the cellular response of rat basophilic leukemia (RBL)-2H3 cells, a granulocyte cell line used extensively in studies of allergy and inflammation, to understand the impact of K-RNT exposure. RBL-2H3 cells express an endogenous, high affinity Fcε receptor (FcεRI), which when bound by IgE in a process called sensitization and subsequently cross-linked by dinitrophenyl-human serum albumin (DNP-HSA), induces degranulation (Gifillan and Tkaczyk, 2006). Degranulation releases a variety of chemical mediators including histamine, serotonin, and β-hexosaminidase providing a sensitive endpoint to examine the effects of K-RNT exposure on immune effector functions (Huang et al., 2009).

Herein, we examined the influence of K-RNT exposure on RBL-2H3 viability and using the IgE-DNP model, assess the direct effects of RNT exposure on IgE/FcεRI-mediated degranulation to determine if they alter the activation of innate immune responses. As well, using a reporter array, we examined various cell signaling pathways related to cellular toxicity to elucidate the cellular mechanisms that mediate cell death reported here and in previous studies. Finally, confocal microscopy studies were performed to gain insight into the physical interactions between RBL-2H3 cells and RNTs. For this purpose, a RNT termed FITC/TBL19-RNT, was synthesized through a co-assembly process of TB-FITC (Fig. 1D) and twin based-butylamine (TB-TBL) (Fig. 1E) in a 1:19 molar ratio, to express the fluorescent marker fluorescein isothiocyanate (FITC), while maintaining a solubility and surface charge profile similar to K-RNTs (Fenniri et al., 2001).

**MATERIALS AND METHODS**

RNT synthesis and characterization. The synthesis of the lysine functionalized G-C motif (Fig. 1A) and self-assembly into a stock solution of K-RNTs (1 g l−1) (Fig. 1C) in nanopure water was performed and extensively characterized according to a previously reported procedure (Fenniri et al., 2001, 2002b). The synthesis of TB-TBL (Fig. 1D) has also been previously reported, while the preparation of TB-FITC (Fig. 1E) will be described in due course. For the preparation of the stock solution of co-assembled FITC/TBL19-RNTs (1:19 molar ratio, 385 mg l−1 total), a solution of TB-TBL in nanopure water was sonicated for 30 s and then transferred to a vial containing TB-FITC. The suspension was sonicated for 5 min, vortexed for 5 s, followed by heating using a heat gun (on high setting) for 1 min. This procedure was repeated twice or until no further dissolution occurred. The yellow suspension was allowed to stand at room temperature in the dark for 2 days to allow for the growth of the RNTs and sedimentation of any undissolved TB-FITC. The yellow supernatant was allowed to stand at room temperature in the fridge in the dark. Aliquots from this stock solution of FITC/TBL19-RNTs were diluted and used for the cell studies.

The hydrodynamic diameter and zeta-potentials of K-RNTs and FITC/TBL19-RNTs at concentrations of 1, 10, 50 mg l−1 and 10, 50 mg l−1, respectively, were determined using dynamic light scattering (DLS; Malvern Instrument Zetasizer Nano ZS, Westborough, Massachusetts). Hydrodynamic radii were measured using 173 backscattering mode and reported as the peak value of >99% intensity. Extensive characterization details of RNTs under a variety of physiological conditions and with various side-group functionalizations have been previously reported and the reader is directed to previous studies by Fenniri et al. (2001, 2002a) and Moralez et al. (2005) for full details. However, a summary profile with pertinent information for this study has been included as Supplementary Table S1.

Cell culture. RBL-2H3 cells were cultured at 37°C and 5% CO2 in filter sterilized (0.22 μm, Corning) Minimum Essential Media (MEM; Hyclone) containing 10% heat-inactivated fetal bovine serum (FBS) (characterized; Hyclone) supplemented with 2mM L-glutamine (Gibco), 100 units ml−1 penicillin (Gibco), and 100 μg ml−1 streptomycin (Gibco) as described previously (Cortes et al., 2014). Human embryonic kidney (HEK) 293T cells were cultured at 37°C and 5% CO2 in filter sterilized (0.22 μm, Corning) DMEM/High glucose (Hyclone) containing 10% heat-inactivated FBS (characterized; Hyclone) supplemented with 2mM L-glutamine (Gibco), 100 units ml−1 penicillin (Gibco), 100 μg ml−1 streptomycin (Gibco), 1mM sodium pyruvate (Gibco), and 1% MEM non-essential amino acid solution (Gibco).

Examination of RBL-2H3 and HEK 293T viability following exposure to K-RNTs. RBL-2H3 cells were seeded in a 96-well plate at a density of 40 000 cells per well and allowed to attach for 1 h (37°C, 5% CO2) before dosing with 1, 10, 50, 100, or 200 mg l−1 K-RNT in MEM for 2, 4, or 24 h (37°C, 5% CO2). We recognize that several dosing metrics have been identified for NMs and provide the conversions between mass per volume, particles per volume and surface area per volume in Supplementary Table S2. Control wells received MEM alone (negative control) or 40 μl of nanopure water (vehicle control). Following exposure, cells were harvested, washed twice with x1 PBS, and resuspended in 200 μl of x1 PBS/proidium iodide (PI) (100 μg ml−1) and analyzed by flow cytometry (Quanta SC, Beckman Coulter). Whole cell populations were gated using side scatter and forward scatter. An increase in PI fluorescence, indicative of cell death, was detected using the FL2 filter. The percentage of viable cells in culture was calculated from the number of cells within the whole population gate that concurrently exhibited low levels of PI fluorescence. Viability is expressed relative to negative controls, calculated as a percentage of viable RNT-exposed cells to viable unexposed cells.

The effects of RNTs on cell viability were also studied using a nonradioactive cell proliferation assay performed according to the manufacturer’s instructions (Cell Titer 96 Aqueous NonRadioactive Cell Proliferation Assay Kit, Promega, Inc., Waltham, Massachusetts).
Effects of K-RNT exposure on intracellular toxicity-related signaling pathways.

To investigate the intracellular signaling events underlying the toxicity observed at higher doses of K-RNTs, highly transfectable human embryonic kidney (HEK)-293T cells were employed and a transfected reporter array was used to identify changes in 10 broad intracellular toxicity-related signaling pathways using Cignal Finder Toxicity 10-Pathway Reporter Array (SABiosciences). This luciferase-based reporter measures changes in intracellular signaling of 10 toxicity-related signaling pathways: (1) p53/DNA damage, (2) hypoxia, (3) NFκB, (4) glucocorticoid receptor, (5) cell cycle/pRB-E2F, (6) MAPK/ERK (mitogen activated protein kinases/extracellular signal-related kinases), (7) MAPK/NK (mitogen activated protein kinases/c-Jun N-terminal kinases), (8) (protein kinase C) PKC/Ca2+ (9) TGFβ, and (10) Myc/Max. Each reporter also consists of a constitutively expressing Renilla construct allowing for transfection efficiencies to be normalized. In addition, the kit includes a negative control (noninducible reporter) and a positive control (constitutively expressing both firefly luciferase and a green fluorescent protein (GFP) construct).

Transfections were performed according to the manufacturer’s instructions using HEK-293T cells seeded in a 96-well plate. Cells were seeded at a density of 25 000 cells/well in 200 µl complete DMEM and incubated overnight (37°C and 5% CO2). Cells were then transfected with 100 ng of Cignal Reporter Constructs. For all 12 Cignal Reporters, a master mix was created consisting of 400 ng of Cignal Reporter, 20 µl of OptiMEM (Invitrogen) and 0.4 µl of Turbofect (Fermentas) per well. This solution was gently mixed and allowed to sit at room temperature for 15 min. Next, 21 µl of master mix was added to each well and the cells were incubated for 24 h (37°C and 5% CO2) at which time, the media was removed and cells were exposed to 50 or 100 mg/l K-RNT in complete DMEM for 6 h (37°C and 5% CO2). Cells were then washed and assayed for firefly and Renilla luciferase activity using Dual-Glo Luciferase Assay System (Promega).

A ratio of luminescence from the experimental reporter (firefly luminescence) to the control reporter (Renilla luminescence) was used to control for transfection efficiency. A quotient of the firefly:Renilla ratio for cells treated with K-RNTs (firefly:Renilla) to the firefly:Renilla ratio for control treatments (firefly:Renilla) was calculated and represents a change in luminescence for RNT exposure compared with control.

Examination of RBL-2H3 degranulation in the presence of K-RNTs.

RBL-2H3 degranulation was measured using the β-hexosaminidase release assay as described previously (Cortes et al., 2012). Briefly, RBL-2H3 cells were seeded into a flat bottom 96-well plate (Costar) at a density of 40 000 cells per well and allowed to attach for 2 h in complete MEM (37°C, 5% CO2). Next, cells were sensitized with 0, 12.5, 25, 50, or 100 ng/ml 1K-RNT in MEM for 2, 4, or 24 h (37°C, 5% CO2), which was the exposure time used for the luciferase reporter assay (see section 2.4 later). Following the exposures, RBL-2H3 or HEK-293T cells were washed with complete media (MEM or DMEM, respectively) and prepared kit reagents were employed and a transfected reporter array was used to investigate the intracellular signaling events.

To determine dose and time required to elicit IgE/FcεRI-mediated degranulation in RBL-2H3 cells, various doses (0, 12.5, 25, 50, 100 ng/ml) of mouse anti-DNP IgE mAb (Sigma-Aldrich) in incomplete Tyrodes buffer (25 mm 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 140 mm NaCl, 1.8 mm CaCl2, 5.6 mm D-glucose, 12 mm NaHCO3, 0.37 mm NaH2PO4, and MgCl2, pH 7.4) for 1 h at 37°C and 5% CO2. Solutions were then removed and the cells washed with 200 µl of Tyrodes buffer containing 0.1% bovine serum albumin (BSA). Cells were then stimulated to degranulate with 0.05 µg/ml DNP-HSA in Tyrodes/BSA buffer heated to 37°C. Negative controls were exposed to Tyrodes buffer alone to measure nonspecific background signal. The efficacy of the assay was verified by use of a positive control, 0.625 µm calcium ionophore A23187 (Sigma-Aldrich). Cells were placed in the incubator for 1 h at 37°C and 5% CO2 and the amount of β-hexosaminidase released by RBL-2H3 cells was then assayed by removing 25 µl of the supernatant and combining it with 100 µl of β-hexosaminidase substrate buffer (2 mm 4-methylumbelliferyl N-acetyl-b-D-glucosaminide (Sigma-Aldrich), 100 mm citrate, pH 4.5) for 30 min at 37°C and 5% CO2. The wash steps and sampling of the supernatant limited the concentration of RNTs present in the final assay components, eliminating potential interference of RNTs with this assay. The reaction was quenched by adding 150 µl of 200 mm L-glutamic acid, pH 10.7. The cleavage of the substrate 4-methylumbelliferone (MUB) with the enzyme β-hexosaminidase by RNT exposure compared with control.

Using the earlier protocol, 2 experimental approaches were devised to investigate different mechanisms by which K-RNTs may affect the degranulatory response of RBL-2H3 cells. The first experiment examined if preexposing RBL-2H3 cells to K-RNTs affected degranulation when subsequently sensitized and stimulated, thereby examining if K-RNTs exposure could affect the long-term function of immune cells to elicit the appropriate inflammatory responses. Here, cells were first exposed to 50 or 100 mg/l K-RNT solutions in complete MEM for 2 h (37°C, 5% CO2) and washed thrice with complete Tyrodes buffer prior to sensitization with IgE. Cells were then stimulated and degranulation was assessed. Control treatments consisted of a 2 h exposure to the ultra pure water vehicle.

In the second experiment, cells were co-exposed to either 50 or 100 mg/l K-RNTs and IgE to examine if the presence of K-RNTs interferes with IgE binding FcεRI during sensitization, a step required to elicit IgE/FcεRI-mediated degranulation in RBL-2H3 cells. Here, during sensitization with 0, 12.5, 25, 50, or 100 mg/ml IgE, cells were simultaneously exposed to 50 and 100 mg/l K-RNT for 1 h at 37°C and 5% CO2. Solutions were then removed and the cells were washed with 200 µl of complete Tyrodes buffer and the protocol was followed as described above. Control treatments consisted of a simultaneous vehicle (ultrapure water) and IgE exposure. 

Wisconsin). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] when bioreduced by cells can gauge cell number by recording a change in absorbance at 490 nm. RBL-2H3 cells were seeded in a 96-well plate at a density of 40 000 cells per well and allowed to attach for 1 h (37°C, 5% CO2) before dosing with 1, 10, 50, 100, or 200 mg/l K-RNT in MEM for 2, 4, or 24 h (37°C, 5% CO2). HEK-293T cells were seeded in a 96-well plate at a density of 25 000 cells per well. Cells were allowed to attach for 2 h before exposing them to 0.5, 1, 5, 10, 50, or 100 mg/l K-RNT in DMEM for 6 h (37°C, 5% CO2), which was the exposure time used for the luciferase reporter assay (see section 2.4 later). Following the exposures, RBL-2H3 or HEK-293T cells were washed with complete media (MEM or DMEM, respectively) and prepared kit reagents were added to the plate for an additional 1 h (37°C, 5% CO2). Absorbance at 490 nm was then measured using a microplate reader (WALLAC 1420, PerkinElmer, Massachusetts). Previous studies have demonstrated that RNTs can interfere with colorimetric assays, including the MTS assay despite the inclusion of several wash steps (Ong et al., 2014). The UV/Vis (ultraviolet/visible) absorption spectra of K-RNTs is provided as Supplemental Figure S1. Pairwise controls containing cells exposed to K-RNTs were used to control for absorbance caused by RNTs alone. Viability was calculated based on the following formula:

Viability (% Control) = (Abs of RNT treated cells – Abs of RNT control) / (Abs of negative control – Abs of background control)

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TABLE 1. Physicochemical Characterization of K- and FITC\textsuperscript{1}/TBL19-RNTs Showing Hydrodynamic Diameter (nm) and Zeta Potential (mV) Diluted to 1, 10, and 50 mg l\textsuperscript{-1} in Ultra Pure H\textsubscript{2}O Reported as Mean ± SD.

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration (mg l\textsuperscript{-1})</th>
<th>Hydrodynamic Diameter (nm)</th>
<th>Polydispersity</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-RNT</td>
<td>1</td>
<td>280 ± 169</td>
<td>0.41 ± 0.12</td>
<td>71 ± 3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>397 ± 136</td>
<td>0.34 ± 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>405 ± 122</td>
<td>0.31 ± 0.05</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>FITC\textsuperscript{1}/TBL19-RNT</td>
<td>10</td>
<td>312 ± 133</td>
<td>0.38 ± 0.07</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

Results

Examination of FITC\textsuperscript{1}/TBL19-RNT interaction with RBL-2H3 cells by confocal microscopy. FITC\textsuperscript{1}/TBL19-RNTs were used to investigate the cellular association of RNTs with RBL-2H3 cells. Glass coverslips (Fisher Scientific) were treated with 70% ethanol, washed with x1 PBS and subsequently UV-irradiated. RBL-2H3 cells were seeded on coverslips in a 6-well plate at a density of 1 × 10\textsuperscript{5} and allowed to grow for 48 h (37°C, 5% CO\textsubscript{2}). After incubation, cells were exposed to 10 mg l\textsuperscript{-1} FITC\textsuperscript{1}/TBL19-RNTs for 2, 4, or 6 h. Cells were washed with antibody staining buffer (phosphate buffered saline, 0.5% bovine serum albumin) antibody staining buffer (ASB). Coverslips were then placed on parafilm containing ASB with 100 ng ml\textsuperscript{-1} IgE mAb for 30 min on ice. Cells were washed with ASB and then placed on parafilm containing ASB with 50 ng ml\textsuperscript{-1} goat anti-mouse IgG R-phycoerythrin (PE)-conjugated staining antibody (Beckman Coulter) at 4°C for 30 min in the dark. Cells were subsequently washed with ASB before being placed in Fixation Buffer (BioLegend) for 20 min at room temperature in the dark. Finally, cells were washed with ASB and mounted on slides using mounting media containing 4',6-diamidino-2-phenylindole (DAPI). Slides were viewed with a Laser Scanning Confocal Microscope (Zeiss LSM 710, objective 40 × 1.3 oilplan-Apochromat) at the Cross Cancer Institute Cell Imaging Facility, Edmonton, Alberta. Images were collected with Zen (2011) software and processed with LSM Image Browser (v. 4.2.0.121, Carl Zeiss). Surface rendering and 3-dimensional (3D) reconstruction of Z-stack images were performed using Imaris software (v. 6.2.2, Bitplane).

Statistical analysis. To investigate the effect of RNT exposure on RBL-2H3 viability, 2-way Analysis of Variance (ANOVA) followed by post hoc Bonferroni test were performed. To determine the effect of RNT exposure on RBL-2H3 degranulation, 2-way ANOVA were performed to determine differences between experimental and control treatments followed by post hoc Sidak’s test. To investigate the effect of RNT exposure on HEK 293T viability, a 1-way ANOVA followed by post hoc Dunnett’s test were performed. All statistical analyses were performed using GraphPad 6.0 statistical software program. Statistical significance was set at p < 0.05; n refers to the number of independent experiments conducted on cultured cells.

Results

Sample Characterization

For this study, K-RNTs self-assembled from the lysine-functionalized G/C motif were characterized as previously described (Fenniri et al., 2001). Representative scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) images of the nanotubes are shown in Figure 1F–1H, which have an average outer diameter of ca. 3.5 nm (Fenniri et al., 2001). DLS measurements shown in Table 1 revealed that the hydrodynamic radii of K-RNTs at concentrations of 10 and 50 mg l\textsuperscript{-1} in ultra pure water were (mean ± SD, n = 4) 397 ± 136 and 405 ± 122 nm, respectively. The measured ζ-potential of K-RNTs at 10 mg l\textsuperscript{-1} was 71 ± 3 mV and this value did not change at a higher concentration of 50 mg l\textsuperscript{-1} (72 ± 2 mV). Unfortunately, it was not possible to characterize RNTs in cell culture media using currently available common techniques such as DLS because the concentrations tested in this complex matrix were below the instruments detection limit. To give the reader additional RNT characterization, Supplementary Table S1 summarizes data from several publications. In our analysis, we use gravimetric measures as an indicator of dose. Because these high aspect ratio RNT materials do not increase in either individual diameter or length with increasing dose, both surface area and molarity will increase linearly with gravimetric dose. The conversion factors for recalculation of dose as either surface area or molarity are provided in Supplemental Table S2.

Effects of RNTs on RBL-2H3 Viability

Exposure to K-RNTs resulted in a significant change in adherent RBL-2H3 viability, with both dose- and time-dependent effects observed. Flow cytometric analysis revealed no significant change in viability for 2, 4, or 24 h exposure to 1, 10, and 50 mg l\textsuperscript{-1} K-RNT (Fig. 2A). However, while exposure to 100 mg l\textsuperscript{-1} K-RNT for 2 and 4 h did not result in a significant change in viability, there was a significantly decreased viability after 24 h of exposure (mean ± S.E.M., n = 4; 84.37 ± 1.64%) compared with control (Fig. 2A). At 200 mg l\textsuperscript{-1} effects of K-RNTs appeared earlier during the exposure with viability significantly decreased to: 81.4 ± 2.6, 83.1 ± 2.7, and 74.8 ± 2.6% of control at 2, 4, and 24 h, respectively (Fig. 2A).

Results using the MTS assay to measure cell proliferation and verify viability results demonstrated that exposure to K-RNTs caused a significant decline in cell viability that was both dose- and time dependent (Fig. 2B). Similar to the flow cytometric viability analysis (Fig. 2A), there was no significant change in RBL-2H3 viability after 2, 4, or 24 h at exposure levels of 1, 10, or 50 mg l\textsuperscript{-1} K-RNT. However, 100 mg l\textsuperscript{-1} of K-RNT after 2, 4, and 24 h resulted in significant declines in viability, compared with controls. Cells exposed to 100 mg l\textsuperscript{-1} had viability significantly decreased to: 82.2 ± 1.7, 76.5 ± 6.2, and 75.4 ± 7.1% of control after 2, 4, and 24 h, respectively (Fig. 2B). Exposure to 200 mg l\textsuperscript{-1} K-RNT further reduced RBL-2H3 viability at 4 and 24 h with 67.2 ± 5.4%, and 66.3 ± 7.5% viability compared with controls (Fig. 2B).

Characterizing Changes in Intracellular Signaling Toxicity-Related Pathways Upon K-RNT Exposure

We sought to investigate the intracellular signaling events underlying the toxicity observed at higher doses of K-RNTs (Fig. 3). To accomplish this, HEK-293T cells were transfected with a series of luciferase-based reporters, each specific for monitoring the transcriptional activity of 10 intracellular signaling pathways related...
to toxicity. During assay optimization, the greatest changes in transcriptional activity were measured after 6 h exposure to the positive control, cadmium selenide nanoparticles (Supplementary Fig. S2). First, we verified that the viability of HEK-293T exposed to K-RNTs for 6 h was similar to that observed for RBL-2H3 cells. HEK-293T cells showed a decrease in viability upon exposure to K-RNTs at doses of 50 mg l⁻¹ (mean ± S.E.M., n = 5; 89 ± 2.9%) and 100 mg l⁻¹ (72 ± 2.31%) (Fig. 3A). Examination of the luciferase activity...
FIG. 3. Exposure to K-RNTs did not change the transcriptional activity of 10 toxicity-related signaling pathways in HEK 293T cells. A, 2.5 x 10^4 HEK 293T cells were exposed to 0.5, 1, 5, 10, 50, or 100 mg L^-1 K-RNT for 6 h. Metabolic activity, as an indicator of cell viability, was measured in cells after exposure using the MTS assay. Absorbance values were measured at 490 nm and compared with control to calculate percent cell viability. Values are means ± SE (n = 8). Letters indicate significantly different values (p < .05, 1-way ANOVA followed by Dunnett’s post hoc comparison). B, 2.5 x 10^4 HEK 293T cells were transfected with 1 of 10 Cignal Reporter Constructs for 24 h. Constructs represent a luciferase reporter gene linked to a specific transcriptional response element for 10 broad, intracellular toxicity-related signaling pathways. Cells were exposed to 50 mg L^-1 K-RNT in growth media for 6 h. Cells were then assayed for luciferase activity as an indicator of intracellular signaling activation. K-RNT treatment is standardized to vehicle control treatment and is expressed as fold-change in luminescence activity. Asterisk indicates significantly different values compared with control (n = 5, p < .05, 1-way ANOVA followed by Dunnett’s post hoc comparison).
after 6 h exposure to 50 mg l⁻¹ K-RNT revealed a general downregulation of multiple toxicity-related pathways with the exception of hypoxia inducible factor, which remained unchanged (Fig. 3B). Luciferase activity after 3 and 12 h RNT exposure showed a similar trend (Supplementary Fig. S2).

Degranulation of RBL-2H3 Cells in Response to RNT Exposure

In the first series of experiments, RBL-2H3 cells were simultaneously sensitized with 0, 12.5, 25, 50, or 100 ng ml⁻¹ IgE and exposed to 50 or 100 mg l⁻¹ K-RNTs. In all treatments tested, there was an increase in the degranulatory response of the cells (Fig. 4A–4C). This effect was observed irrespective of the concentration of IgE (12.5, 25, 50, or 100 ng ml⁻¹) or concentration of K-RNT (50 or 100 mg l⁻¹) tested (Fig. 4A and 4B). The presence of K-RNTs at either 50 or 100 mg l⁻¹ resulted in an increased degranulatory response, ranging from (mean ± S.E.M., n = 5) 119 ± 3.9% to 140 ± 7.2% of controls (Fig. 4C). It should be noted that when IgE was not present (0 ng ml⁻¹), exposure to either 50 or 100 mg l⁻¹ K-RNT still resulted in a significantly elevated β-hexosaminidase release (126.6 ± 3.9% and 120 ± 3.8%, respectively).

The second experiment examined if preexposure to either 50 or 100 mg l⁻¹ K-RNTs for 2 h affects the ability of RBL-2H3 cells to degranulate when subsequently washed, sensitized, and stimulated. Preexposure to 50 mg l⁻¹ of K-RNT showed a potentiated degranulatory response starting at higher doses of IgE (25 mg l⁻¹ and above), relative to controls (Fig. 5A). Cells preexposed to 100 mg l⁻¹ of K-RNT for 2 h displayed a potentiated degranulatory response at all concentrations of IgE tested (mean ± S.E.M., n = 5) 12.5 ng ml⁻¹: 123 ± 4.3%; 25 ng ml⁻¹: 129 ± 3.6%; 50 ng ml⁻¹: 126 ± 1.6%; and 100 ng ml⁻¹: 123 ± 1.5%) (Fig. 5B). Of note, cells that were preexposed to 50 and 100 mg l⁻¹ K-RNT but were not sensitized with IgE (0 ng ml⁻¹ IgE) did not have an elevated degranulatory response (Fig. 5C). This contrasts with the previous experiment when K-RNT and IgE were exposed simultaneously during sensitization (Figs. 4C and 5C).

Examination of FITC¹/TBL₁₉⁻RNT Interaction With RBL-2H3 Cells by Confocal Microscopy

Confocal laser scanning microscopy of FITC¹/TBL₁₉⁻RNTs demonstrated that RNTs bind to RBL-2H3 cells. When RBL-2H3 cells were exposed to 10 mg l⁻¹ FITC¹/TBL₁₉⁻RNTs for 4 or 6 h, extensive co-localization between RNTs and FcR was observed (Fig. 6). Micrographs showing DAPI fluorescence (Fig. 6A–6C), FcR fluorescence (Fig. 6D–6F), and FITC¹/TBL₁₉⁻RNT fluorescence (Fig. 6G–6I) suggest that FITC⁻RNTs are associated with FcR as demonstrated through co-localized fluorescence (Fig. 6J–6O). This association remained despite the extensive washing of RBL-2H3 cells during confocal preparation, suggesting a strong affiliation between the two.

Using the fluorescence of DAPI, a nuclear stain, as a reference point for the intracellular compartment, FITC¹/TBL₁₉⁻RNT fluorescence is observed within the cell interior, visualized through 3D reconstruction of Z-stack images (Fig. 6P and 6Q). This suggests FITC¹/TBL₁₉⁻RNTs can be internalized by RBL-2H3 cells after just 2 h of exposure.

Discussion

Effects of K-RNT on Immune Cell Viability

Cell viability was assessed through a combination of flow cytometric analysis and the MTS assay. RBL-2H3 cells showed a dose dependent decrease in cell viability with increasing doses of K-RNTs after 2 and 4 h of exposure with significant decreases in metabolic activity at 100 and 200 mg l⁻¹ K-RNT. By comparison, Journeay et al. (2008) demonstrated a significant reduction in the viability of the pulmonary epithelial cell line Calu-3 after 24 h exposure to 50 mg l⁻¹ K-RNTs. Similarly, a human macrophage cell line, U937, demonstrated a significant decrease in viability after 24 h exposure to 50 mg l⁻¹ K-RNTs (Journeay et al., 2009). Although a significant change in RBL-2H3 cell viability at 50 mg l⁻¹ K-RNT was not observed, the epithelial cell line HEK 293 did exhibit a significant decrease in cell viability after 6 h of exposure to 50 and 100 mg l⁻¹ K-RNTs. Differential susceptibility of several cell lines has been reported in studies on metal toxicity (Tan et al., 2008). Pfaller et al. (2009) in their investigation of immunomodulatory effects of NMs noted significant differences in sensitivity of A549, Jurkat, and THP-1 cell lines. Our results, together with previous findings, highlight the need to use a variety of models and viability assays when investigating the biocompatibility of new materials (Zhao et al., 2013).

With demonstrated changes in viability upon K-RNT exposure, an investigation into the mechanisms of observed cell death was warranted. The use of a luciferase-based reporter system has been used with success to survey a broad list of potential mechanisms of zinc oxide and platinum nanoparticle toxicity for further study (Rallo et al., 2011). In general, it was found that after 6 h of K-RNT exposure, the transcriptional activity related to a broad variety of intracellular signaling pathways was downregulated with the exception of hypoxia inducible factor, which remained unchanged. However, no significant changes in transcriptional activity were observed. Many of these candidate signaling pathways play important roles in apoptosis including PKC/Ca²⁺, Myc/Max and p53/DNA damage. The lack of induction of these pro-apoptotic pathways by K-RNT exposure suggests the possibility of nonapoptotic mechanisms of cell death such as regulated or unregulated necrosis (Sun and Wang, 2014).

Using FITC¹/TBL₁₉⁻RNTs, the cellular association and subsequent internalization of the nanotubes in RBL-2H3 cells was confirmed. This is the first demonstration of the cellular uptake of RNTs using confocal imaging. Confocal micrographs show co-localization between IgE labeled FcR and FITC¹/TBL₁₉⁻RNTs, suggesting these NMs are associating with the cellular membrane. The presence of FITC¹/TBL₁₉⁻RNTs, despite extensive washing, demonstrates a strong association between this material and the cell membrane surface receptors. In computer simulations, functionalizing gold NMs with positive surface charges resulted in increased lipid bilayer adherence when compared with negative and hydrophobic surface functionalizations (Lin et al., 2010). It is likely that the high level of FITC¹/TBL₁₉⁻RNT and cell membrane interaction results from the attraction between the positively charged nanotubes (Table 1) and negatively charged cell membranes. 3D reconstruction and surface renderings of Z-stacks also suggest the internalization of FITC¹/TBL₁₉⁻RNTs after 2 h exposure. Such strong affiliation of RNTs with the membrane could potentially contribute to a loss of membrane integrity, causing cell death through a nonregulated necrotic process. With a lack of candidates identified from the reporter assay, our lab is currently investigating if necrosis could be the mechanism of RNT toxicity observed at higher doses.

Effects of K-RNT on RBL-2H3 Cell Degranulation

Endpoints beyond viability are being recognized as important in the toxicological evaluation of NMs (Ortega et al., 2013). Here, we demonstrate for the first time an immediate degranulatory event by RBL-2H3 cells when exposed to K-RNTs. Interestingly,
RBL-2H3 cells increased release of β-hexosaminidase when co-exposed to K-RNTs during IgE sensitization. RBL-2H3 cells were seeded at $4 \times 10^4$ into a 96-well plate and allowed to rest for 2 h. Cells were simultaneously sensitized with IgE (0, 12.5, 25, 50, or 100 ng ml$^{-1}$) and exposed to either (A) 50 or (B) 100 mg l$^{-1}$ K-RNT. Control cells were simultaneously sensitized with IgE (0, 12.5, 25, 50, or 100 ng ml$^{-1}$) and exposed to vehicle (ultra pure H$_2$O). Negative controls were exposed to Tyrodes buffer alone (no IgE) to measure nonspecific background signal. After washing, cells were subsequently stimulated to degranulate with dinitrophenyl-human serum albumin (DNP-HSA). Supernatant was collected and β-hexosaminidase activity assayed. The relative fluorescent units (RFUs) for each treatment was standardized to our negative control to calculate the fold change in β-hexosaminidase activity over background. C, Summary, showing the percent increase in β-hexosaminidase activity for K-RNT treatment compared with control for each level of IgE sensitization. Values are means ± SE ($n=5$). Asterisk indicates significantly different values compared with control ($p<.05$, 2-way ANOVA followed by Sidak’s post hoc comparison).

FIG. 4. RBL-2H3 cells increased release of β-hexosaminidase when co-exposed to K-RNTs during IgE sensitization. RBL-2H3 cells were seeded at $4 \times 10^4$ into a 96-well plate and allowed to rest for 2 h. Cells were simultaneously sensitized with IgE (0, 12.5, 25, 50, or 100 ng ml$^{-1}$) and exposed to either (A) 50 or (B) 100 mg l$^{-1}$ K-RNT. Control cells were simultaneously sensitized with IgE (0, 12.5, 25, 50, or 100 ng ml$^{-1}$) and exposed to vehicle (ultra pure H$_2$O). Negative controls were exposed to Tyrodes buffer alone (no IgE) to measure nonspecific background signal. After washing, cells were subsequently stimulated to degranulate with dinitrophenyl-human serum albumin (DNP-HSA). Supernatant was collected and β-hexosaminidase activity assayed. The relative fluorescent units (RFUs) for each treatment was standardized to our negative control to calculate the fold change in β-hexosaminidase activity over background. C, Summary, showing the percent increase in β-hexosaminidase activity for K-RNT treatment compared with control for each level of IgE sensitization. Values are means ± SE ($n=5$). Asterisk indicates significantly different values compared with control ($p<.05$, 2-way ANOVA followed by Sidak’s post hoc comparison).
exposure to K-RNTs increased $\beta$-hexosaminidase release, with and without IgE sensitization (Fig. 4C) suggesting these materials can augment IgE-mediated release of granular products, but can also induce degranulation in RBL-2H3 cells independent of normal immune activating processes. Previous reports in Calu-3 pulmonary epithelial cells and U937 cells, a human macrophage cell line, demonstrated that exposure to $50 \text{mg L}^{-1}$ K-RNT for 1 and 6 h induced cytokine secretion (Journeay et al., 2008).

FIG. 5. RBL-2H3 cells showed increased degranulation after preexposure to K-RNTs. RBL-2H3 cells were seeded at $4 \times 10^4$ into a 96-well plate and allowed to rest for 2 h. Cells were then exposed to (A) 50 or (B) $100 \text{mg L}^{-1}$ K-RNT for 2 h. Cells were washed to remove K-RNTs before sensitization with 0, 12.5, 25, 50, or $100 \text{ng mL}^{-1}$ IgE and subsequent treatment with DNP-HSA to stimulate degranulation. Supernatant was collected and $\beta$-hexosaminidase activity assayed. The relative RFUs for each treatment was standardized to our negative control to calculate the fold change in $\beta$-hexosaminidase activity over background. C. Summary, showing the percent increase in $\beta$-hexosaminidase activity compared with control. Values are means ± SE ($n=5$). Asterisk indicates significantly different values compared with control ($p < .05$, 2-way ANOVA followed by Sidak’s post hoc comparison).
Together these findings suggest K-RNTs may be proinflammatory, capable of eliciting an immune response through the earlier endpoints. Similar proinflammatory effects on mast cells have been reported for other high-aspect ratio NMs, including multi-wall carbon nanotube (Katwa et al., 2012). RBL-2H3 cells have also been demonstrated to degranulate due to a variety of nonimmunological stimuli (Passante and Frankish, 2009). For example, Fowlkes et al. (2013) showed that mechanical loading in RBL-2H3 cells accomplished through RGD-binding integrin receptors caused secretion of β-hexosaminidase. With our

**FIG. 6.** Confocal micrographs showing association of FITC1/TBL19-RNTs with RBL-2H3 cells. Cells were stained for FcεR using IgE primary antibody and goat anti-mouse IgG PE-conjugated secondary antibody before being fixed. Nuclei were stained using DAPI. Laser scanning confocal micrographs with DAPI fluorescence (a, b, c), FcεR fluorescence (d, e, f), FITC-RNT fluorescence (g, h, i), fluorescence overlay (j, k, l), and bright-field overlay (m, n, o) of RBL-2H3 cells exposed to vehicle control for 4 h (top) or 10 mg l⁻¹ FITC1/TBL19 for 4 (middle) or 6 (bottom) h. Scale bars are 10 μm. Surface rendering and 3-dimensional (3D) reconstruction of Z-stack images suggest FITC-RNTs are internalized in RBL-2H3 cells after 2 h of exposure. (p, q) RBL-2H3 cells were exposed to 10 mg l⁻¹ FITC1/TBL19-RNTs for 2 h and subsequently washed, stained for FcεR using IgE primary antibody and goat anti-mouse IgG PE-conjugated secondary antibody before being fixed. Nuclei were stained with DAPI. Surface rendering and 3D reconstruction of z-stack fluorescence was performed using Imaris software. FcεR fluorescence (red) and FITC1/TBL19-RNT fluorescence (green) shown without (p) and with (q) DAPI fluorescence (blue). Full color version available online.
confocal results demonstrating a strong interaction with RBL-2H3 cell membranes, the observed increase in degranulation upon K-RNT exposure could be attributed to such physical stimulation.

To further investigate the proinflammatory response to K-RNTs, we tested the effect of preexposing RBL-2H3 to K-RNT on their ability to degranulate. In contrast to the previous experiment, residual unbound K-RNT was washed-off prior to sensitization with IgE. This step was essential to establish whether unbound K-RNTs were interfering with IgE binding its receptor, FcεRI. It is well known that many NMs nonspecifically bind to proteins in solution and thereby alter either their activity or affect their ability to bind to their cognate receptors (MacCormack et al., 2012, Stueker et al., 2014). Moreover, with confocal microscopy demonstrating a strong affilation of K-RNTs with the cellular membrane, we wanted to determine if the noted effects were most likely due to K-RNTs remaining bound to the cells. We found that following 2 h preexposure to K-RNTs and subsequently washing away unbound K-RNT, cells were still able to elicit an IgE-mediated degranulatory response. However, unexpectedly, the cells demonstrated a significant increase in IgE mediated release of β-hexosaminidase compared with untreated cells.

To date, a variety of stimuli have been shown to induce degranulation in RBL-2H3 cells. It is possible that RNTs could be affecting membrane integrity in RBL-2H3 cells. Carbon NMs have been implicated in several studies to destabilize cellular membranes (Tahara et al., 2012). If such a mechanism occurs in RBL-2H3 cells, the release of a variety of intracellular components, including β-hexosaminidase would result. However, because cells were washed prior to sensitization, we removed these factors and therefore suggest that K-RNTs are acting directly on the cell membrane and eliciting a potentiation of the IgE mediated degranulatory response. Our confocal microscopy demonstrated co-localization of RNTs with FcεRI. Therefore, it is possible RNTs potentiate the degranulatory response by increasing FcεRI cross-linking. Previous reports have suggested NMs can alter receptor cross-linking in RBL-2H3 cells, either promoting or inhibiting degranulation based largely on NM architecture. For example, gold nanoparticles larger than 19.8 nm, and coated with cell-activating antigens, promoted FcεRI cross-linking and activation and were potent effectors of RBL-2H3 degranulation; alternatively, antigen-coated gold NMs smaller than 19.8 nm competitively inhibited degranulation (Huang et al., 2009). Interestingly, gold NMs alone did not elicit or alter a degranulatory response. RNTs are a high-aspect ratio NM with a diameter of 3.5 nm; however, they can be up to several 100-μm long. Given this large architecture, it is possible RNTs could be promoting FcεRI cross-linking and inducing degranulation in RBL-2H3 cells.

In conclusion, we have demonstrated that K-RNTs interact directly with the membrane of immune cells and can be internalized after exposure. These interactions affect viability and alter the ability of these cells to elicit receptor-mediated responses such as degranulation. In addition, at high doses, K-RNTs can elicit degranulatory responses irrespective of IgE mediated stimulation. Currently, we are trying to elucidate the nature of this interaction, the relative importance of membrane associated versus internalized K-RNTs, and the mechanism mediating this response. These questions are essential as we seek to understand the nature of the interactions between RNTs and cells and are important for tailoring RNTs for a variety of biomedical applications. For example, Fine et al. (2009) demonstrated that the biocompatibility of vascular stents could be improved with a 10 mg l⁻¹ coating of K-RNTs, resulting in enhanced endothelial cell adherence. The utility of RNTs for such applications can be advanced by minimizing negative interactions with the immune system, ultimately improving biocompatibility.

SUPPLEMENTARY DATA

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

FUNDING

This work was supported by the Natural Sciences and Engineering Research Council of Canada-National Research Council of Canada-Business Development Bank of Canada-Environment Canada (NSERC-NRC-BDC-EC Nanotechnology Initiative Grant Number NNBPJ 380151-08) and Alberta Innovates Technology Futures Nanoworks Grant (Grant Number PAB01015). J.D.E. was supported by an NSERC Vanier Canadian Graduate Scholarship and an Alberta Innovates Graduate Student Scholarship. V.A.O. is supported by NSERC Postgraduate Scholarship-Doctoral and the Alberta Innovates Health Solutions Graduate Studentship.

ACKNOWLEDGMENTS

The authors thank Biological Sciences Aquatic Facilities for all their help and assistance, Geraldine Barron at the Cross Cancer Cell Imaging Facility for her help with confocal microscopy and Dr Jon Veinot for advice and access to DLS analytical facilities.

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