Supporting Information

Instillation *versus* Inhalation of Multiwalled Carbon Nanotubes: Exposure-Related Health Effects, Clearance, and the Role of Particle Characteristics

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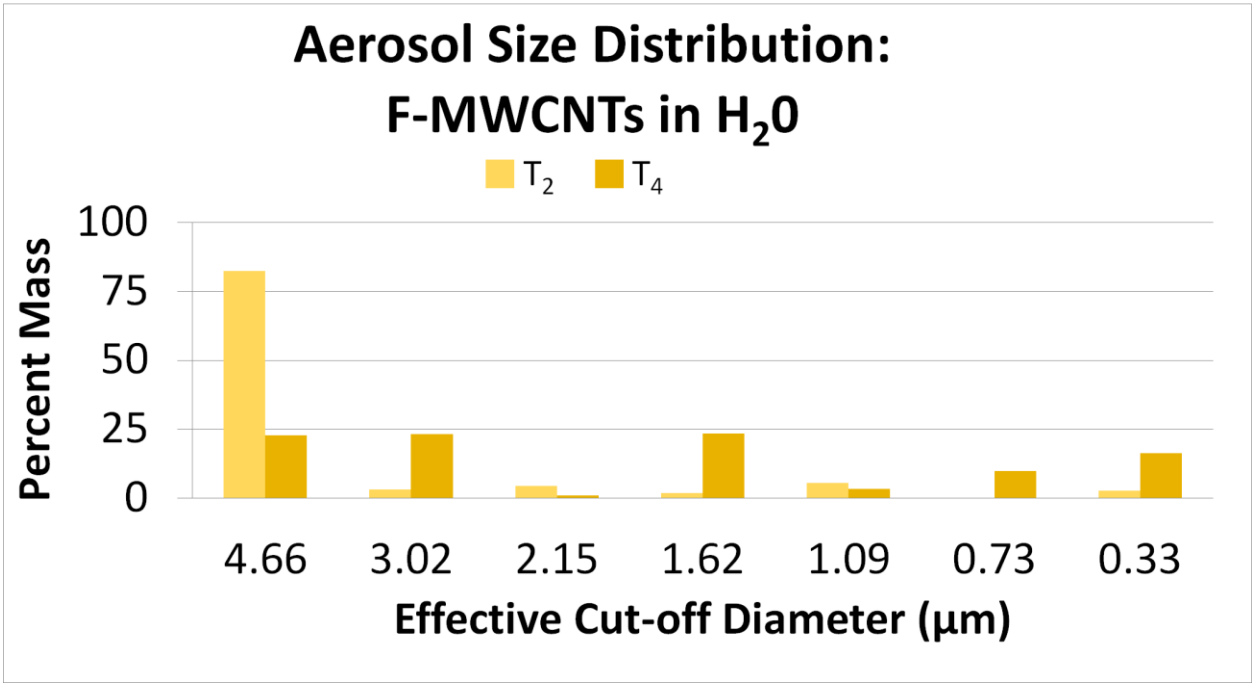

Figure S1. Aerosolized F-MWCNTs in water exhibited a range of droplet sizes. Panels show results from cascade impactor sampling during a six-hour inhalation exposure to F-MWCNTs in water. The cascade impactor had 7 stages with effective cut-off diameters ranging from 4.66 µm on stage 1, to 0.33 µm on stage 7. Samples were taken at multiple time-points corresponding to two (T_2) and four hours (T_4) into the exposure.

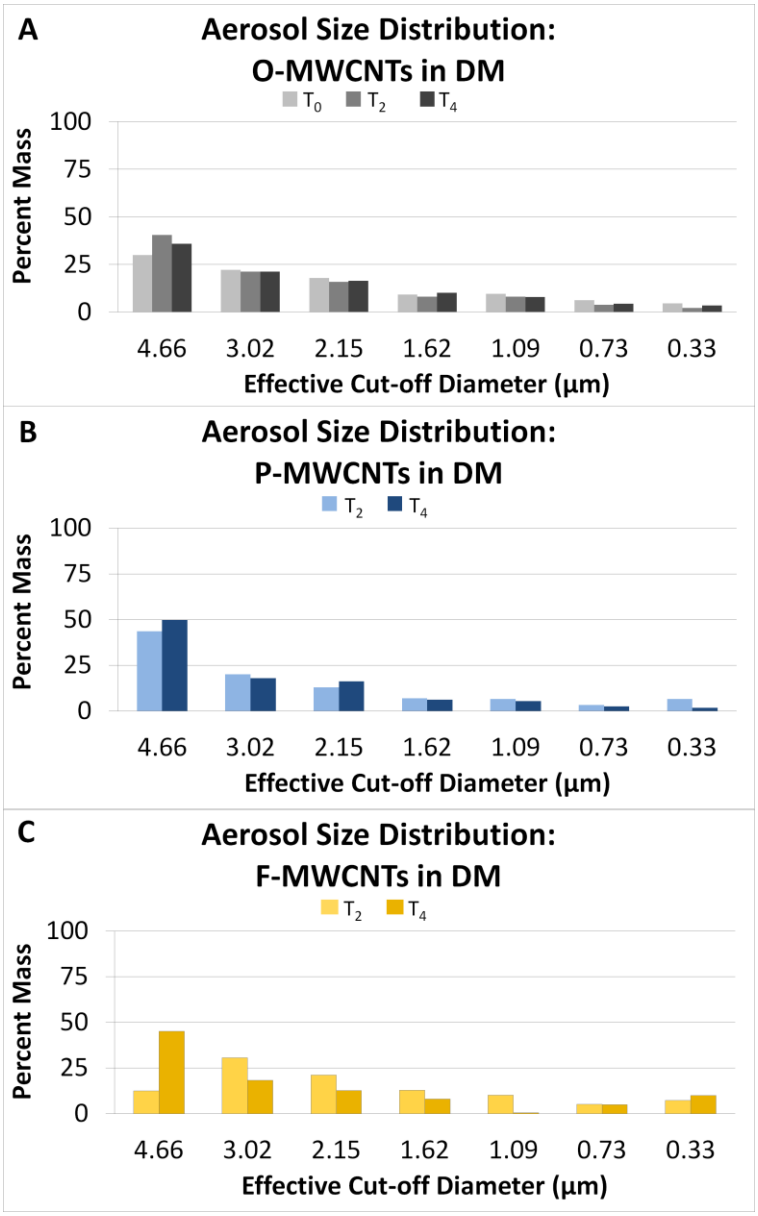

Figure S2. Aerosolized MWCNTs in dispersion media (DM) produced normally-distributed droplet sizes. Panels show results from cascade impactor sampling during six-hour inhalation exposures to either O-, P-, or F-MWCNTs (panels A-C, respectively) suspended in DM. The cascade impactor had 7 stages with effective cut-off diameters ranging from 4.66 µm on stage 1, to 0.33 µm on stage 7. Samples were taken at multiple time-points corresponding to zero (T_0), two (T_2) or four hours (T_4) into the exposure for panels A -C.

MWCNT formulation and time post exposure were significant factors post IT/inhalation of MWCNTs suspended in DM. Total cell numbers (10^4) were significantly higher at post instillation Day 1 (*M* = 209.20) than Day 21 (*M* = 182.64) (Table S1), and upon O- (*M* = 211.59) *versus* P-MWCNT (*M* = 180.34) instillation. Instilled F-MWCNTs produced an intermediate response that was not significantly different from O- or P-MWCNTs. For inhaled MWCNTs in DM, total cell numbers (10^4) were higher at Day 21 (*M* = 129.08) *versus* Day 1 (*M* = 107.60), and upon inhalation of O-MWCNTs (*M* = 132.65) *versus* P-MWCNTs (*M* = 105.08) (Table S1). Inhalation of F-MWCNTs in DM produced intermediate numbers of cells (*M* = 117.30), which were not significantly different from those post O- or P-MWCNT inhalation.

Table S1. Significant Post Hoc Comparisons of Main Effects: BALF Total Cells (10^4) post Instillation or Inhalation of MWCNTs in Dispersion Media

Compared Factor(s)	Group I (A)	Group II (B)	Mean Difference (A-B)	Standard Error Difference (A-B)	*p* - value	*df*	LCL	UCL
Time post IT	Day 1	Day 21	28.23	10.15	0.01	1	8.10	48.35
Instilled Formulation	O-	P-	31.24	12.38	0.05	2	1.84	60.65
Time post Inhalation	Day 1	Day 21	21.47	7.90	0.01	1	5.66	37.28
Inhaled Formulation	O- in DM	P- in DM	27.57	9.60	0.05	2	4.49	50.65

df = degrees of freedom. LCL and UCL = lower confidence limit, and upper confidence limit, respectively. O-, P- = original, and purified multi-walled carbon nanotubes, respectively. IT = intratracheal instillation, and DM = dispersion media.

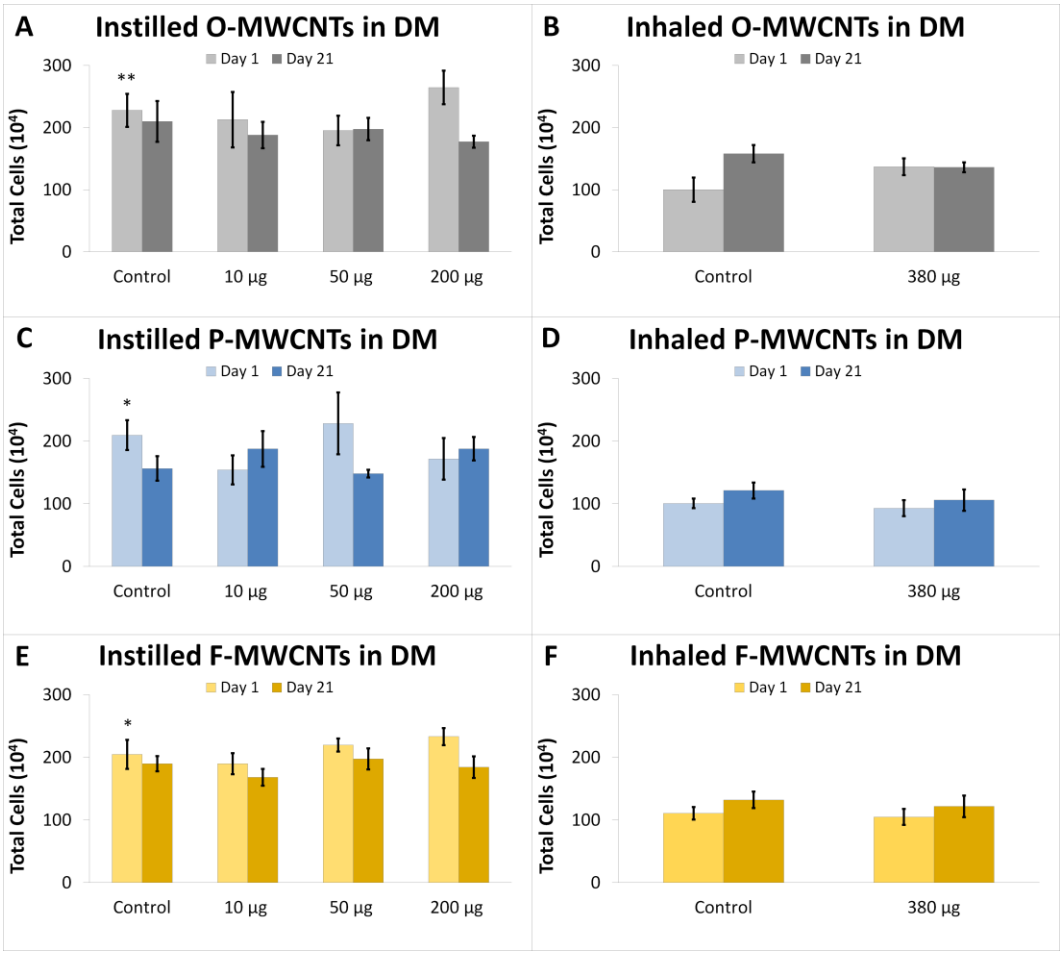

Figure S3. Instillation produces more BALF cells than inhalation. Total cells in BALF at Day 1 (left panels) and Day 21 (right panels) post intratracheal instillation (IT) or inhalation. "Control" animals were instilled with 250 µL of DM for IT studies, or maintained in a filtered air environment for inhalation studies. "Exposed" animals got O-MWCNTs (A & B), P-MWCNTs (C & D), or F-MWCNTs (E & F) suspended in dispersion media (DM) *via* IT or inhalation, or F-MWCNTs suspended in water (H_20) *via* inhalation. Results are from ANOVA considering dose (control *versus* exposed), time (Day 1 *versus* Day 21), and particle formulation/administration method. Asterisks (*) indicate significant differences (*$p \leq 0.05$, **$p \leq 0.01$) between groups sacrificed on the same day, but exposed *via* different administration methods (Instillation *versus* Inhalation with DM).

Overall, the total number of neutrophils in BALF was higher at post IT Day 1 (M = 461.20) than Day 21 (M = 69.82) (Table S2), and control animals (M = 102.53) exhibited significantly less inflammation than those instilled with MWCNTs (Table S2).

Table S2. Significant Post Hoc Comparisons of Main Effects: Square root-transformed Number of Neutrophils post IT

Compared Factor	Group I (A)	Group II (B)	Mean Difference (A-B)	Standard Error Difference (A-B)	p-value	df	LCL	UCL
Time	Day 1	Day 21	391.37	27.21	CON	1	337.46	445.29
Dose	200 µg	Control	380.98	39.49	CON	3	277.96	484.00
Dose	200 µg	10 µg	265.75	39.49	CON	3	162.74	368.77
Dose	200 µg	50 µg	225.25	39.49	CON	3	122.23	328.27
Dose	50 µg	Control	155.73	37.43	0.001	3	58.08	253.39
Dose	10 µg	Control	115.23	37.43	0.05	3	17.57	212.88

CON represents "convincing" findings at $p < 0.0001$. df = degrees of freedom. LCL and UCL = lower confidence limit, and upper confidence limit, respectively.

When the interaction of time and dose was analyzed, total polymorphonuclear cells (PMNs: neutrophils) in BALF was significantly higher for animals instilled with 200 µg MWCNTs (*M* = 860.38) in contrast to other doses (Table S3), at Day 1 specifically; and by Day 21, all MWCNT-instilled animals had significantly less neutrophils than at Day 1 (Table S3).

Table S3. Significant Post Hoc Comparisons of Interactions: Square root-Transformed Number of Neutrophils post IT

Compared Factors	Group I (A)	Group II (B)	Mean Difference (A-B)	Standard Error Difference (A-B)	p-value	df	LCL	UCL
Time & Dose	Day 1, 200 µg	Day 1, Control	702.02	56.65	CON	3	526.96	877.08
Time & Dose	Day 1, 200 µg	Day 1, 10 µg	479.56	56.65	CON	3	304.50	654.62
Time & Dose	Day 1, 200 µg	Day 1, 50 µg	415.16	56.65	CON	3	240.11	590.22
Time & Dose	Day 1, 50 µg	Day 1, Control	286.86	53.79	CON	3	120.65	453.06
Time & Dose	Day 1, 10 µg	Day 1, Control	222.45	53.79	0.01	3	56.25	388.66
Time & Dose	Day 1, 200 µg	Day 21, 200 µg	753.75	58.61	CON	3	572.63	934.87
Time & Dose	Day 1, 50 µg	Day 21, 50 µg	373.93	52.94	CON	3	210.34	537.51
Time & Dose	Day 1, 10 µg	Day 21, 10 µg	326.14	52.94	CON	3	162.55	489.72

CON represents "convincing" findings at $p < 0.0001$. *df* = degrees of freedom. LCL and UCL = lower confidence limit, and upper confidence limit, respectively.

Overall, neutrophilia was higher at Day 1 (*M* = 42.22) than Day 21 (*M* = 16.14) (Table S4), and filtered-air control animals (*M* = 12.24) exhibited significantly less inflammation than those exposed to aerosolized MWCNTs in DM (*M* = 46.12) (Table S4). Inhalation of MWCNTs in DM (*M* = 68.21) *versus* filtered air (*M* = 16.22) produced significant increases in neutrophils recovered from BALF at Day 1 (Table S4); however, this inflammation resolved by Day 21 (Table S4). Inhalation of F-MWCNTs in water did not affect neutrophils in BALF in comparison to the filtered air control (data not shown).

Table S4. Significant Post Hoc Comparisons: Square root-Transformed Number of Neutrophils post Inhalation

Compared Factor(s)	Group I (A)	Group II (B)	Mean Difference (A-B)	Standard Error Difference (A-B)	p-value	df	LCL	UCL
Main Effects								
Time	Day 1	Day 21	26.08	7.35	0.001	1	11.36	40.79
Dose	Control	Exposed	33.88	7.35	CON	1	19.16	48.59
Interactions								
Time & Dose	Day 1, 380 µg	Day 1, Control	51.99	10.48	CON	1	24.28	79.71
Time & Dose	Day 1, 380 µg	Day 21, 380 µg	59.95	10.48	CON	1	32.24	87.67

CON represents "convincing" findings at $p < 0.0001$. *df* = degrees of freedom. LCL and UCL = lower confidence limit, and upper confidence limit, respectively.

Table S5. Significant Post Hoc Comparisons: Square root-Transformed Number of MWCNT-Positive Macrophages post IT

Compared Factor(s)	Group I (A)	Group II (B)	Mean Difference (A-B)	Standard Error Difference (A-B)	*p* - value	*df*	LCL	UCL
Main Effects								
Time	Day 1	Day 21	300.41	39.64	CON	1	221.9	378.9
Dose	10 µg	Control	158.03	54.54	0.05	3	15.75	300.3
Dose	50 µg	Control	465.05	54.54	CON	3	322.8	607.3
Dose	200 µg	Control	453.84	57.54	CON	3	303.7	603.9
Dose	50 µg	10 µg	307.01	54.54	CON	3	164.7	449.3
Dose	200 µg	10 µg	295.8	57.54	CON	3	145.7	445.9
Interactions								
Time & Dose	Day 1, 10 µg	Day 1, Control	272.73	78.37	0.05		30.6	514.9
Time & Dose	Day 1, 200 µg	Day 1, 10 µg	404.43	82.54	CON		149.4	659.5
Time & Dose	Day 1, 50 µg	Day 1, Control	636.41	78.37	CON		394.3	878.6
Time & Dose	Day 1, 50 µg	Day 1, 10 µg	363.67	78.37			121.5	605.8
Time & Dose	Day 1, 200 µg	Day 1, Control	677.16	82.54	CON		422.1	932.2
Time & Dose	Day 1, 10 µg	Day 21, 10 µg	275.12	77.13	0.01		36.8	513.5
Time & Dose	Day 1, 50 µg	Day 21, 50 µg	388.44	77.13	CON		150.1	626.8
Time & Dose	Day 1, 200 µg	Day 21, 200 µg	492.37	85.40	CON		228.5	756.3
Time, Dose, & Formulation	Day 1, 50 µg, O-	Day 1, 10 µg, O-	615..85	131.42	.01		125.4	1106
Time, Dose, & Formulation	Day 1, 200 µg, O-	Day 1, 10 µg, O-	851.53	137.84	CON		337.2	1366

CON = "convincing" findings, $p < 0.0001$. *df* = degrees of freedom. LCL and UCL = lower confidence limit, and upper confidence limit, respectively. O-, P-, F- = original, purified, and functionalized multi-walled carbon nanotubes, respectively.

Semi-Quantitative Histopathology Scoring Rubric

Type of Pathology	Score			
	0	1	2	3
Alveolitis	Normal. Thin alveolar walls, with very few free macrophages in the lumen. No inflammatory cells.	Similar to 0 score with more free macrophages in the alveolar lumen. No PMNs.	Atypical cellularity in the walls and/or lumen of the alveoli with the majority of the alveolar spaces still clear of free cells. Over-represented cell types include macrophages, monocytes, and/or PMNs.	Thickened alveolar walls. Marked influx of mixed cells (phagocytes and/or PMNs) into the alveolar lumen forming large cellular agglomerates which occupy much of the airspace.
Bronchiolitis	Normal respiratory epithelium, 1 cell-layer thick. Normal smooth muscle and submucosal layers.	Mild influx of macrophages and/or monocytes to the airway submucosa, but no PMNs.	Slightly thickened airway due to moderate influx of PMNs and/or phagocytes into the submucosa. PMNs encompass <15% of influxing cells.	Marked influx of inflammatory cells into the submucosal layer causing pronounced thickening of the airway. A high percentage of PMNs may be present, but is not necessary.
Perivascular Inflammation	Normal vascular endothelium.	Mild influx of a few macrophages and/or monocytes to the region, but no PMNs. Nearly all of the connective tissue is still visible.	Moderate PMN and/or phagocyte infiltrates with much of the connective tissue still visible.	Marked mixed cellular infiltrates such that much of the connective tissue is obscured by influxing cells. A high percentage of PMNs may be present.
Particle Agglomerates	No particle agglomerates.	Obvious particle agglomerate with little/no increase in vicinal cellularity.	Obvious particle agglomerate with moderate increase in vicinal cellularity. Phagocyte and/or PMN influx. Small cellular aggregates possible.	Obvious particle agglomerate surrounded by large, focal cellular infiltrates.
Pleural Inflammation	Little/no cells at the pleura.	Slightly increased cellularity at the pleura. No PMNs.	Moderately increased cellularity with PMNs and/or phagocytes.	Severe influx of cells to the pleura. A high percentage of PMNs may be present along with foamy macrophages.

Figure S4. Semi-Quantitative Histopathology Scoring Rubric

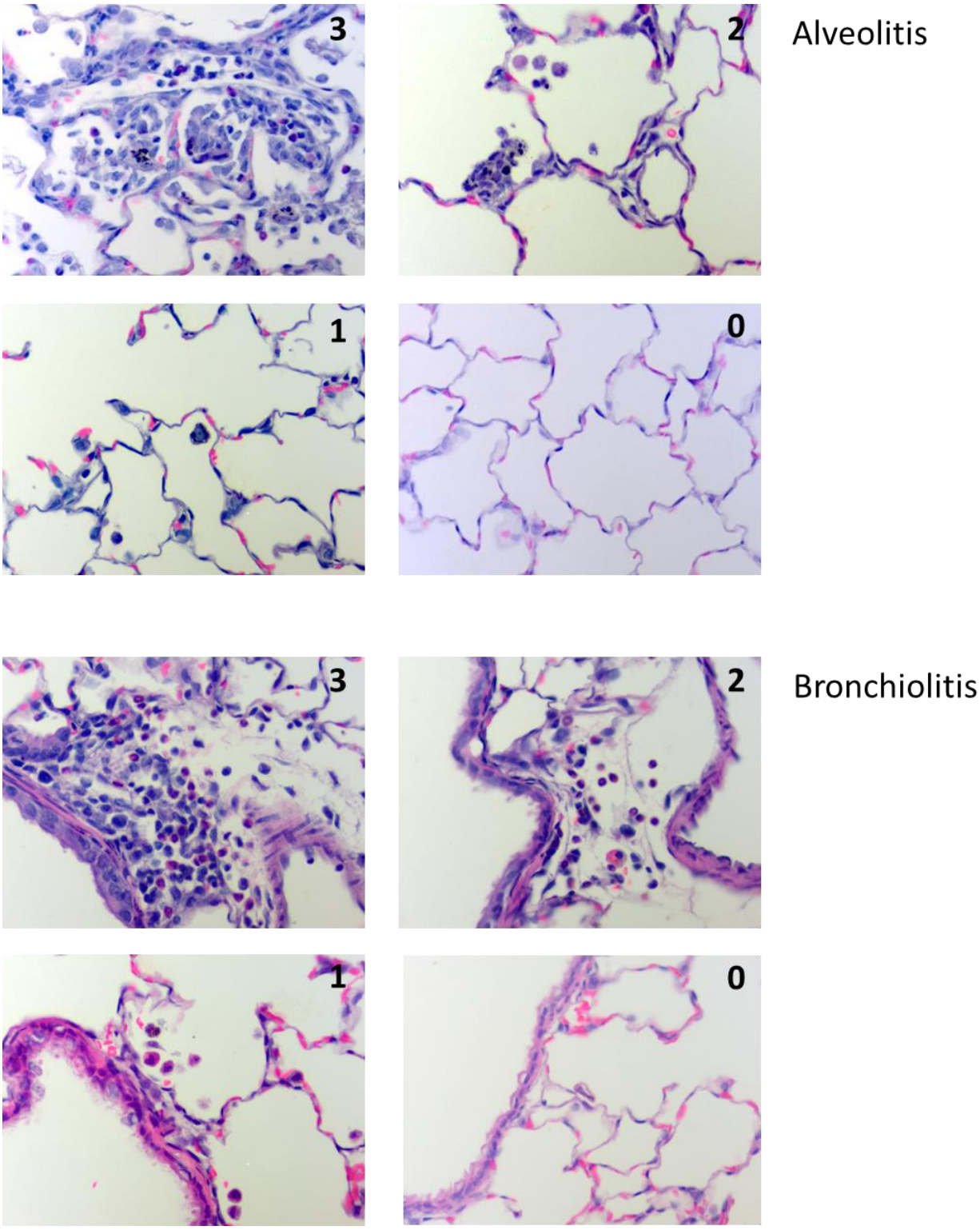

Figure S5. Illustrated Histopathology Scoring Reference: Part I

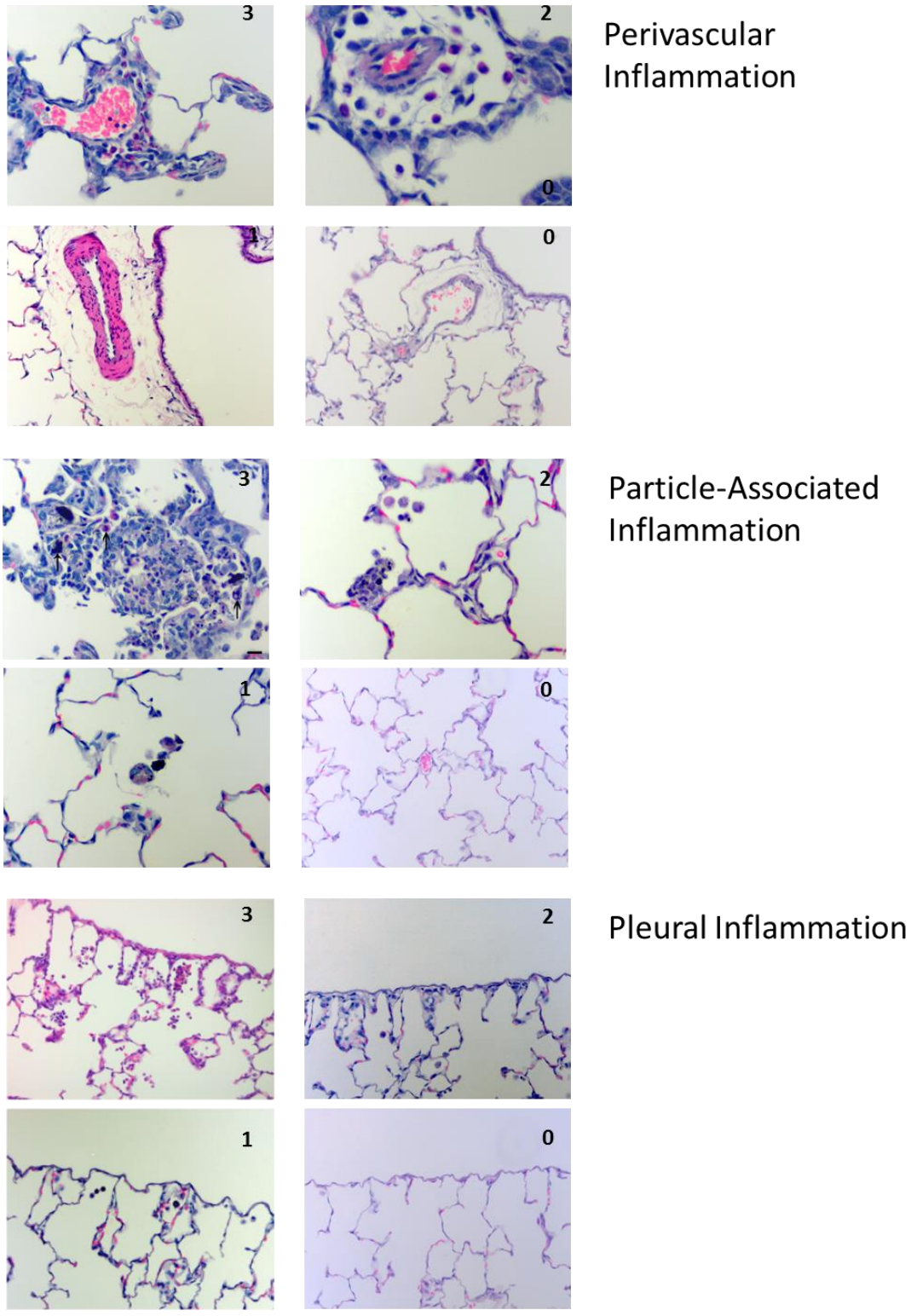

Figure S6. Illustrated Histopathology Scoring Reference: Part II

Table S6. Significant Post Hoc Comparisons of Main Effects: Day 21 Histopathology post IT

Pathology Compared	Group I Mean (A)	Group II Mean (B)	Mean Difference (A-B)	Standard Error Difference (A-B)	*p* - value	*df*	LCL	UCL
Main Effect = Formulation								
Bronchiolitis	O-	F-	0.92	0	CON	2	0.52	1.31
Bronchiolitis	P-	F-	1.04	0	CON	2	0.62	1.46
Perivascular Inflammation	O-	F-	0.50	0.19	0.05	2	0.03	0.97
Pleural Inflammation	O-	F-	1.00	0.18	CON	2	0.56	1.44
Pleural Inflammation	P-	F-	1.04	0.19	CON	2	0.57	1.51
Main Effect = Dose								
Particle-Associated Inflammation	200 µg, M = 0.83	Control, M =0.28	0.56	0.25	0.05	1	.	.

CON = "convincing" findings, $p < 0.0001$. *df* = degrees of freedom. LCL and UCL = lower confidence limit, and upper confidence limit, respectively. O-, P-, F- = original, purified, and functionalized multi-walled carbon nanotubes, respectively.

Table S7. Significant Post Hoc Comparisons of Main Effects: Day 21 Histopathology post Exposure (Instillation *versus* Inhalation)

Pathology Compared	Group I (A)	Group II (B)	Mean Difference (A-B)	Standard Error Difference (A-B)	p-value	df	LCL	UCL
Main Effect = Exposure Method								
Alveolitis	Instilled O-	Inhaled O- in DM	1.39	0.23	CON	6	0.71	2.07
Alveolitis	Instilled P-	Inhaled P- in DM	1.18	0.25	CON	6	0.45	1.91
Alveolitis	Instilled F-	Inhaled F- in DM	2.14	0.23	CON	6	1.46	2.82
Bronchiolitis	Instilled O-	Inhaled O- in DM	1.39	0.20	CON	6	0.81	1.97
Bronchiolitis	Instilled P-	Inhaled P- in DM	1.51	0.22	CON	6	0.89	2.14
Pleural Inflammation	Instilled O-	Inhaled O- in DM	1.83	0.08	CON	6	1.60	2.07
Pleural Inflammation	Instilled P-	Inhaled P- in DM	1.88	0.09	CON	6	1.62	2.13
Pleural Inflammation	Instilled F-	Inhaled F- in DM	0.83	0.08	CON	6	0.60	1.07
Perivascular Inflammation	Instilled O-	Inhaled O- in DM	0.86	0.13	CON	6	0.50	1.23
Perivascular Inflammation	Instilled P-	Inhaled P- in DM	0.53	0.14	CON	6	0.13	0.92

df = degrees of freedom. LCL and UCL = lower confidence limit, and upper confidence limit, respectively. O-, P-, F- = original, purified, and functionalized multi-walled carbon nanotubes, respectively. DM = dispersion media. CON = "convincing" findings, $p \leq 0.0001$.

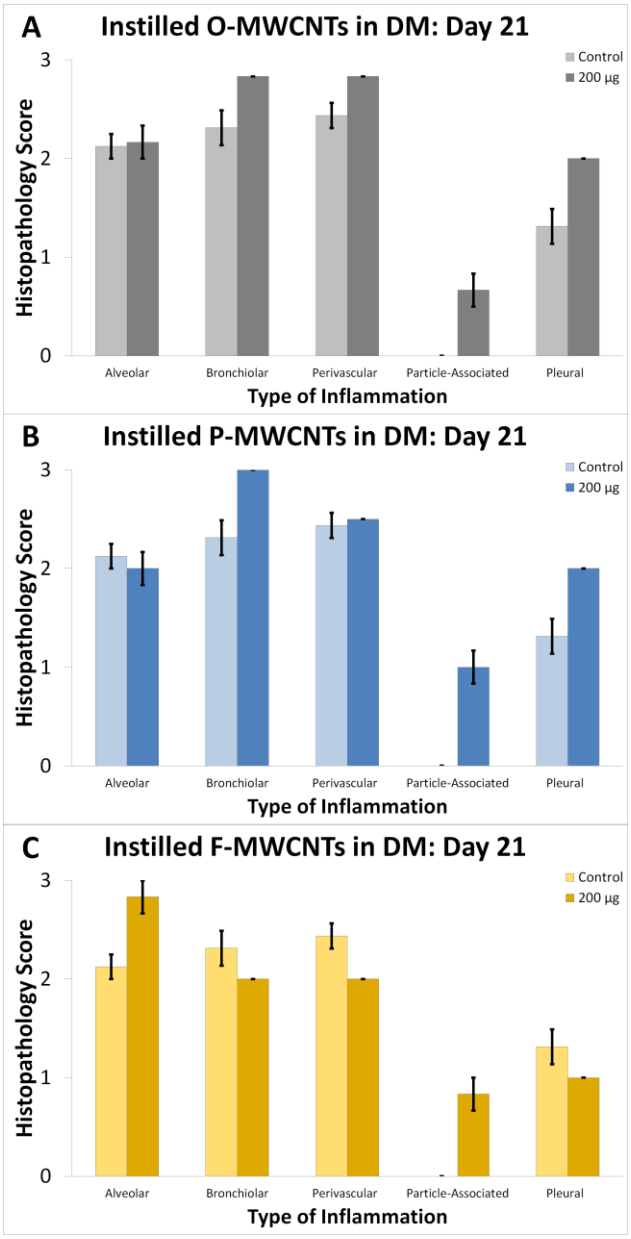

Figure S7. Histopathology Resulting from MWCNT Instillation of Resolved by Day 21.

Results from semi-quantitative scoring are shown for O-MWCNTs (A), P-MWCNTs (B), and F-MWCNTs (C) for Day 21. Results are from an ANOVA model considering dose and particle formulation.