124771-JCI-INS-CC-TR Supplemental Material

Supplemental Methods

Animal Husbandry and Telemetry. Rhesus macaques were born and housed at the Tulane National Primate Research Center (Covington, LA), which is US Department of Agriculture-licensed and fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Subcutaneous radiotelemetry transmitters combined with sensors capable of detecting biopotential signals of an electrocardiogram as well as thermistor-type sensors capable of detecting temperature signals (T34G-8; Konigsberg Instruments) were surgically implanted under aseptic conditions in 8 of the 12 vaccinated macaques and both control macaques before the start of the study. Animals determined to be in respiratory distress and those that survived for 21 d after exposure to ricin were euthanized by an overdose of sodium pentobarbital, consistent with the recommendation of the American Veterinary Medical Association's Panel on Euthanasia, and submitted for necropsy. All methods were approved by the Tulane Institutional Animal Care and Use Committee (IACUC).

Treatment of Rhesus macaques. At 4 h or 12 hours post-exposure, designated animal groups received a single intravenous administration of huPB10 by slow infusion at an individualized unit dose of 10 mg/kg. Sham-treated animals were administered saline for injection at the four-hour time point. Treated animals were observed for signs of adverse reactions to the antibody during administration and throughout anesthesia recovery period. Animals were bled just before and 24 h following aerosol challenge. Blood was also collected when the animals either succumbed to intoxication or 21 d after challenge, when the experiment was terminated.

RT aerosolization, dosing, and calculation. Aerosolization, dosing, and delivery of ricin were performed as described (17). Inductive plethysmography that measures volume of air breathed by each individual animal per minute was performed just before the ricin exposure. Ricin was dissolved in 10 mL sterile phosphate buffer saline to the desired concentration for each animal based on plethysmography data obtained 2 d

before the exposure. Aerosols were generated directly into a head-only chamber using a Collision three jet-nebulizer (BGI) with fully automated management control system (Biaera Technologies, Hagerstown, MD) all within a Class III biological safety cabinet housed within the TNPRC high-containment (BSL-3) laboratories. The nebulizer operated at 18 lb/inch^2 equating to a flow of 7.5 L/min and produced 3.0E+04 particles per cc with a mass median aerodynamic diameter of ~1.4 μm. Each discrete aerosol exposure lasted 10 minutes (per animal). Air samples were continuously obtained during the exposure and the protein concentrations of these samples were determined using a micro-BSA protein assay kit (Thermo Scientific). The aerosol concentrations were determined and the inhaled dose of RT for each animal was calculated by multiplying the empirically determined aerosol exposure concentration (μg/liter of air) in the chamber by volume of air estimated to have been breathed by the animal (via results of plethysmography just before exposure). The LD_{50} of ricin is 5.8 μg/kg body weight and the target dose for this experiment was set at the equivalent of three LD_{50}s (≈18 μg/kg). The mean inhaled dose of ricin across all animals was 4.4 ± 1.4 LD_{50}s.

Tissue collection and histological analysis. After gross necropsy, tissues were collected in neutral buffered zinc-formalin solution (Z-Fix Concentrate, Anatach). Tissues were processed, sectioned, and stained as previously described (4).

Supplemental Tables

Table S1. Characteristics of huPB10 used in study

Test Parameters	Test Method	Result
Concentration	UV Absorbance	19.7 mg/mL
Appearance	Visible	clear, liquid
Physical/chemical	pH Determination	5.6
Purity	SDS-PAGE	> 99%
Osmolality	Micro-Osmometer	412 mOS/kg
Bioburden	Bioburden Testing	0 CFU/mL
Aggregates	Size Exclusion HPLC	1.90%
Potency	ELISA	20.0 mg/mL
Safety	Endotoxin	0.2 EU/mg
Binding affinity FAb	SPR	0.1 nM

Table S2. Cytokines, chemokines and growth factors in serum and BAL fluids in NHPs before and after RT Exposure							
		Serum (pg/ml)			BAL (pg/ml)		
Marker		**Pre Mean (SD)**	**Post Mean (SD)**	**Sig.**	**Pre Mean (SD)**	**Post Mean (SD)**	**Sig.**
EGF	Control	24.42 (25.79)	60.32 (35.16)	ns	OOR	OOR	
	12 h	48.11 (25.55)	68.26 (20.62)				
	4 h	65.17 (40.53)	79.23 (51.57)				
Eotaxin	Control	145.7 (81.76)	308.8 (226.07)	**	2.08 (1.81)	22.32(0)	****
	12 h	226.71 (93.3)	238.55 (110.35)		1.48 (0.59)	31.54(24.33)	
	4 h	168.55 (51.56)	228.01 (101.19)		1.79 (1.03)	9.47(7.09)	
GCSF	Control	82.45 (57.45)	960.56 (446.44)	****	51.58 (4.71)	113.9(0)	
	12 h	60.62 (14.35)	53.62 (5.46)	ns	53.91 (2.52)	165.25(203.54)	ns
	4 h	120.46 (87.63)	87.19 (44.08)	ns	51.42 (1.6)	58.67(3.43)	
GMCSF	Control	0.79 (0.07)	8.05 (3.22)	****	0.9 (0)	4.62(0)	
	12 h	6.24 (11.43)	6.37 (11.82)	ns	0.9 (0.15)	6.08(1.74)	****
	4 h	1.55 (1.16)	1.42 (0.84)	ns	1 (0.14)	2.75(0.81)	**
IFNG	Control	16.34 (14.84)	39.02 (17.24)	ns	9.38 (4.31)	8.33(0)	
	12 h	18.02 (9.46)	19.2 (13.81)		7.58 (4.12)	17.51(6.72)	****
	4 h	55.5 (96.74)	24.88 (33.52)		7.94 (1.83)	9.46(2.04)	ns
IL1B	Control	6.91 (1.07)	19.83 (8.96)	***	3.08 (0.57)	14.28(0)	
	12 h	10.82 (3.63)	10.66 (3.57)	ns	2.84 (0.76)	13.5(7.28)	****
	4 h	9.91 (2.03)	9.21 (1.16)	ns	2.83 (0.35)	8.07(5.19)	
IL1RA	Control	64.45 (2.09)	10236.15 (5777.8)	****	350.94 (218.76)	784.96(0)	
	12 h	157.75 (41.1)	658.56 (571)	**	389.57 (182.86)	2033.87(1452.68)	****
	4 h	424.59 (385.78)	486.84 (386.8)	ns	542.7 (280.29)	954.58(200.28)	
IL4	Control	10.78 (0.85)	16.79 (0.85)	ns	2.22 (0.86)	5.21(0)	
	12 h	7.78 (1.7)	7.05 (0.66)		2.33 (1.08)	7.58(2.77)	***
	4 h	13.91 (10.34)	9.22 (5.27)		1.97 (0.81)	3.18(1.01)	
IL5	Control	0.76 (0.11)	2.44 (0.85)	**	0.61 (0.06)	1.54(0)	
	12 h	2.2 (3.65)	2.36 (4.14)	ns	0.66 (0.06)	2.23(0.58)	****
	4 h	1.38 (1.59)	0.93 (0.62)	ns	0.61 (0.03)	0.87(0.16)	**
IL6	Control	<0.15 (>0)	11480.01 (13781.71)	****	6.79 (2.94)	1227.86(0)	
	12 h	<4.5 (>7.88)	30.4 (15.97)	***	5.61 (3.23)	3624.85(4992.29)	****
	4 h	<2.36 (>1.93)	21.91 (11.08)	**	5.1 (1.42)	245.22(160.02)	****
IL8	Control	66.05 (55.11)	17.41 (10.61)	***	OOR	OOR	
	12 h	106.38 (72.66)	<7.232 (>6.81)				
	4 h	125.69 (94.11)	23.88 (17.82)				
IL10	Control	2.12 (0.38)	5.7 (0.45)	ns	4.77 (0.44)	5.62(0)	
	12 h	6.2 (8.34)	6.72 (9.2)		4.73 (0.48)	8.11(2.75)	***
	4 h	5.58 (4.85)	3.94 (1.44)		4.66 (0.15)	4.93(0.26)	
IL12	Control	213.88 (76.76)	157.24 (21.3)	ns	OOR	OOR	

	12 h	193.94 (103.41)	116.75 (70.6)				
	4 h	940.17 (1580.7)	537.61 (824.06)				
ITAC	Control	97.18 (89.13)	81.72 (83.47)		27.63 (16.69)	14.72(0)	
	12 h	81.41 (30.96)	60.78 (37.75)	ns	24.13 (9.91)	73.27(40.05)	****
	4 h	94.68 (29.58)	88.09 (53.62)		22.36 (4.27)	85.91(36.89)	
MCP1	Control	264.65 (145.35)	3961.54 (3806.14)	***	217.82 (216.44)	186.51(0)	
	12 h	371.68 (156.45)	464.76 (235.77)	ns	212.57 (218.17)	3443.33(2964.01)	****
	4 h	279.43 (58.43)	453.72 (175.17)	ns	69.05 (61.77)	825.31(417.45)	
MDC	Control	190.63 (104.86)	180.1 (49.81)		157.27 (20.4)	162.21(0)	
	12 h	314.13 (172.27)	288.82 (156.34)	ns	156.37 (12.44)	878.8(732.01)	***
	4 h	714.1 (292.66)	712.17 (286.86)		162.16 (9.55)	709.91(255.63)	
MIF	Control	50.47 (58.19)	193.54 (156.16)		542.08 (210.65)	1121.5(0)	
	12 h	85.03 (50.83)	120.67 (18.81)	**	662.29 (157.49)	648.89(328)	ns
	4 h	96.53 (29.08)	131.14 (53.98)		563.96 (81.1)	943.56(185.48)	
MIP1A	Control	14.74 (3.54)	25.53 (5.25)		18.06 (0.71)	23.9(0)	
	12 h	18.34 (0.64)	18.6 (2.15)	ns	17.95 (0.87)	31.53(14.39)	***
	4 h	27.72 (25.98)	21.58 (11.9)		18.19 (0.34)	28.84(9.78)	
VEGF	Control	OOR	OOR		8.67 (6.32)	5.02(0)	
	12 h				6.94 (5.7)	10.14(2.37)	ns
	4 h				5.92 (2.76)	6.03(1.67)	
IP10	Control	OOR	OOR		1.67 (0.55)	2.47(0)	
	12 h				1.41 (0.27)	3.77(1.01)	***
	4 h				1.62 (0.27)	2.11(0.4)	ns
RANTES	Control	1655.57 (190.3)	1781.57 (149.72)		OOR	OOR	
	12 h	2231.2 (275.93)	2108.77 (264.27)	ns			
	4 h	2063.94 (491.1)	2048.85 (496.15)				

Mean concentrations (pg/ml) and standard deviations are shown for serum and BAL fluids from all three groups of animals in the study. Samples were collected 7 days prior to and 1-day post-RT challenge. When concentrations were below the measurable range of the standard curve they were replaced with the lowest measured value, as indicated in red text (e.g., IL6, IL8). Statistical significance of changes in pre- versus post-RT challenge for each analyte was determined by two-way repeated measures ANOVA. P-values were corrected with the Benjamini, Krieger, and Yekutieli method to control false discovery rate (ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$). In the case of significant interaction, pre- and post-challenge values were compared for each group via Tukey's multiple comparisons test. Only those analytes with measurable concentrations are shown; OOR indicates that >3 values were out of range for either serum or BALF, and thus the values was excluded.

Figure S1

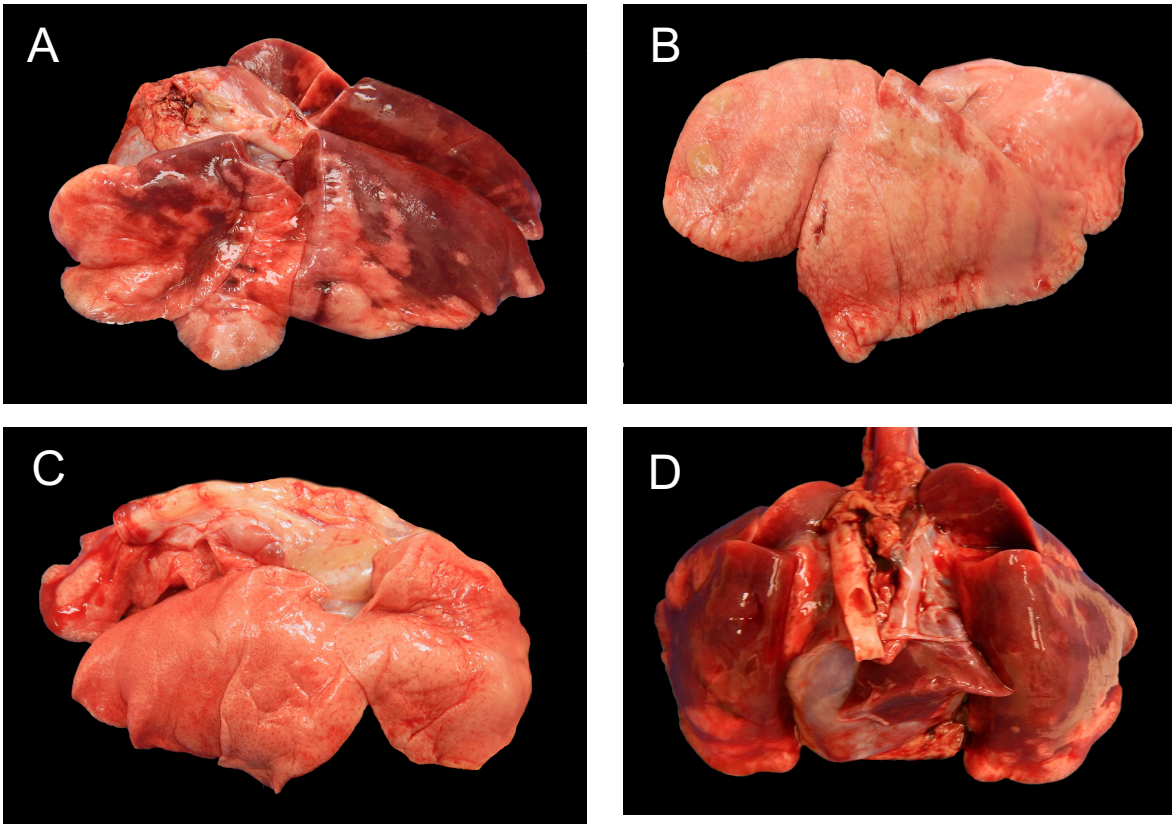
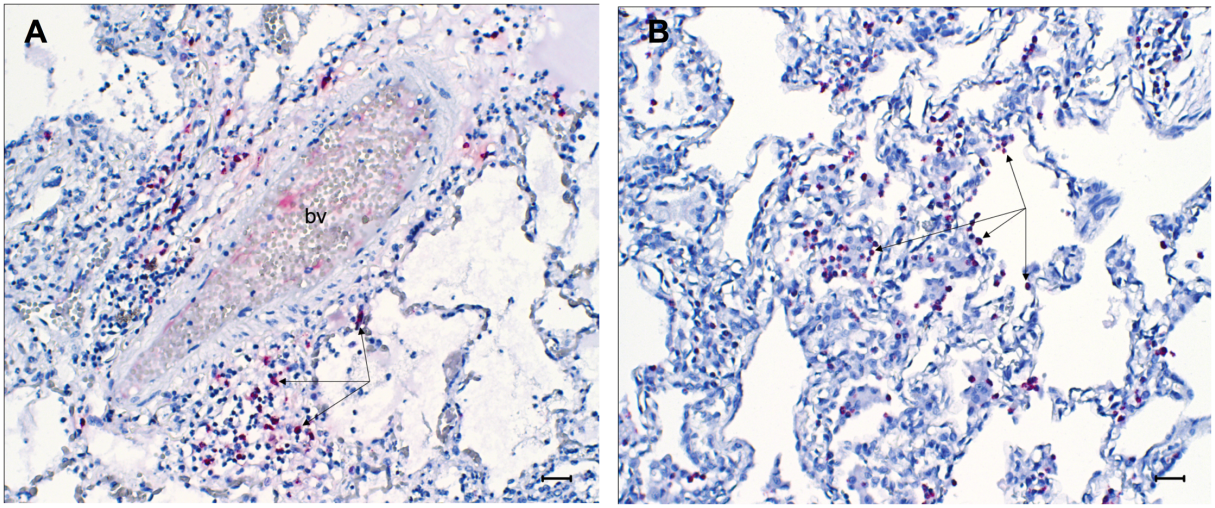


Figure S1. Gross pathology associated with RT exposure and huPB10 intervention. (A) Representative lungs from RT challenge. Sham-treated animals showed hallmarks of inflammation, marked edema, corresponding hemorrhage; grossly, wet weight was 3-times normal with coalescing hemorrhage. (B-C) Lungs from animals treated with huPB10 at 4 h that survived until day 14 when experiment was terminated. There was remarkably little pulmonary damage. Lung wet weights were essentially normal. (D) Lungs from RT challenged animal that was treated with huPB10 at 12 hours and succumbed to RT intoxication. The tissue had mild to moderate inflammation with edema, and punctate hemorrhage evident; gross lung weight was approximately 2x with clear signs of hemorrhage. Original magnification at 10x.

Figure S2



Supplement Figure 2. IHC analysis of lungs of ricin-treated and huPB10 intervention animals. (A) In ricin-treated animals, immunohistochemistry (IHC) with an eosinophil major basic protein antibody (crimson staining; arrows) revealed a mixed inflammatory infiltrate of eosinophils and PMNs in animals treated with RT. (B) In animals treated with huPB10 at 4 h, there were multifocal areas of moderate numbers of eosinophils, primarily in areas of fibrosis. Blood vessels, bv. Original magnification 20x; bar = 20 μm.

Figure S3

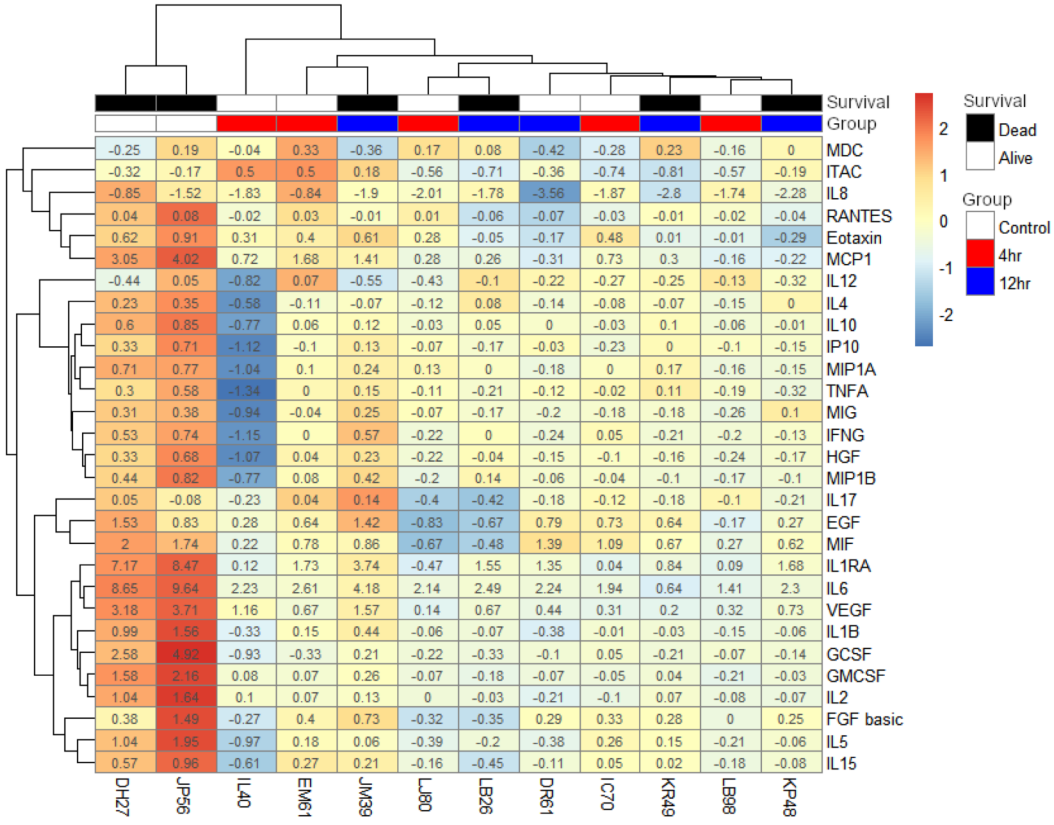


Figure S3. Scaled heatmap and cluster analysis of log_2-fold changes of inflammatory markers in serum of individual macaques following RT challenge. Heatmaps visualizing log_2-fold changes in cytokines, chemokines and growth factors in serum. Both individual animals and cytokines are arranged by hierarchical clustering, represented by dendrograms on top and to the right of the heatmaps, respectively. Surviving animals are marked in white in the top bar above the heatmap, while dead animals are in black. In the bar below this, the 4-hour group of animals are marked in red, the 12-hour group in blue, and the control group in green. Fold change values are centered and scaled for each analyte by first subtracting the mean fold change from each value and then dividing by the standard deviation of that analyte.

Figure S4

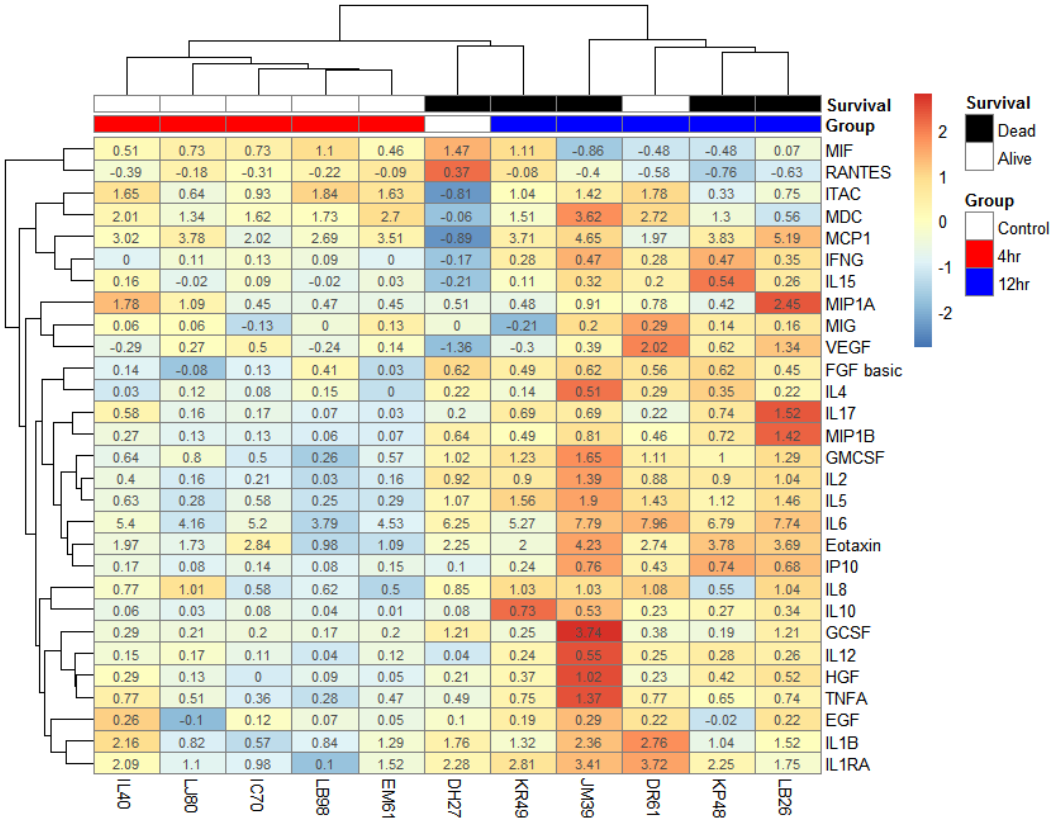


Figure S4. Scaled heatmap and cluster analysis of log2-fold changes of inflammatory markers in BALF of individual macaques following RT challenge. Heatmaps visualizing log_2-fold changes in cytokines, chemokines and growth factors in BAL. Both individual animals and analytes are arranged by hierarchical clustering, represented by dendrograms on top and to the right of the heatmaps, respectively, as described in the legend to Figure S3.