Supplemental Information

A STING-Adjuvanted Outer Membrane Vesicle Nanoparticle Vaccine against *Pseudomonas aeruginosa*

Elisabet Bjånes[1,#], Nishta Krishnan[2,#], Truman Koh[1], Anh T.P. Ngo[1], Jason Cole[1], Joshua Olson[1], Ingrid Cornax[1], Chih-Ho Chen[1], Natalie Chavarria[1,†], Samira Dahesh[1,†], Shawn M. Hannah[1,†], Alexandra Stream[1,†], Jiaqi Amber Zhang[1,†], Hervé Besançon[1,†], Daniel Sun[1], Siri Yendluri[1], Sydney Morrill[1,], Jiarong Zhou[2], Animesh Mohapatra[2], Ronnie H. Fang[2], Victor Nizet[1,3]

[1]Division of Host-Microbe Systems and Therapeutics, Department of Pediatrics, University of California San Diego, La Jolla, California, USA

[2]Aiiso Yufeng Li Family Department of Chemical and Nano Engineering, University of California San Diego, La Jolla, California, USA

[3]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, California, USA

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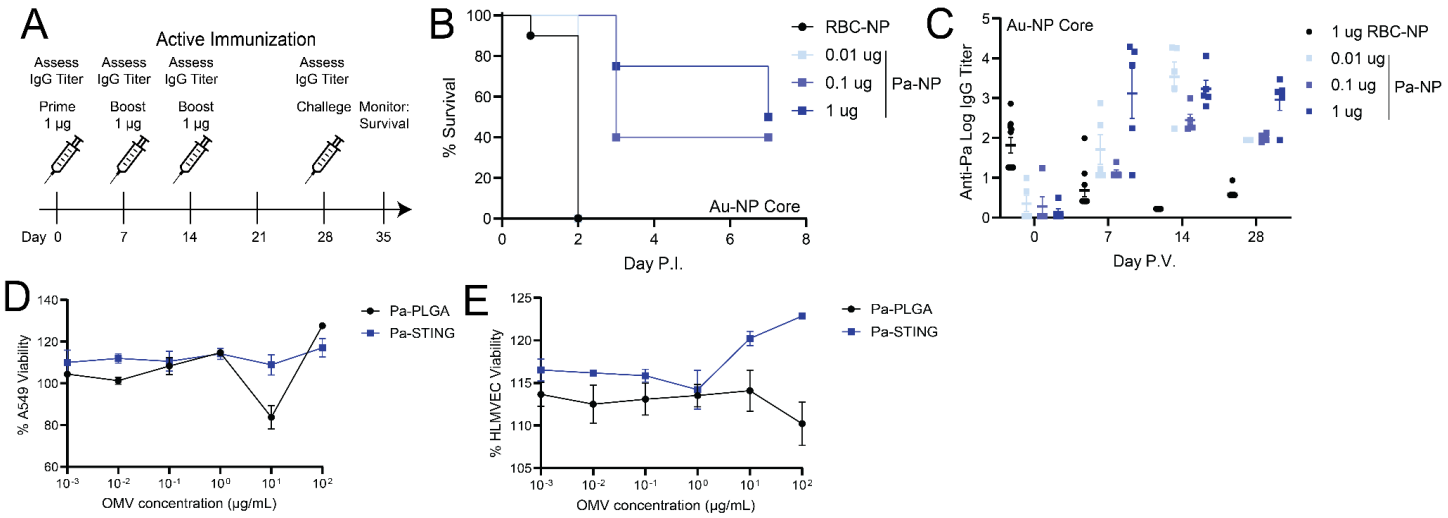
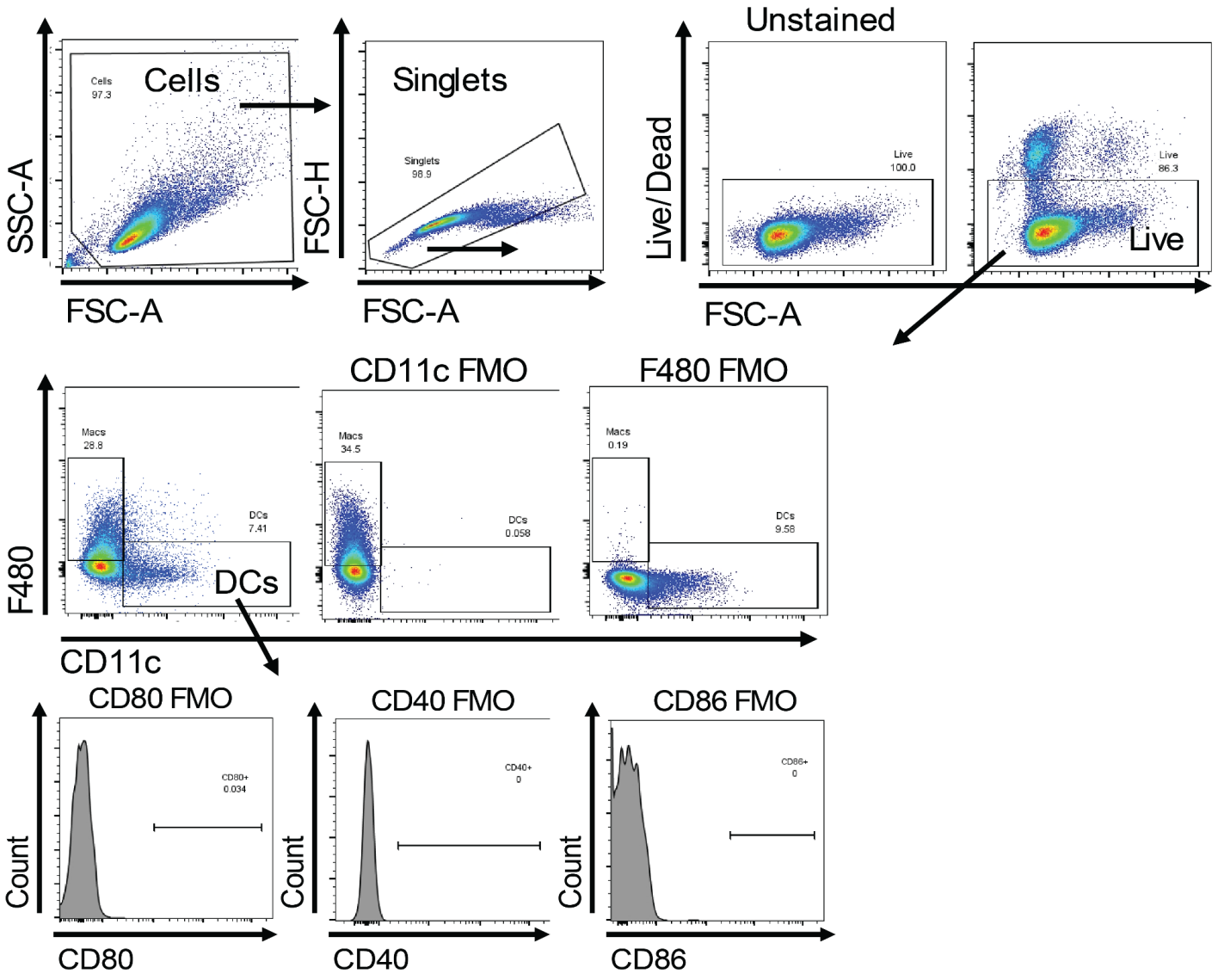
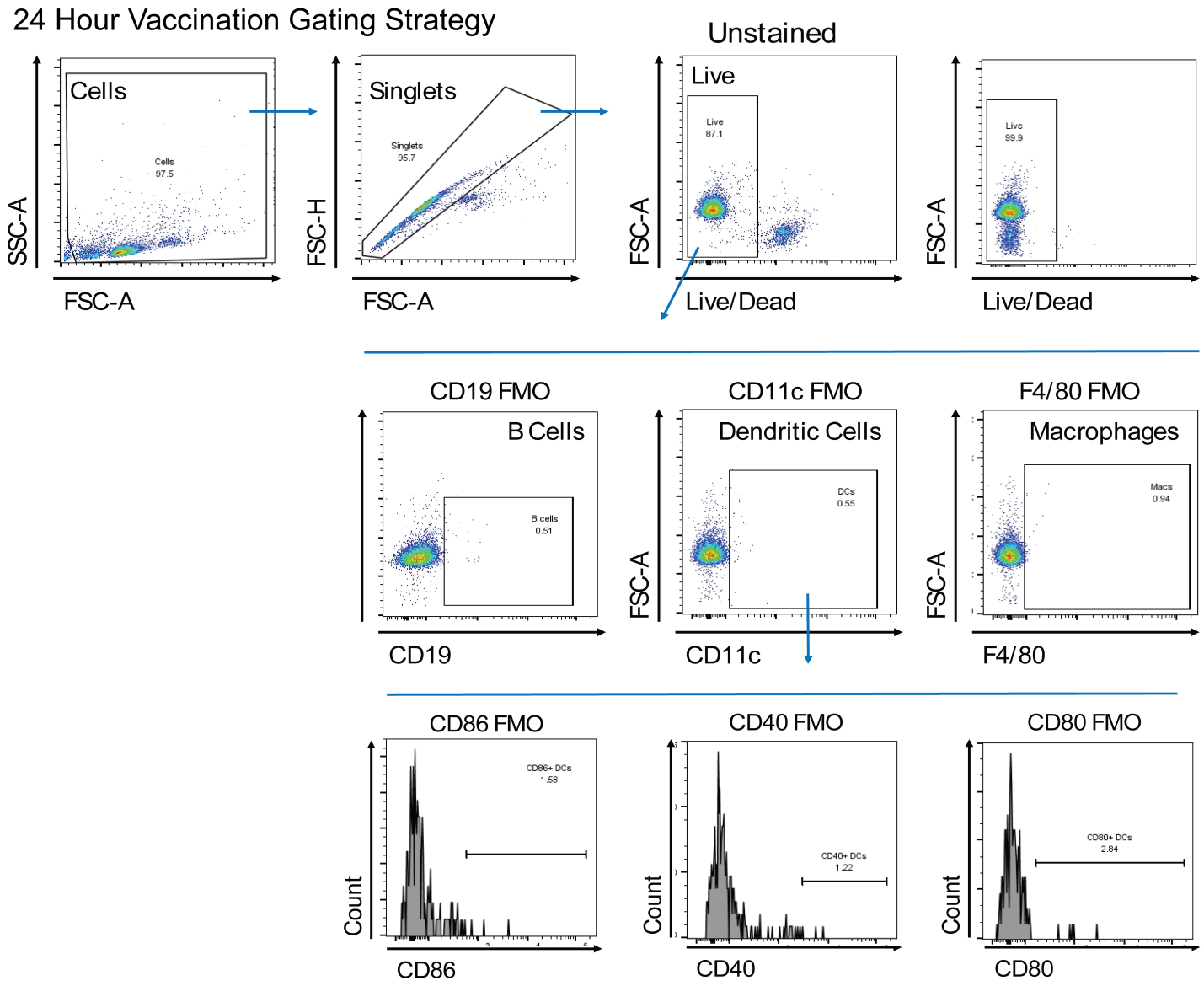


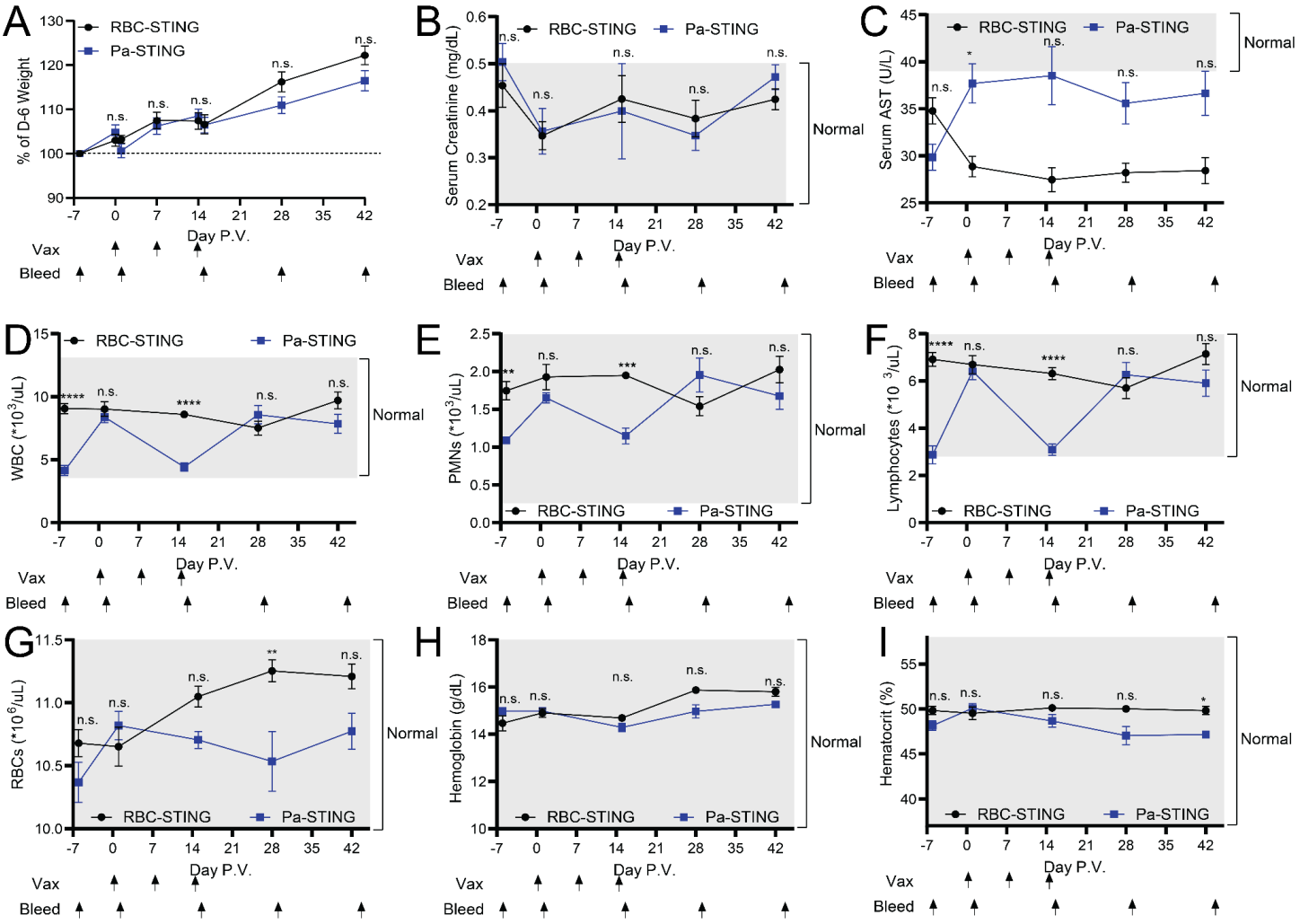
Supplemental Figure 1: Pa-NP vaccination with a gold core provides partial protection against lethal pneumonia. A) Active immunization scheme with RBC-NP and Pa-NP formulated with a gold core (Au-NP), Mice were immunized subcutaneously with 1 µg RBC-NP or Pa-NP. B) Mortality curves in mice vaccinated with 1 µg RBC-NP or Pa-NP and intratracheally infected with ~1 x 10^7 CFUs PA14 pneumonia. Mice were monitored for mortality for seven days. C) Anti-Pa IgG titers from B). Titers were assessed by mandibular cheek bleeding and ELISAs on day 0, 7, 14, and 28. Means ± SEM. n=5/group, representative of one experiment. D) % A549 or E) % human microvascular endothelial cell (HMVEC) viability after incubation with Pa-STING or Pa-PLGA particles for 48 hours. Cells were incubated with increasing concentrations of Pa-STING or Pa-PLGA particles and viability was measured by CytoTox 96 non-radioactive cytotoxicity assay. Representative of three independent experiments.



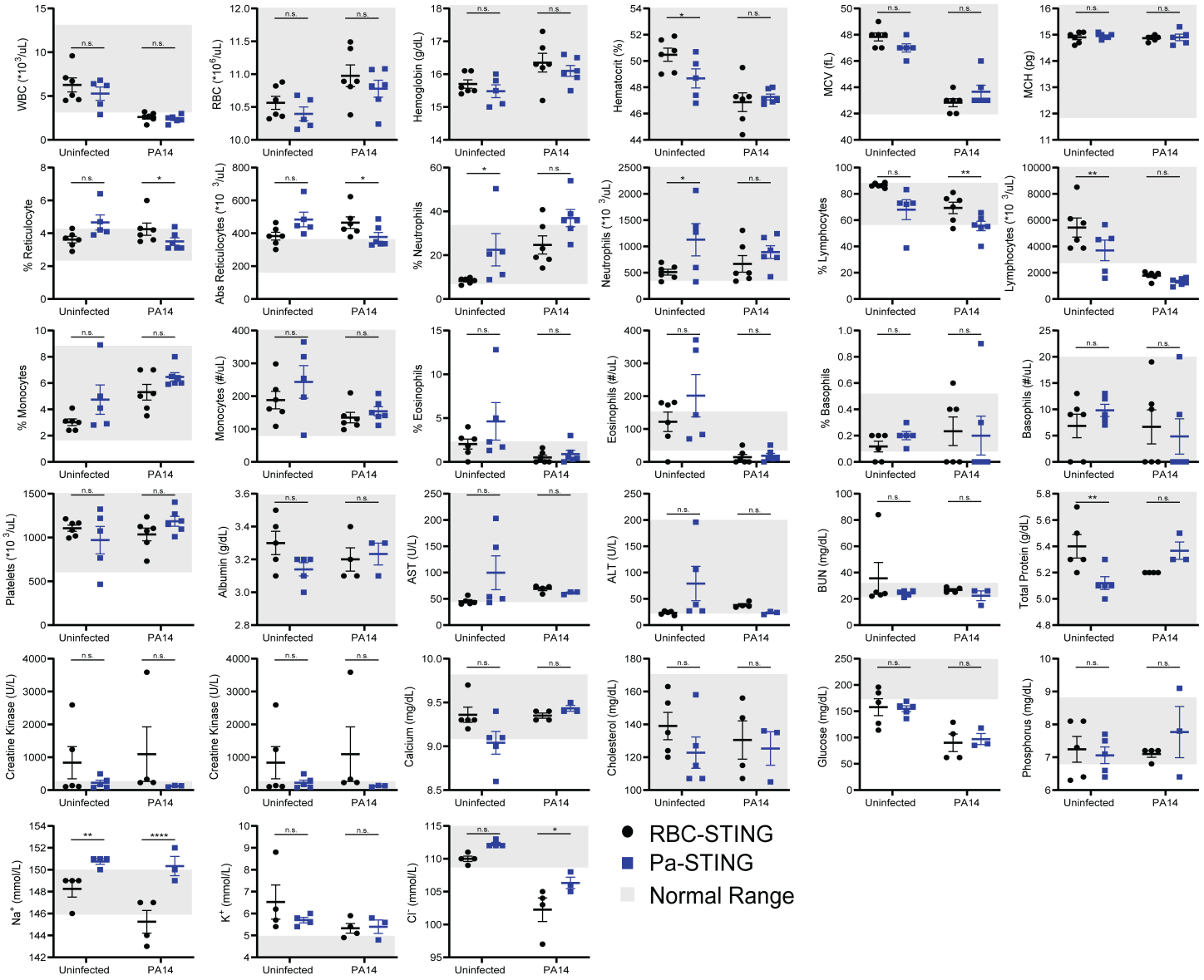
Supplemental Figure 2: Gating strategy for dendritic cell activation. For antigen presenting cell analysis, bone marrow derived dendritic cells were stimulated for 48 hours, harvested, stained and analyzed on a BD FACS Canto II. Cells were gated cells, singlets, live, $CD11c^{+}/F4/80^{-}$. Dendritic cells were further gated on CD86, CD40, and CD86. Gates were drawn with single-stained and unstained controls and with fluorescence minus ones.



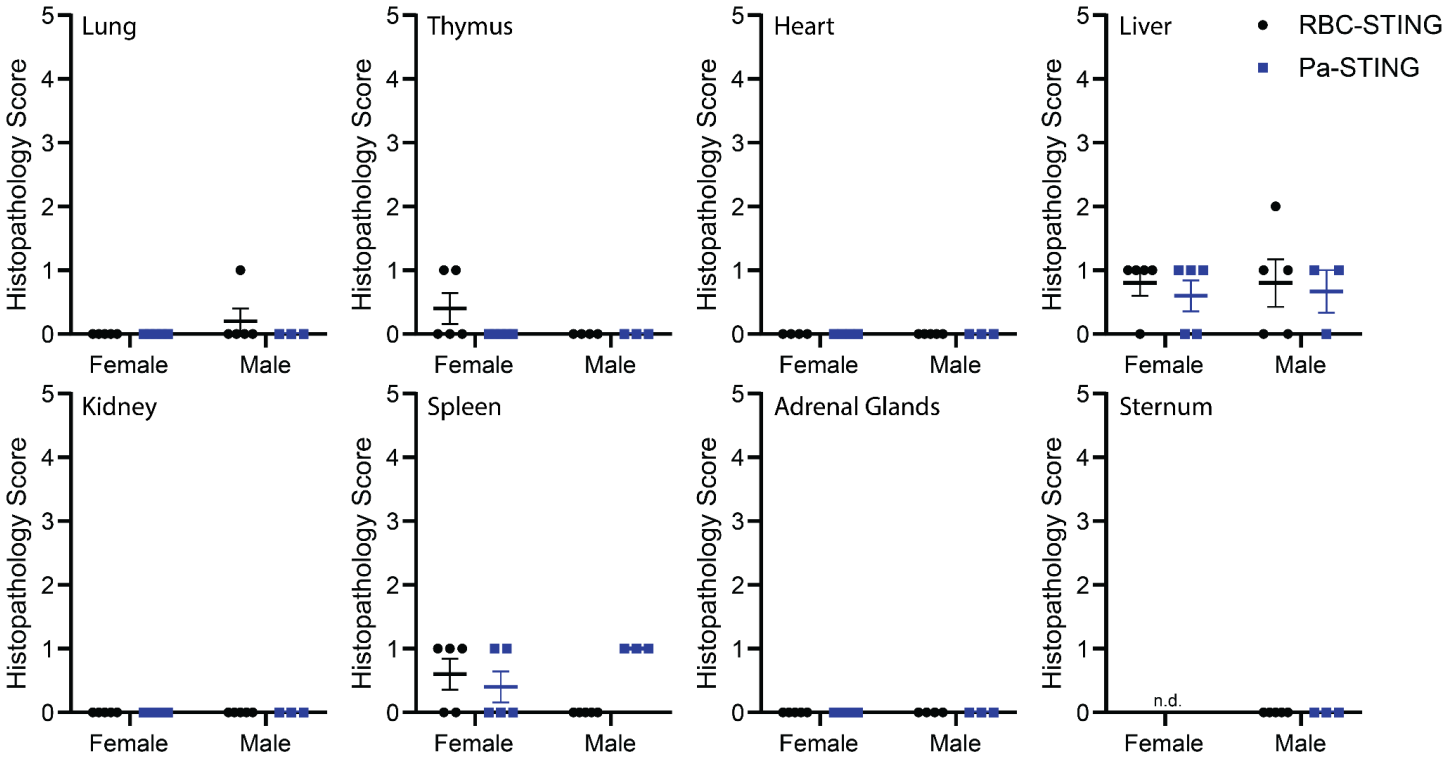
Supplemental Figure 3: Gating Strategy for ILN Flow Cytometry. For antigen presenting cell analysis, inguinal lymph nodes were processed for single cell isolation, stained and analyzed on a BD FACS Canto II. Cells were gated cells, singlets, live. B cells were gated $CD19^{+}$. Dendritic cells were gated $CD11c^{+}$. Macrophages were gated $F4/80^{+}$. Dendritic cells were further gated on CD86, CD40, and CD86. Gates were drawn with single-stained and unstained controls and fluorescence minus ones.



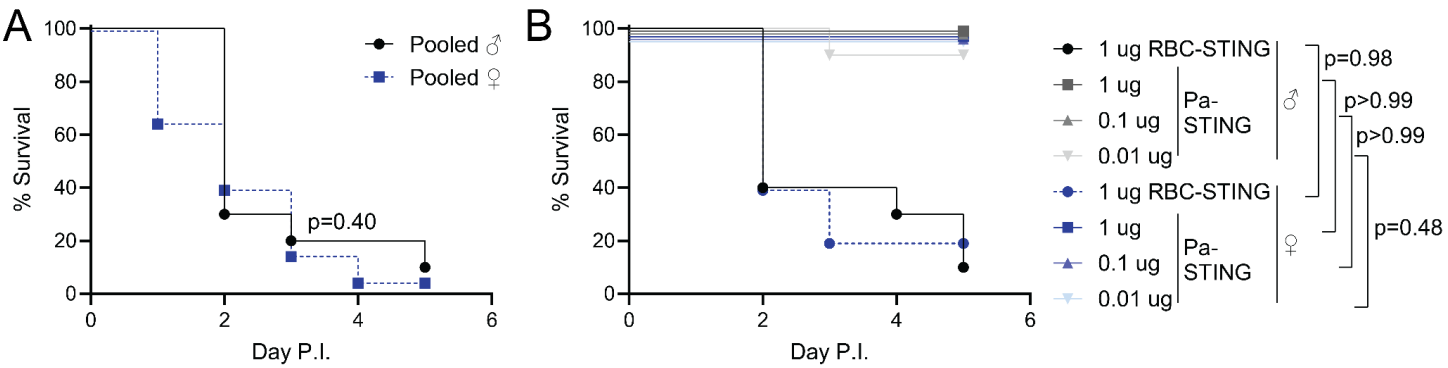
Supplemental Figure 4: Pa-STING vaccination does not induce toxicity in the hematology compartment. A) Percent weight gain during vaccination with 1 µg RBC-STING or Pa-STING. Mice were weighed on days -6, 0, 1, 7, 14, 15, 28, and 42. Serum B) creatinine and C) aspartate aminotransferase (AST) levels from mice vaccinated with 1 µg RBC-STING or PA-STING on days 0, 7, 14. Creatinine and AST levels were analyzed by Hemovet on days -6, 1, 15, 28, and 42. D-I) Hematology results from mice vaccinated with 1 µg RBC-STING or PA-STING on days 0, 7, 14. D) white blood count, E) polymorphonuclear cells (PMNs), F) lymphocytes, G) red blood cells (RBCs), H) hemoglobin, and I) hematocrit were analyzed by Hemovet on days -6, 1, 15, 28, and 42. Means ± SEM. n=5-10/group. (A-I) Two-way ANOVA with Sidak's multiple comparison's post-test. n.s. not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data is listed in Supplemental Table 1 and Supplemental Data File.



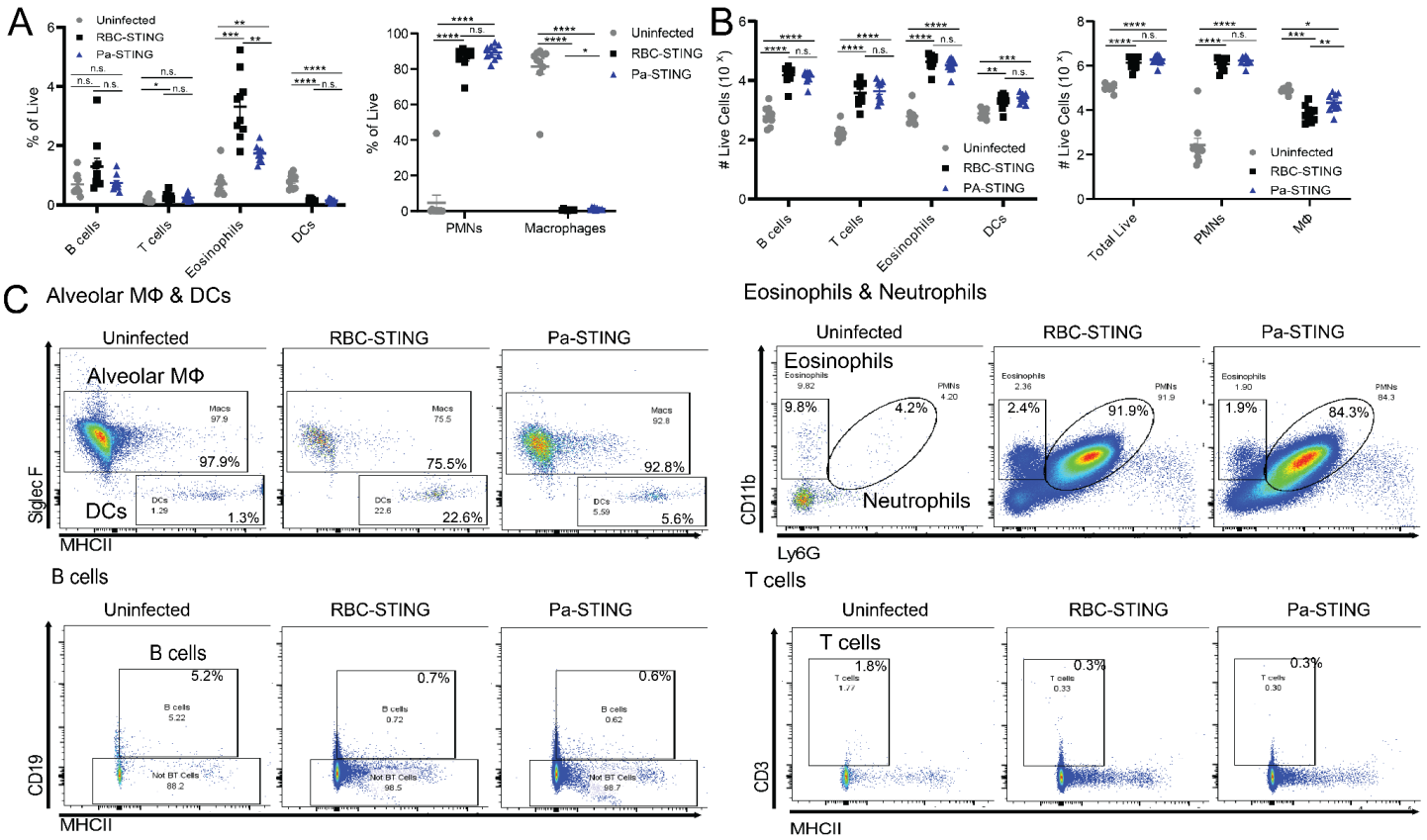
Supplemental Figure 5: Comprehensive serum chemistry and hematology from RBC-STING and Pa-STING vaccinated mice. Mice were vaccinated with 1 µg RBC-STING or Pa-STING on days 0, 7, and 14. Mice were infected with 0.5-1 x 10^7 CFUs PA14 or left uninfected on day 28. On Day 29 blood was collected by submandibular cheek bleeding and serum was isolated. Whole blood and serum were sent for comprehensive serum chemistry and hematology by IDEXX Technologies. Some samples were pooled from two mice to reach the minimum volume necessary. The grey box indicates the normal expected range for B6 mice. Means ± SEM. n=5-6/group, two independent experiments pooled. Mixed model two-way ANOVA with uncorrected Fisher's LSD post-test. n.s. not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data is listed in the Supplemental Data File.



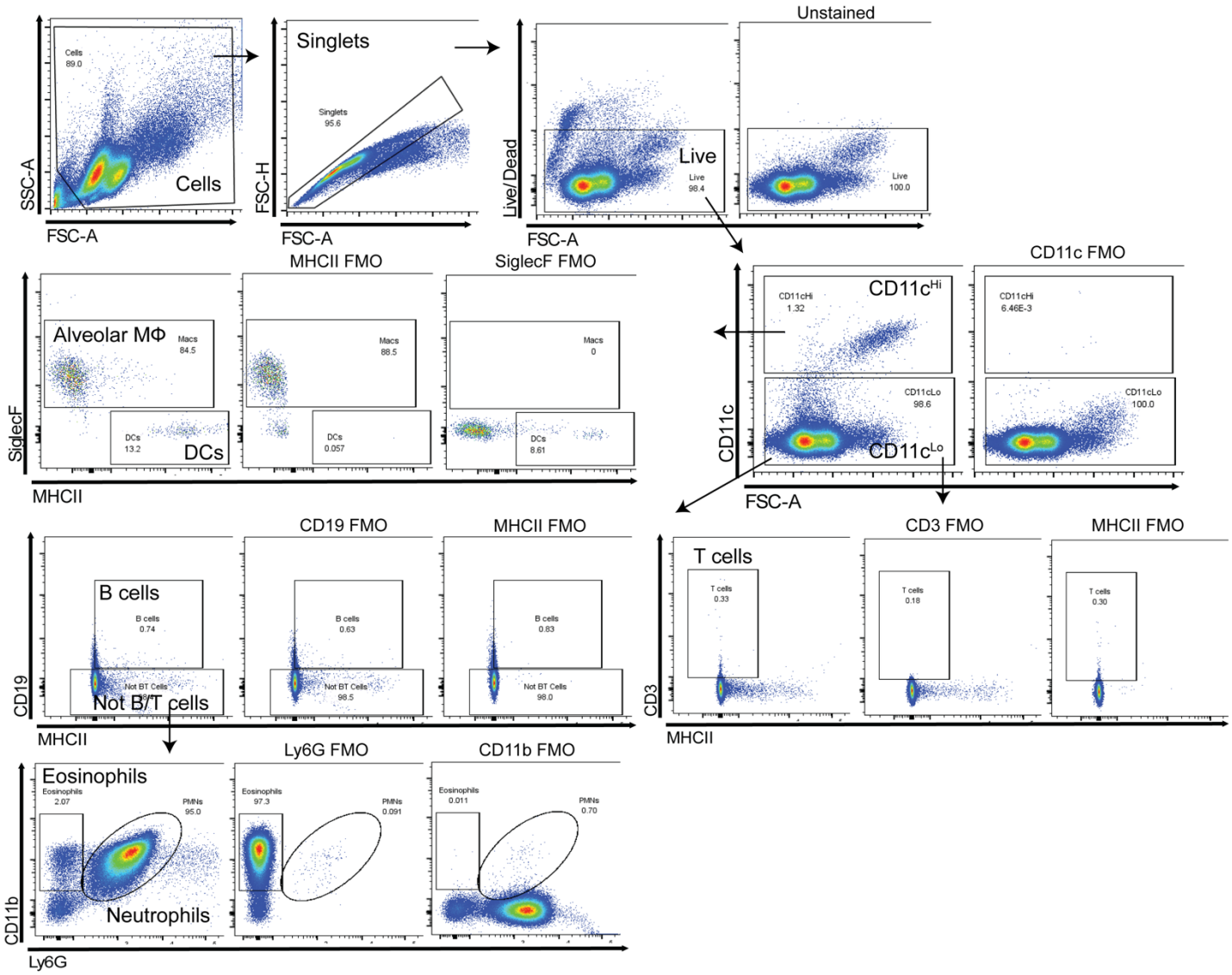
Supplemental Figure 6: Histopathology of RBC-STING and Pa-STING vaccinated mice. Mice were vaccinated with 1 μg RBC-STING or Pa-STING on days 0, 7, and 14. On Day 29 mice were humanely euthanized, the lungs were perfused with 10% formalin and removed along with the thymus, heart, liver, kidneys, spleen, adrenal glands, and sternum. Organs were fixed for 24 hours in 10% formalin, rinsed and stored to 70% EtOH prior to paraffin block embedding, sectioning, and H&E staining by the UCSD Biorepository and Tissue Technology Shared Resources. Sections were blindly scored by a board-certified veterinary pathologist. Two independent experiments pooled, n = 3-6/group.



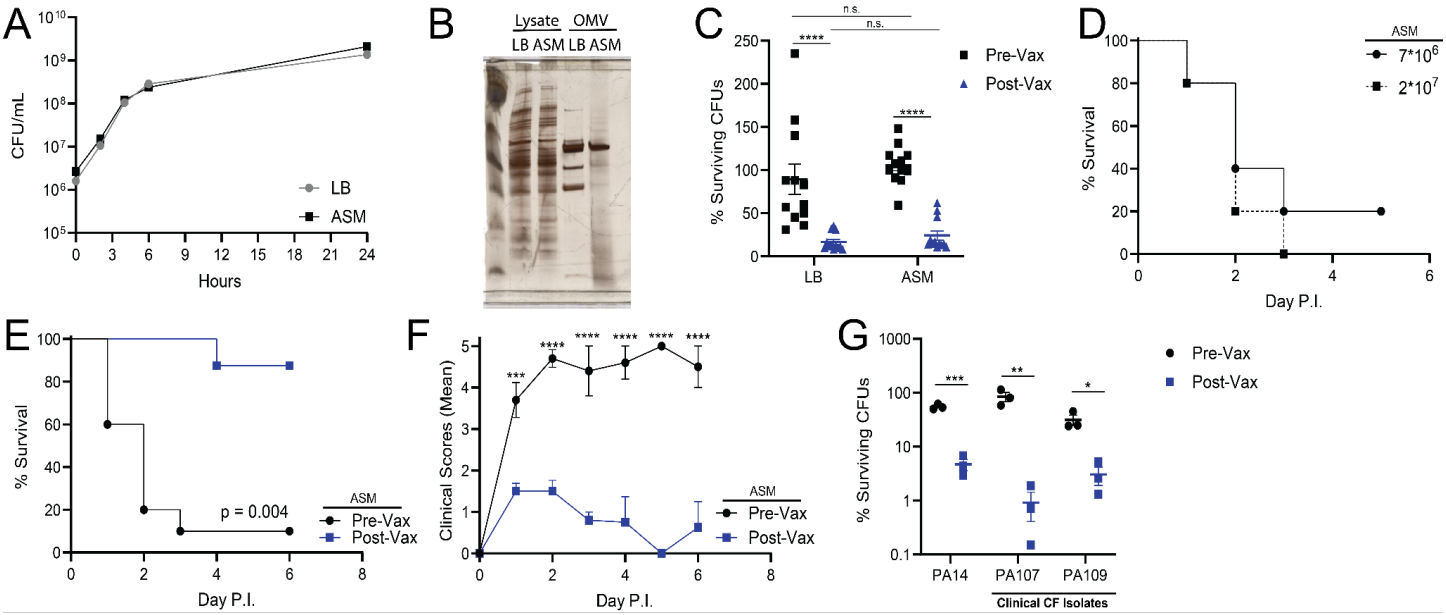
Supplemental Figure 7: No sex differences were observed in unvaccinated and vaccinated mice following intratracheal infection to PA14. A) Male and female survival data was pooled from **Figure 4B** and **4C** and analyzed using Kaplan-Meier (Log-rank). Median survival: male = 2.0 days, female = 2.0 days. B) Male and female survival data pooled from **Figure 4D** and **4E** and analyzed using Kaplan-Meier (Log-rank). Median survival: Male RBC-STING = 2.0 days, Female RBC-STING = 2.0 days. All other conditions had indeterminate median survival times.



Supplemental Figure 8: Immune infiltrating cells in BAL in RBC-STING and Pa-STING vaccinated and PA14 infected mice. Mice were vaccinated with 1 µg RBC-STING or Pa-STING weekly on days 0, 7 & 14, and infected with ~0.5-1 x 10^7 CFUs PA14 intratracheally on Day 28. RBC-STING vaccinated uninfected mice were used as a control. 20 hours p.i., mice were humanely euthanized and lung immune infiltrates were collected by BAL. Cells were stained, fixed and analyzed by flow cytometry. **A)** Quantification of cell populations as a percent of total live cells. **B)** Quantification of total number of live cells. A) & B) n = 10/group, two independent experiments pooled. Means ± SEM. **C)** Representative flow plots depicting cell populations in **A)** and **B)**. A gating strategy is depicted in **Supplemental Figure 9**. (A & B) Mixed model two-way ANOVA with Tukey's or Sidak's multiple comparisons post-test. n.s. not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



Supplemental Figure 9: Gating Strategy for BAL Flow Cytometry. For BAL infiltrating cell analysis, mice were humanely euthanized 24 hours post infection. BAL was collected, spun, stained and analyzed on a BD FACS Canto II. Gating strategy was based on the method by Van Hoecke *et al (1)*. Cells were gated cells, singlets, live, CD11c. Alveolar macrophages were gated $CD11c^{Hi}$, Siglec $F^{+}/MHCII^{-}$. Dendritic cells were gated $CD11c^{Hi}$, Siglec $F^{-}/MHCII^{+}$. T cells were gated $CD11c^{Lo}$, $CD3^{+}/MHCII^{-}$. B cells were gated $CD11c^{Lo}$, $CD19^{+}/MHCII^{+}$. Eosinophils were gated $CD11c^{Lo}$, $CD19^{-}$, $Ly6G^{-}/CD11b^{+}$. Neutrophils were gated $CD11c^{Lo}$, $CD19^{-}$, $Ly6G^{+}/CD11b^{+}$. Gates were drawn with single-stained and unstained controls and FMOs.



Supplemental Figure 10: Pa-STING vaccination provides protection against PA14 grown in artificial sputum media. A) Growth curves of PA14 grown in Luria broth (LB) or artificial sputum media (ASM). Representative of 2 independent experiments. B) Protein loading of LB vs ASM grown PA14 whole cell lysates and OMVs. ~10 μg of protein or lysate measured by BCA assay was loaded per lane and visualized with silver stain. C) Opsonophagocytic killing of PA14 grown in either LB or ASM by healthy human neutrophils incubated with pre-vax or post-vax rabbit serum. Percent surviving colony forming units (CFUs) relative to starting inputs is graphed. Pooled means of technical replicates from 2 independent experiments ± SEM. D) Survival curve of unvaccinated mice infected intratracheally with 0.7-2 x 10^7 CFUs PA14 grown in ASM. E) Survival curves from mice passively vaccinated with pre-vax or post-vax serum 72 hours prior to intratracheal infection with ~0.5-1 x 10^7 CFUs PA14 grown in ASM media. Mice were monitored twice daily for 5-7 days. n = 8-10/group, two independent experiments pooled. F) Clinical scores (means ± SEM) from mice passively immunized with pre-vax or post-vax serum and infected intratracheally with PA14 grown in ASM media. G) Opsonophagocytic killing of PA14 or clinical cystic fibrosis isolates PA107 and PA109 by healthy human neutrophils incubated with pre-vax or post-vax rabbit serum. Percent surviving colony forming units (CFUs) relative to starting inputs is graphed. C) Mixed model two-way ANOVA with Tukey's multiple comparisons post-test. E) Kaplan Meier (Log-Rank) test. F) Mixed model two-way ANOVA with Sidak's multiple comparisons post-test. G) Unpaired two-tailed Student's *t*-test. n.s. not significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplemental Table 1: Pa-STING vaccination does not induce toxicity in the hematology compartment.

WBC (10^3/uL)						WBC (%)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	9.056	0.4076	4.13	0.39563	<0.0001	-6	9.056	0.4076	4.13	0.39563	<0.0001
1	9.022	0.57995	8.376	0.45369	0.9225	1	9.022	0.57995	8.376	0.45369	0.9225
15	8.594	0.26322	4.42	0.33839	0.0002	15	8.594	0.26322	4.42	0.33839	0.0002
28	7.514	0.55218	8.566	0.72709	0.6261	28	7.514	0.55218	8.566	0.72709	0.6261
42	9.706	0.67084	7.844	0.75556	0.1151	42	9.706	0.67084	7.844	0.75556	0.1151
PMNs (10^3/uL)						PMNs (%)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	1.747	0.12195	1.089	0.03411	0.0084	-6	19.333	1.14094	27.41	1.9646	0.0524
1	1.928	0.16791	1.652	0.06472	0.595	1	21.291	0.94163	19.804	0.55751	0.7103
15	1.949	0.04534	1.148	0.10427	0.001	15	22.736	0.61595	26.064	1.53537	0.4018
28	1.54	0.12759	1.955	0.2262	0.184	28	20.512	0.76905	22.667	1.42202	0.7289
42	2.026	0.17707	1.675	0.17499	0.3405	42	20.854	0.88364	21.352	0.64677	0.9956
Lymphocytes (10^3/uL)						Lymphocytes (%)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	6.914	0.28951	2.869	0.38045	<0.0001	-6	76.42	0.86052	68.193	2.35228	0.103
1	6.695	0.39156	6.408	0.36339	0.9919	1	74.355	1.00503	76.47	0.37278	0.4245
15	6.314	0.259	3.089	0.24714	<0.0001	15	73.383	0.72448	69.798	1.98386	0.5558
28	5.7	0.4427	6.265	0.52641	0.864	28	75.784	0.78991	73.253	1.47895	0.6316
42	7.14	0.45044	5.909	0.5629	0.1736	42	73.766	1.34174	75.354	0.66602	0.8658
Monocytes (10^3/uL)						Monocytes (%)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	0.336	0.07231	0.138	0.00644	0.0013	-6	3.624	0.67849	3.415	0.38315	0.9997
1	0.345	0.03581	0.289	0.03367	0.7859	1	3.83	0.26487	3.412	0.22111	0.7801
15	0.278	0.03019	0.146	0.02561	0.0543	15	3.245	0.3721	3.309	0.47748	>0.9999
28	0.252	0.01356	0.258	0.02663	>0.9999	28	3.416	0.29992	3.043	0.2993	0.925
42	0.345	0.03138	0.223	0.03393	0.088	42	3.518	0.12141	2.788	0.25565	0.2001
Eosinophils (10^3/uL)						Eosinophils (%)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	0.041	0.01427	0.025	0.00447	>0.9999	-6	0.449	0.14985	0.688	0.121	0.7648
1	0.4	0.26968	0.268	0.0142	0.8233	1	0.4	0.26968	0.268	0.0142	0.9948
15	0.039	0.0156	0.031	0.00678	>0.9999	15	0.483	0.20024	0.656	0.09921	0.9578
28	0.018	0.00339	0.072	0.02601	0.9958	28	0.25	0.05753	0.828	0.29223	0.4698
42	0.151	0.05073	0.03	0.00474	0.8695	42	1.459	0.41514	0.426	0.08549	0.2911
Basophils (10^3/uL)						Basophils (%)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	0.015	0.00652	0.01	0.00354	0.9958	-6	0.172	0.06486	0.293	0.07957	0.7979
1	0.012	0.00718	0.004	0.00187	0.9656	1	0.125	0.06762	0.043	0.00982	0.8244
15	0.012	0.00957	0.007	0.00122	0.9958	15	0.156	0.11646	0.174	0.03562	>0.9999
28	0.001	0.001	0.018	0.00735	0.5483	28	0.036	0.01111	0.21	0.08404	0.4321
42	0.043	0.02004	0.004	0.001	0.0078	42	0.401	0.169	0.078	0.01158	0.4974
RBCs (10^6/uL)						Platelets (10^3/uL)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	10.679	0.10612	10.368	0.16004	0.4325	-6	608.1	16.9192	420.5	32.9981	0.0117
1	10.652	0.1544	10.819	0.11111	0.9094	1	643.9	16.1736	635.2	21.9696	0.9992
15	11.05	0.08204	10.706	0.0677	0.3255	15	688.1	21.7373	585.6	63.7465	0.6502
28	11.253	0.08773	10.534	0.23711	0.0023	28	768.2	15.9833	727.3	67.833	0.9878
42	11.209	0.09861	10.774	0.14135	0.1252	42	705.7	24.6939	669.1	52.768	0.9824
Hemoglobin (g/dL)						Hematocrit (%)					
	RBC		PA				RBC		PA		
Day	Mean	± SEM	Mean	± SEM	p value	Day	Mean	± SEM	Mean	± SEM	p value
-6	14.47	0.32117	14.98	0.14883	0.6779	-6	49.81	0.47021	48.16	0.54139	0.2303

1	14.9	0.18908	14.98	0.09028	0.9982	**1**	49.52	0.70331	50.08	0.53024	0.9803
15	14.69	0.06782	14.29	0.17847	0.3721	**15**	50.11	0.36242	48.66	0.70735	0.4668
28	15.87	0.06819	14.97	0.27776	0.1395	**28**	50.01	0.40847	47.04	1.00603	0.1791
42	15.8	0.19685	15.27	0.13657	0.2736	42	49.81	0.457	47.16	0.38968	0.0119

MCV (fL)						MCH (Pg)					
	RBC		PA				RBC		PA		
Day	**Mean**	**± SEM**	**Mean**	**± SEM**	**p value**	**Day**	**Mean**	**± SEM**	**Mean**	**± SEM**	**p value**
-6	46.65	0.39718	46.46	0.24052	0.9974	-6	13.57	0.36042	14.45	0.12748	0.3034
1	46.48	0.27046	46.3	0.08367	0.9824	1	13.99	0.14612	13.84	0.14089	0.9624
15	45.34	0.21413	45.44	0.54369	>0.9999	15	13.31	0.09798	13.35	0.16733	>0.9999
28	44.44	0.16233	44.67	0.51904	0.9972	28	14.1	0.07906	14.22	0.1437	0.9658
42	44.45	0.23184	43.81	0.42024	0.7277	42	14.1	0.16508	14.19	0.2176	0.999

Serum Creatinine (mg/dL)						Serum AST (U/L)					
	RBC		PA				RBC		PA		
Day	**Mean**	**SEM**	**Mean**	**SEM**	**p value**	**Day**	**Mean**	**SEM**	**Mean**	**SEM**	**p value**
-6	0.45357	0.04669	0.50357	0.03929	0.9434	-6	34.7672	1.39227	29.8479	1.4087	0.1756
1	0.34643	0.02963	0.35625	0.04877	>0.9999	1	28.8573	1.10811	37.691	2.08453	0.0458
15	0.425	0.05018	0.39911	0.10125	0.9998	15	27.4607	1.28956	38.5227	3.09373	0.0934
28	0.38304	0.03885	0.34732	0.03171	0.9679	28	28.2072	1.01453	35.5961	2.19798	0.1159
42	0.42411	0.02223	0.47143	0.02628	0.6874	42	28.4201	1.39846	36.6436	2.33341	0.1004

Serum ALT (U/L)					
	RBC		PA		
Day	**Mean**	**SEM**	**Mean**	**SEM**	**p value**
-6	11.6581	0.98838	11.4282	0.61796	>0.9999
1	9.16728	0.25087	7.90693	2.22286	0.9901
15	8.59246	0.30922	8.00912	0.41975	0.83
28	7.30231	1.52722	9.53345	0.64389	0.7332
42	9.43551	0.61964	11.5432	0.78617	0.3051

Means ± SEM for hematology analysis conducted on plasma obtained by submandibular cheek bleeding from mice vaccinated with 1 µg RBC-STING or Pa-STING subcutaneously on days 0, 7, and 14. Plasma and serum were obtained on day -6, 1, 15, 28, and 42. Serum creatinine and aspartate aminotransferase (AST) were assessed by colorimetric kits according to the manufacturer's instructions. All other values were obtained by Hemavet analysis.

REFERENCE

1. Van Hoecke L, Job ER, Saelens X, Roose K. Bronchoalveolar lavage of murine lungs to analyze

inflammatory cell infiltration. *J Vis Exp*. 2017;(123):e55398.