Supplemental Figures

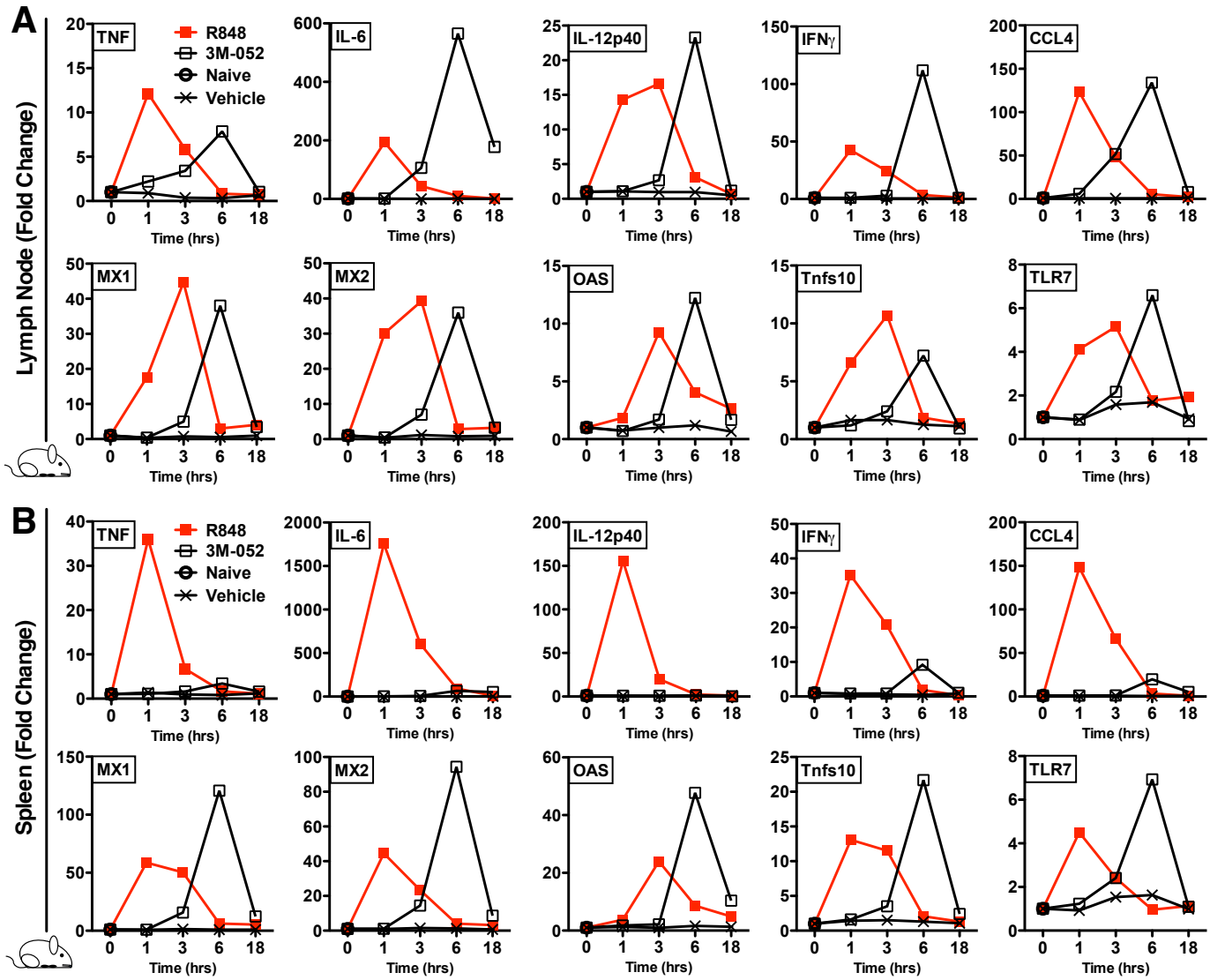
TLR7/8 Adjuvant Overcomes Newborn Hyporesponsiveness to Pneumococcal Conjugate Vaccine at Birth

Dowling *et al*., JCI Insight, 2016

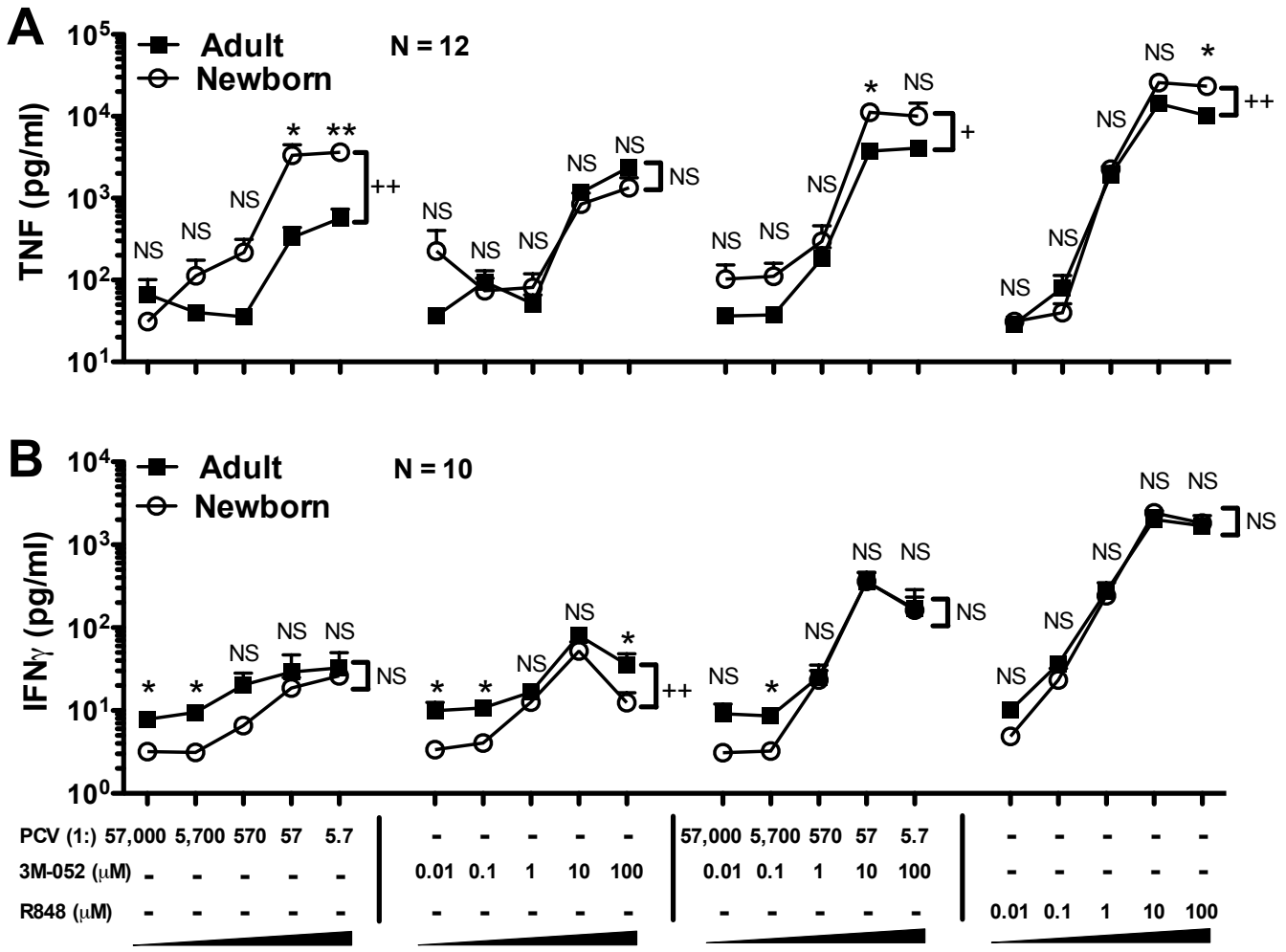
Supplemental Table 1. 3M-052 oil-in-water emulsion (O/W) size details.

Name	TLR	Ave. Diameter (nm)	PdI Index	Endotoxin-LAL Assay (EU/ml)
O/W (vehicle)	N/A	129	0.23	< 1
3M-052 (0.01 mg/kg) O/W	7/8	134	0.18	< 1
3M-052 (0.1 mg/kg) O/W	7/8	141	0.19	< 1

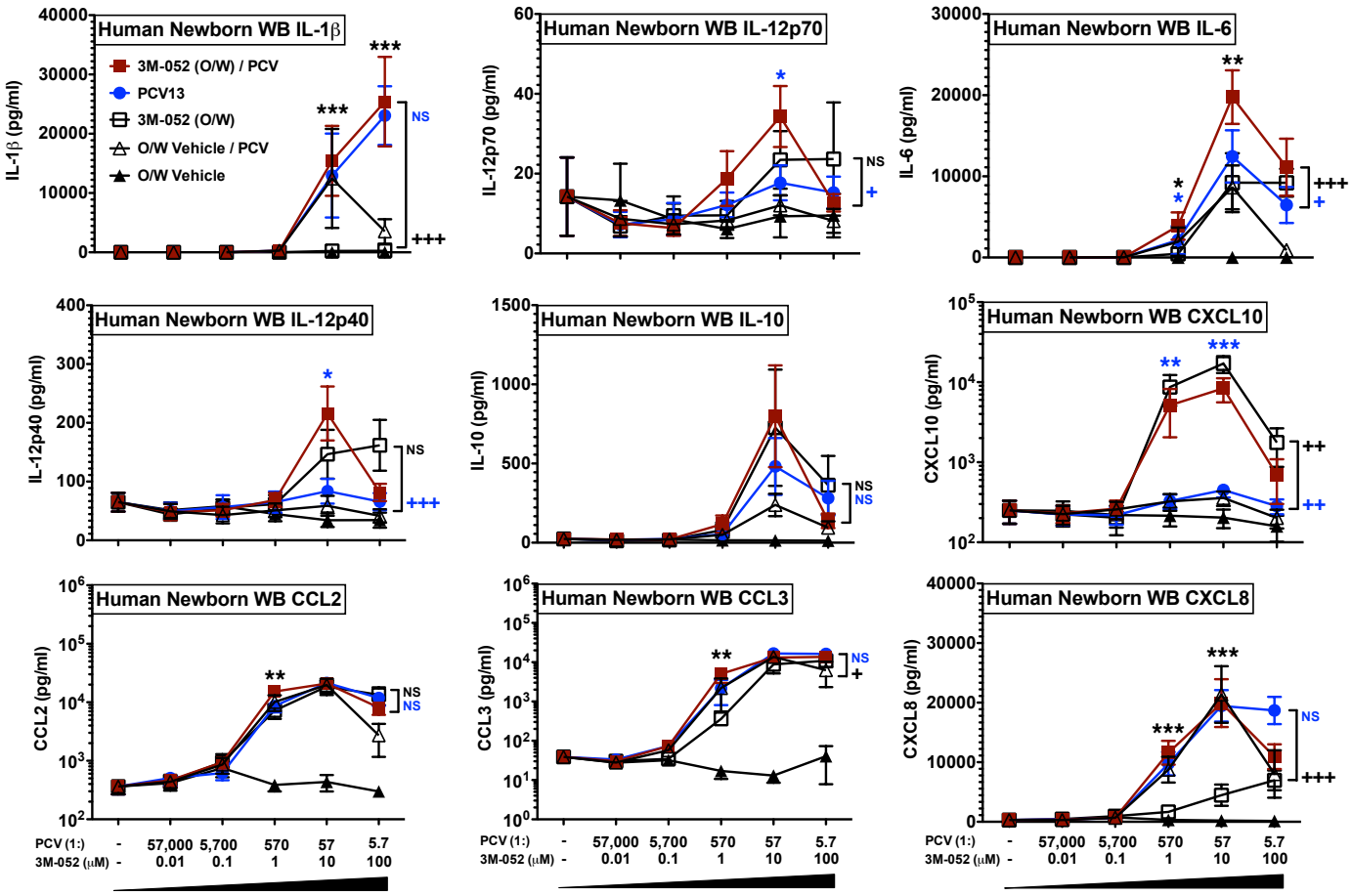
PdI: Polydispersity; TLR: Toll-like receptor; LAL: Limulus amebocyte lysate; EU: Endotoxin Units; O/W: Oil-in-Water.



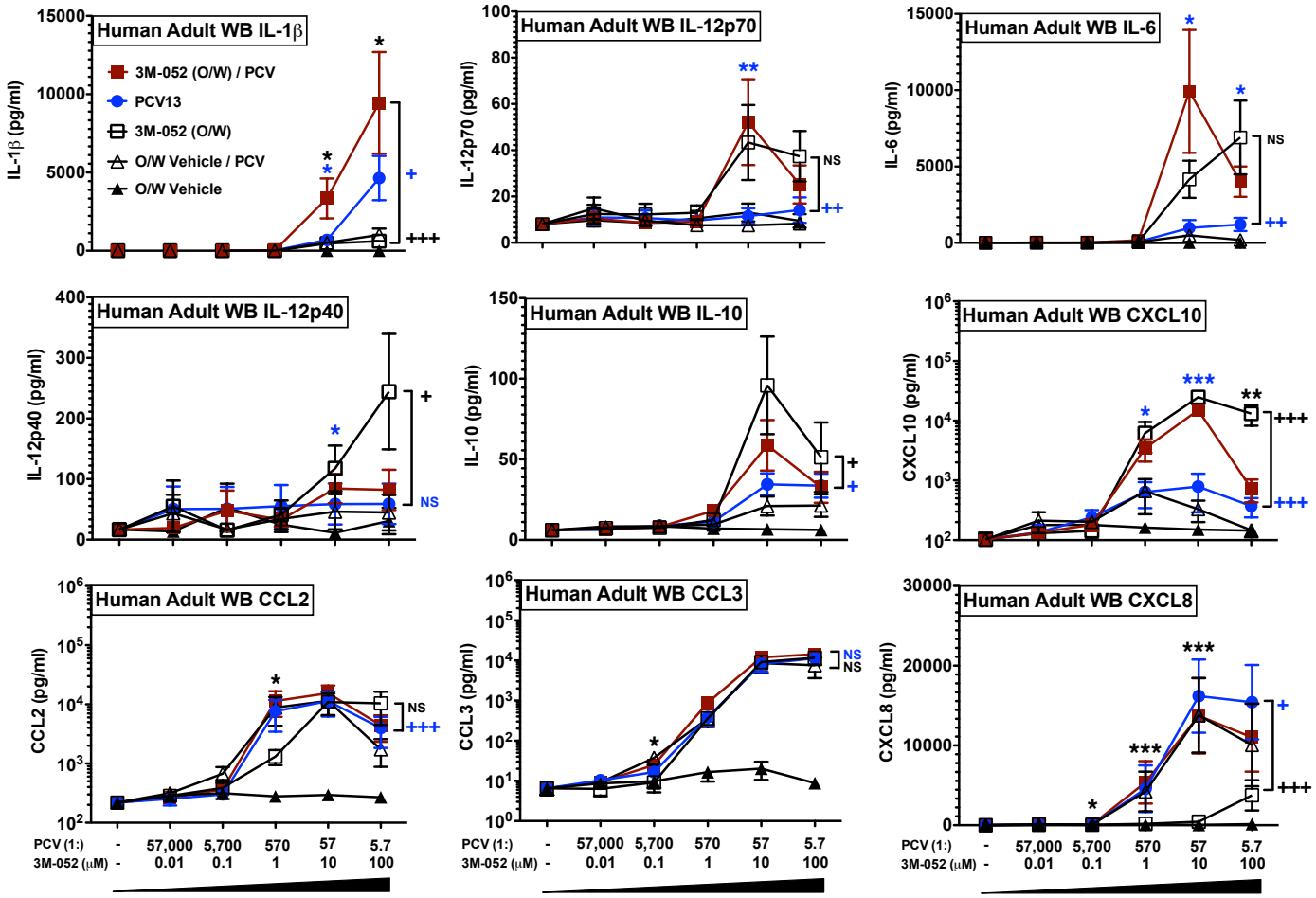
Supplemental Figure 1. Cytokine and IFN-inducible gene expression following free or lipidated TLR7/8 imidazoquinoline subcutaneous injection. Mouse mRNA expression is depicted in (A) draining lymph nodes (brachial and axillary), and (B) spleen post a single subcutaneous injection of 3M-052 or R848 formulated (both 1 mg/kg, (20 µg/mouse)) in oil-in-water emulsion (O/W) (vehicle) to the scruff of the neck. Data represents relative fold-change gene expression (i.e., treatment relative expression / untreated relative expression) (n = 3).



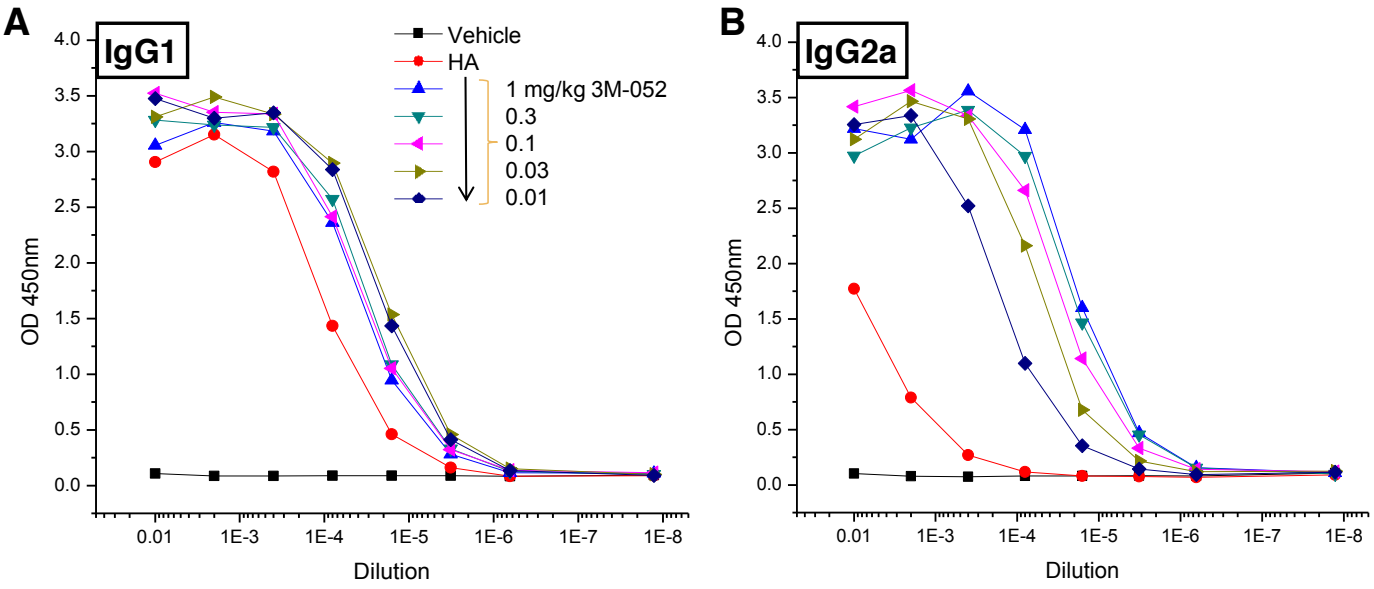
Supplemental Figure 2. Addition of 3M-052 to PCV13 enhances TNF and IFNγ responses in newborn cord blood. Human neonatal and adult blood was cultured for 6 hours with sterile buffer control (RPMI, not shown), PCV13 (1:5.7 – 57,000 v/v), 3M-052 or R848 (both 0.01, 0.1, 1, 10, 100 μM), or (PCV13+3M-052). Supernatants were collected for ELISA and multiplex assay. Mean ± SEM of agonist-induced cytokine production are shown for (A) TNF (n = 12) and (B) IFNγ (n = 10). For comparisons between overall groups (e.g., newborn vs. adult), two-way repeated measures ANOVA for non-parametric sample populations were applied and statistical significance denoted as +$p < 0.05$, ++$p < 0.01$. For comparison at individual concentrations, the unpaired Mann-Whitney test was applied and statistical significance denoted as *$p < 0.05$, **$p < 0.01$, or NS (not significant). Results represent means ± SEM.



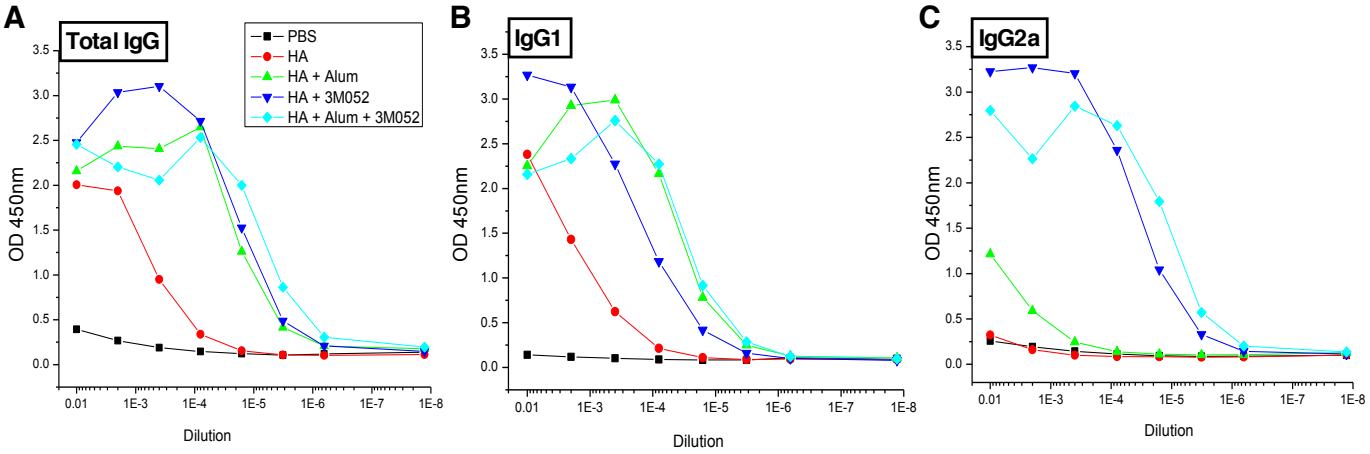
Supplemental Figure 3. Human newborn whole blood cytokine responses to 3M-052, PCV13, and (PCV13 + 3M-052). Human cord blood cultured for 6 hours with O/W vehicle, PCV13 alone (1:5.7 – 57,000 v/v), 3M-052 alone (0.01, 0.1, 1, 10, 100 μM) and concentration dependent combinations of each. Supernatants were collected for ELISA and multiplex assay. Mean ± SEM of agonist-induced cytokine production are shown (n = 8 – 10). For comparisons between overall groups (e.g., PCV13 vs. (PCV13 + 3M-052)), two-way repeated measures ANOVA for non-parametric sample populations were applied and statistical significance denoted as +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$. For comparison at individual concentrations, the unpaired Mann-Whitney test was applied and statistical significance denoted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Results represent means ± SEM, with p values indicating significance as compared to that colored group.



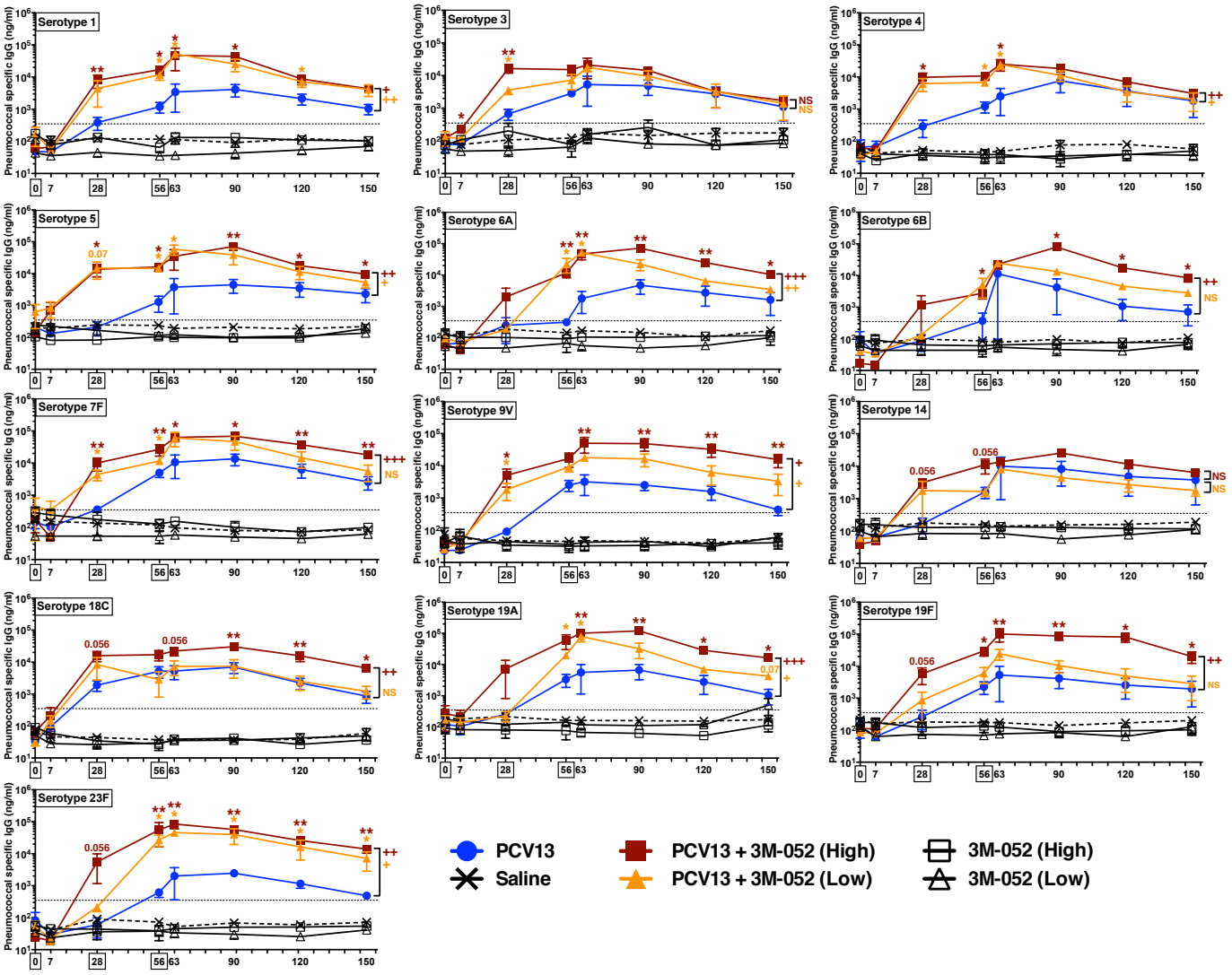
Supplemental Figure 4. Human adult whole blood cytokine responses to 3M-052, PCV13 and (PCV13 + 3M-052). Human adult blood cultured for 6 hours with O/W vehicle, PCV13 alone (1:5.7 - 57,000 v/v), 3M-052 alone (0.01, 0.1, 1, 10, 100 μM) and concentration dependent combinations of each. Supernatants were collected for ELISA and multiplex assay. Mean ± SEM of agonist-induced cytokine production are shown (n = 8 – 10). For comparisons between overall groups (e.g., PCV13 vs. (PCV13 + 3M-052)), two-way repeated measures ANOVA for non-parametric sample populations were applied and statistical significance denoted as +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$. For comparison at individual concentrations, the unpaired Mann-Whitney test was applied and statistical significance denoted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Results represent means ± SEM, with p values indicating significance as compared to that colored group.



Supplemental Figure 5. 3M-052 enhances antigen-specific IgG levels while also skewing the response towards Th1 (IgG2a induction). Balb/c mice were immunized by subcutaneous injection (scruff of neck) with a 10 µg dose of influenza hemagglutinin (HA) alone, or in combination with 0.01, 0.03, 0.1, 0.3, or 1 mg/kg 3M-052 three times (prime, boost, boost) 14 days apart. Serum was collected at Day 77 (21 days after the final immunization) for measurement of HA-specific serum Ig levels by ELISA. Production of both serum (A) IgG1 and (B) IgG2a, suggest induction of a mixed Th1/Th2-response following immunization (n = 5).



Supplemental Figure 6. Addition of 3M-052 augments Th1-responses to alum adjuvanted influenza hemagglutinin antigen. Addition of 3M-052 to Alum-adjuvanted HA antigen markedly enhances IgG2a Ab production. Balb/c mice were immunized by subcutaneous injection (scruff of neck) with a 10 µg dose of influenza hemagglutinin (HA) alone or in combination with Alum or 0.1 mg/kg 3M-052 three times (prime, boost, boost) 14 days apart. The results depict median HA-specific serum (A) IgG, (B) IgG1, and (C) IgG2a levels measured by ELISA on Day 77, which was 21 days after the final immunization (n = 5).

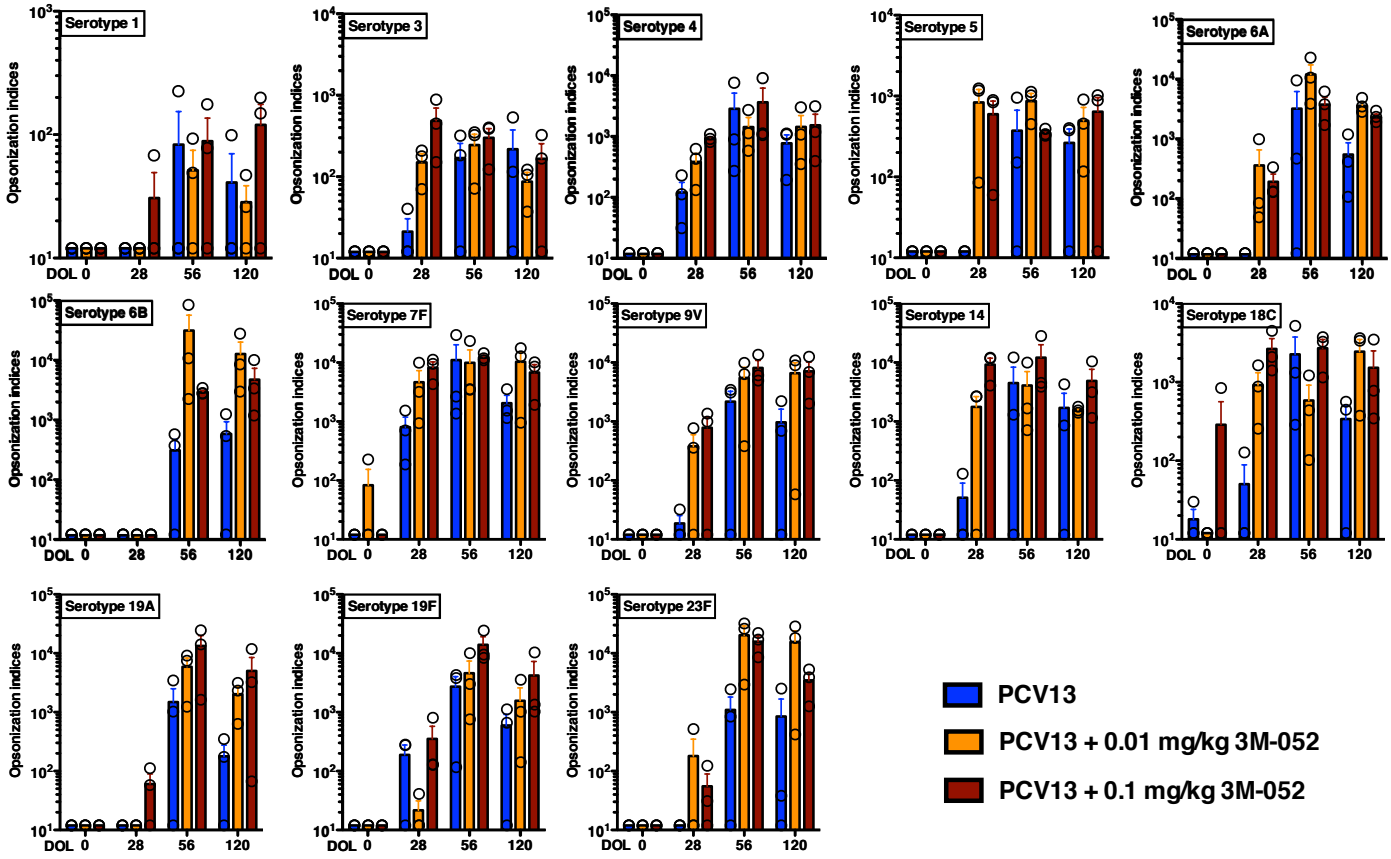


Supplemental Figure 7. Addition of a TLR7/8 agonist accelerates serotype-specific antibody responses to PCV13 in a dose dependent manner. Thirteen PCV13 serotypes are shown. Neonatal and infant rhesus macaques were immunized at DOL0, 28, and 56 with saline control, PCV13 alone, 3M-052 alone (0.01 or 0.1 mg/kg), or (PCV13 + 3M-052 (0.01 or 0.1 mg/kg)). Peripheral blood was collected at the indicated time-points to obtain serum for anti-pneumococcal serotype titers by polysaccharide-IgG binding microarray (n = 3 - 5 per group) run in triplicate. Horizontal broken line indicates 0.35 µg/ml, the WHO recommended reference Ab concentration of IgG used as a correlate of protective levels in humans. For comparisons between overall groups (i.e., PCV13 vs. (PCV13 + 3M-052)), two-way repeated measures ANOVA for non-parametric sample populations were applied and statistical significance denoted as +p < 0.05, ++p < 0.01, or NS (not significant). For comparison at individual time-points (i.e. PCV13 vs. (PCV13 + 3M-052) at DOL28), unpaired Mann-Whitney test was applied at each time-point. Results represent means ± SEM, with statistical significance denoted as *p < 0.05, **p < 0.01, ***p < 0.001.

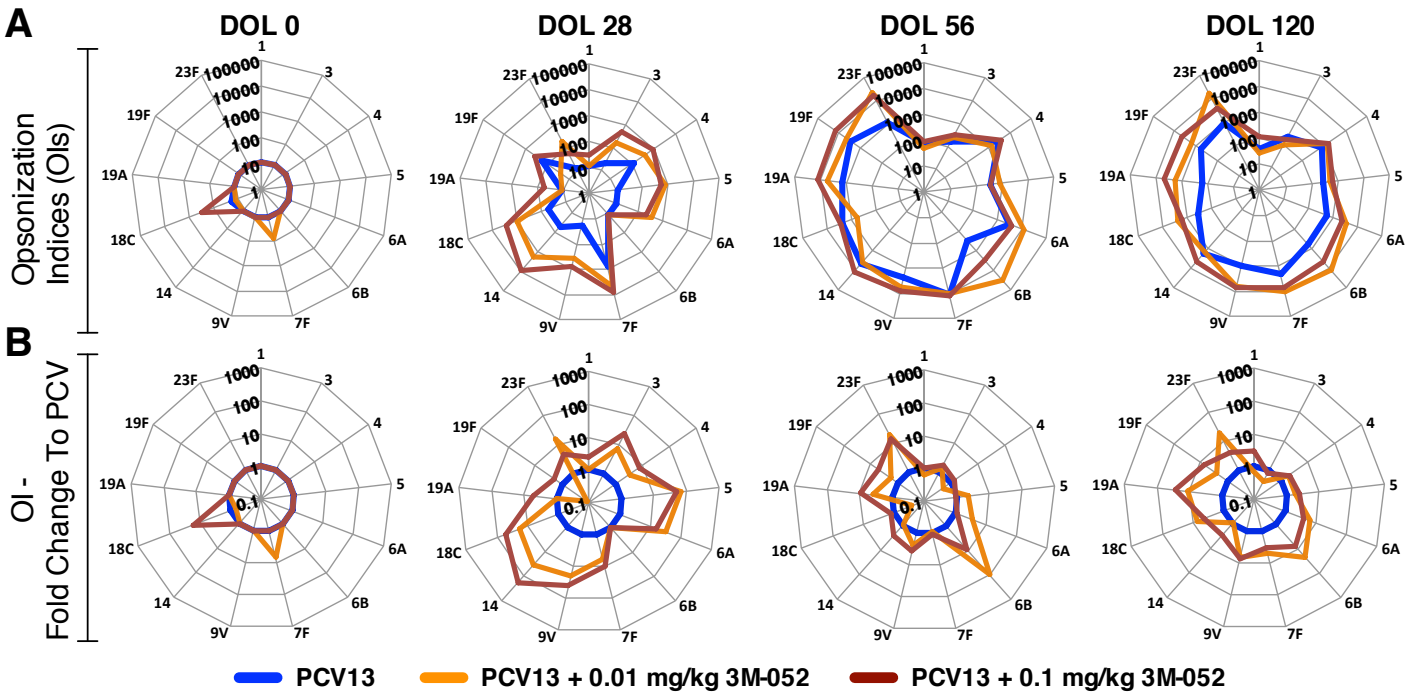
Supplemental Table 2. Longitudinal non-human primate study design overview

DOL	0	2	7	14	28	30	35	42	56	58	63	70	90	120	150	180	360
Vaccination	x				x				x								
Micro-chipping/Tattooing	x																
Weight monitoring	x		x	x	x	x	x		x		x		x	x	x	x	x
Physical Exam	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Representative Photography	x	x	x	x	x	x	x	x	x	x	x	x					
Phlebotomy	x		x		x	x	x		x		x		x	x	x	x	x
Muscle Biopsy*	x	x				x				x							
LN Biopsy*			x								x						

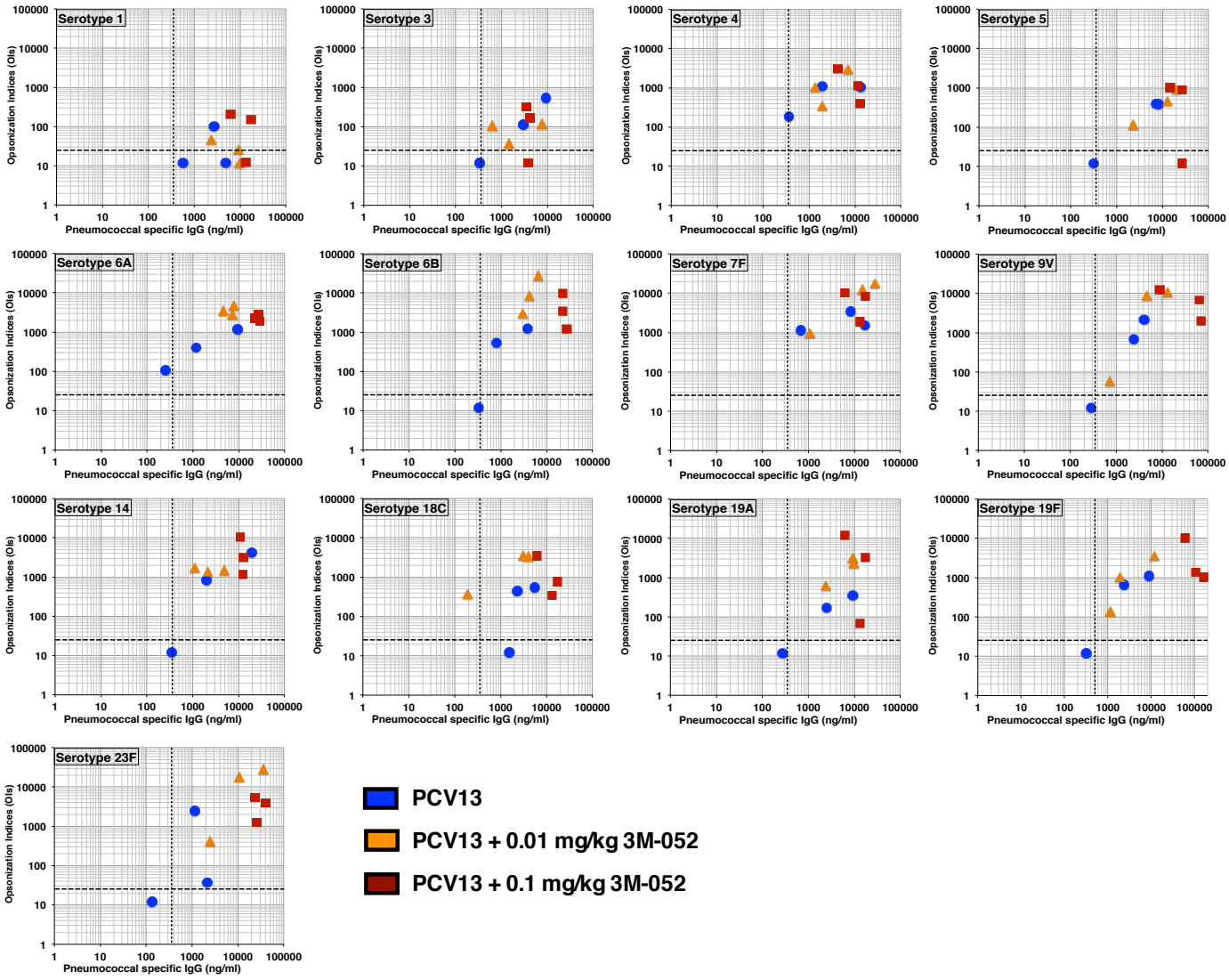
Neonatal and infant NHP were vaccinated on DOL0 (within 24 hours of birth), DOL28, and DOL56. Phlebotomy, routine physical exams, weight measurements were performed up to DOL360. Representative photography for each animal was performed up to DOL70. * Indicates sub study group of animals ($n = 8$) that underwent muscle and lymph node biopsies.



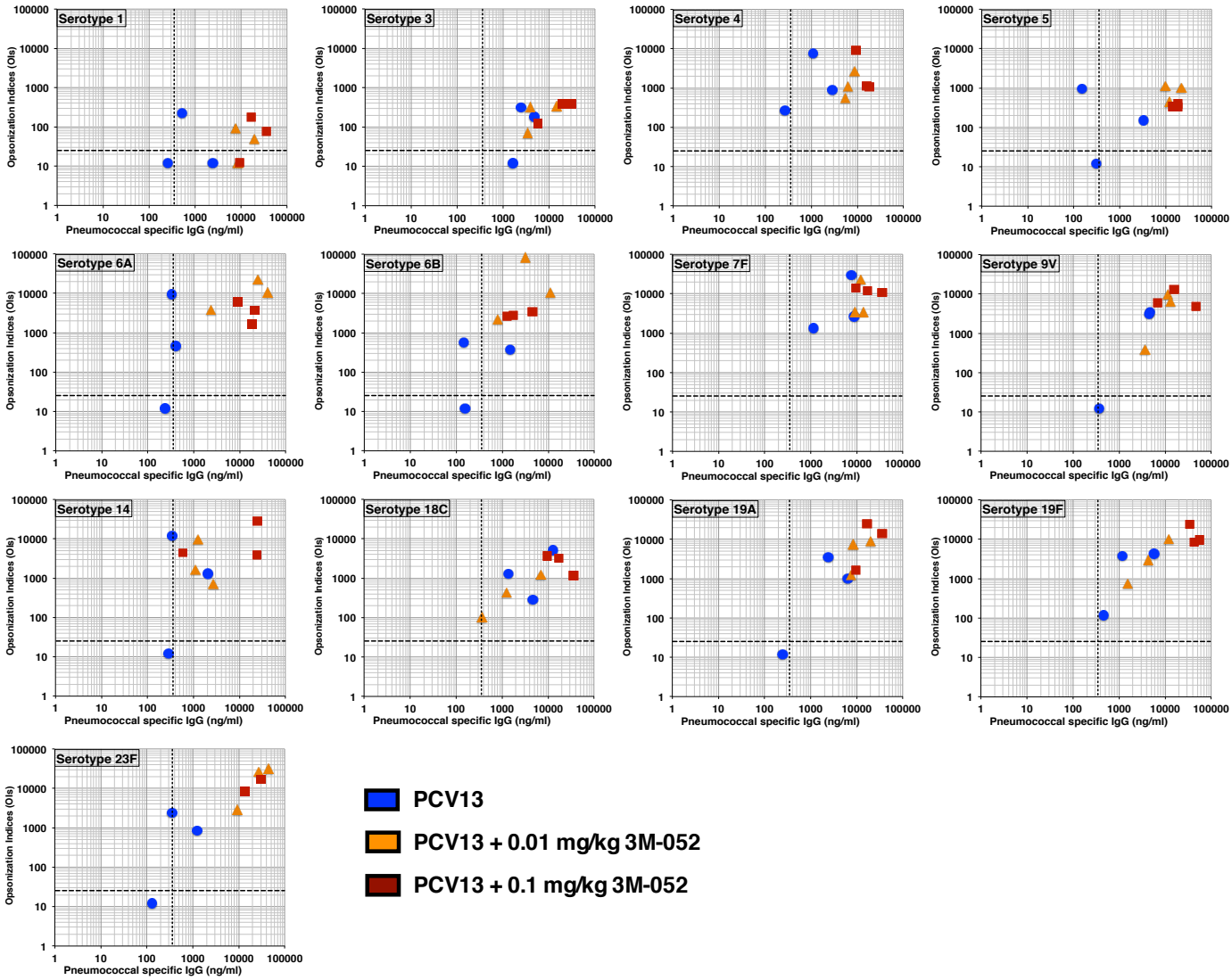
Supplemental Figure 8. TLR7/8 adjuvantation markedly accelerates and enhances serotype-specific pneumococcal opsonophagocytic killing capacity in neonatal serum. Neonatal and infant rhesus macaques were immunized at DOL0, 28, and 56 with either PCV13 alone or (PCV13 + 3M-052). Peripheral blood was collected at the indicated time-points to obtain serum for measurement of IgG concentrations and opsonization indicies (OIs) as described in Methods. Geometric mean titers of serotype-specific opsonophagocytic killing activity from n = 3 rhesus macaques per treatment group are shown. Samples with undetectable OIs were assigned an OI of 12. Results represent means ± SEM.



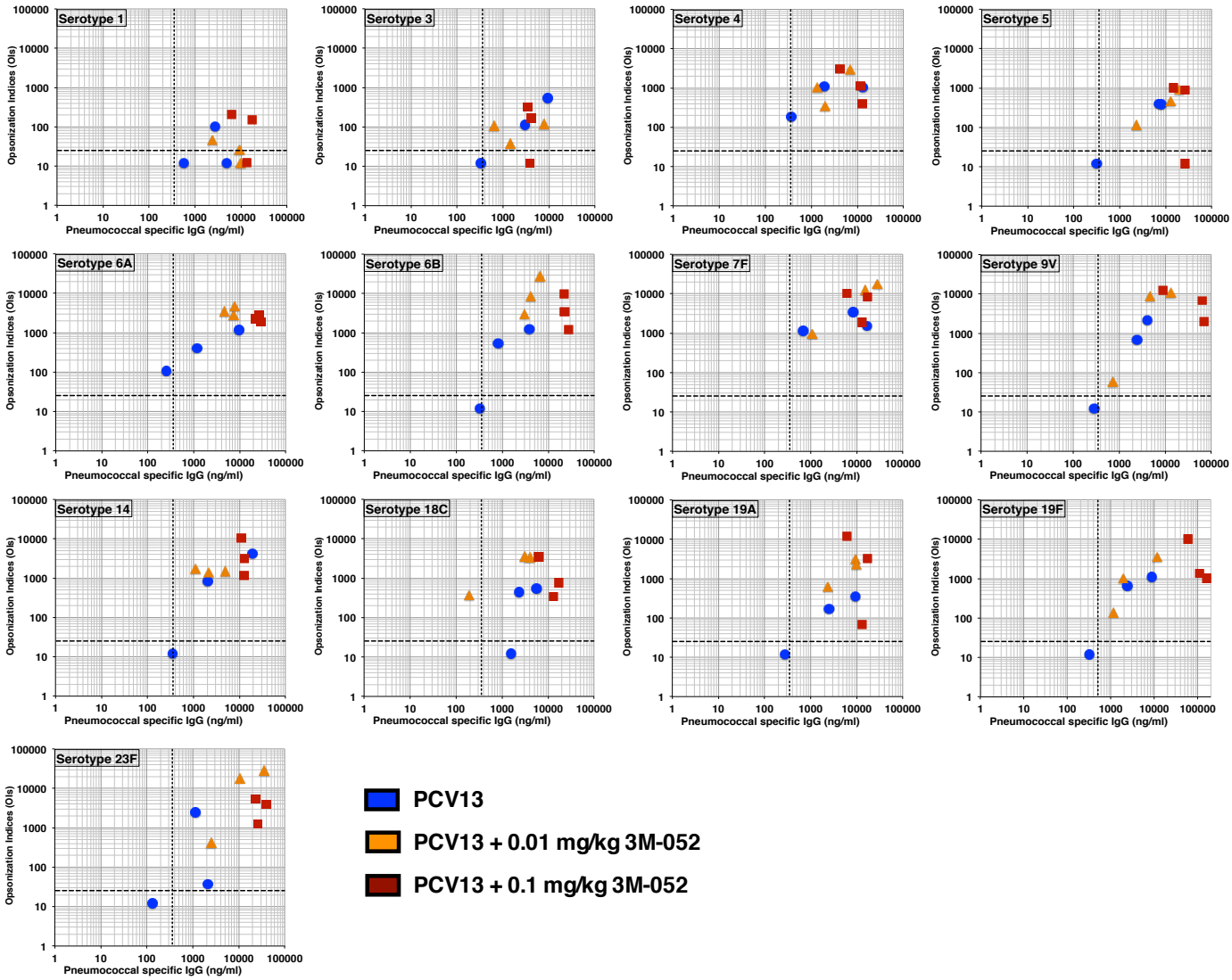
Supplemental Figure 9. TLR7/8 adjuvantation dramatically accelerates and enhances serotype-specific pneumococcal opsonophagocytic killing capacity in neonatal serum. Neonatal and infant rhesus macaques were immunized at DOL0, 28, and 56 days with either PCV13 alone or PCV13 co-administered with 3M-052. Peripheral blood was collected at the indicated time-points to obtain serum. Average geometric mean titers of serotype-specific opsonophagocytic killing activity from rhesus macaques per treatment group (n = 3). The results are expressed as opsonization indices (Ois), defined as the interpolated dilution of serum that kills 50% of bacteria. Samples identified as negative in the assay (i.e., samples having no functional activity detected) were assigned an OI of 12. Radar plot analysis of all 13 serotypes tested, including raw OI (A) and fold-change analysis (B) at DOL0, 28, 56, and 120. After a single dose of (PCV13 + 3M-052), all immunized infants exceeded this level for all 13 serotypes tested.



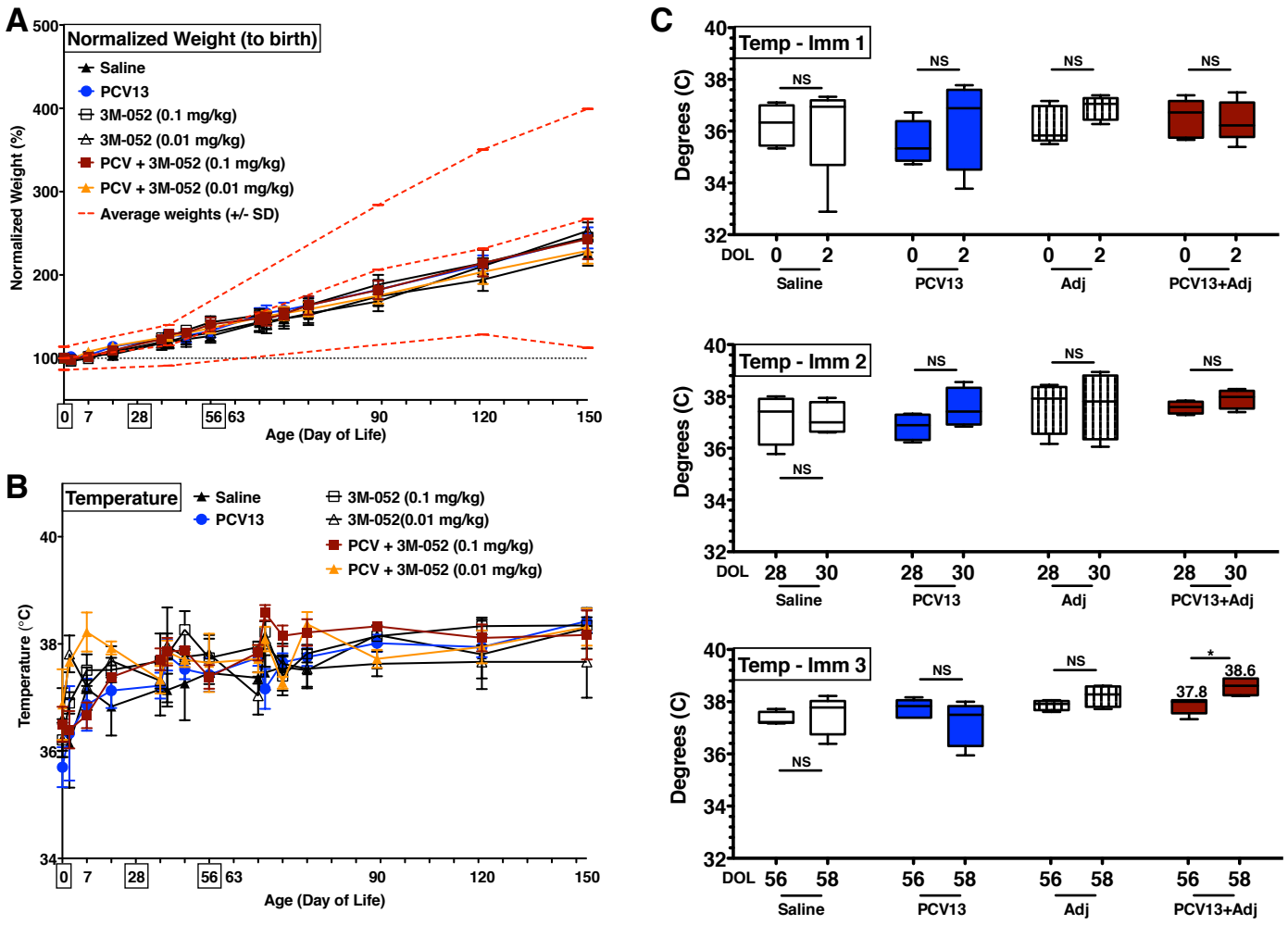
Supplemental Figure 10. Day 28 opsonophagocytic killing activity corresponds with accelerated serotype-specific antibody responses to TLR7/8 agonist-adjuvanted pneumococcal conjugate vaccine. Neonatal and infant rhesus macaques were immunized at DOL0 with either PCV13 alone or (PCV13 + 3M-052). Peripheral blood was collected at the indicated time-points to obtain serum for measurement of IgG concentrations and opsonization indicies (OIs) as described in Methods. Day 28 post-first immunization OIs (y-axis) are plotted as a function of IgG concentrations (x-axis) depicted as geometric mean titers (n = 3 per treatment group). Samples with undetectable OIs were assigned an OI of 12. Results represent means ± SEM.



Supplemental Figure 11. TLR7/8 agonist-adjuvantation of PCV13 enhances Day 56 opsonophagocytic killing activity. Neonatal and infant rhesus macaques were immunized at DOL0 and 28 with either PCV13 alone or (PCV13 + 3M-052). Peripheral blood was collected at the indicated time-points to obtain serum for measurement of IgG concentrations and opsonization indicies (OIs) as described in Methods. Day 56 post-first immunization OIs (y-axis) are plotted as a function of IgG concentrations (x-axis) depicted as geometric mean titers (n = 3 per treatment group). Samples with undetectable OIs were assigned an OI of 12. Results represent means ± SEM.



Supplemental Figure 12. TLR7/8 agonist-adjuvantation of PCV13 enhances Day 120 opsonophagocytic killing activity. Neonatal and infant rhesus macaques were immunized at DOL0, 28, and 56 with either PCV13 alone or (PCV13 + 3M-052). Peripheral blood was collected at the indicated time-points to obtain serum. Day 120 post-first immunization OIs (y-axis) are plotted as a function of IgG concentrations (x-axis) depicted as geometric mean titers (n = 3 per treatment group). Samples with undetectable OIs were assigned an OI of 12. Results represent means ± SEM.



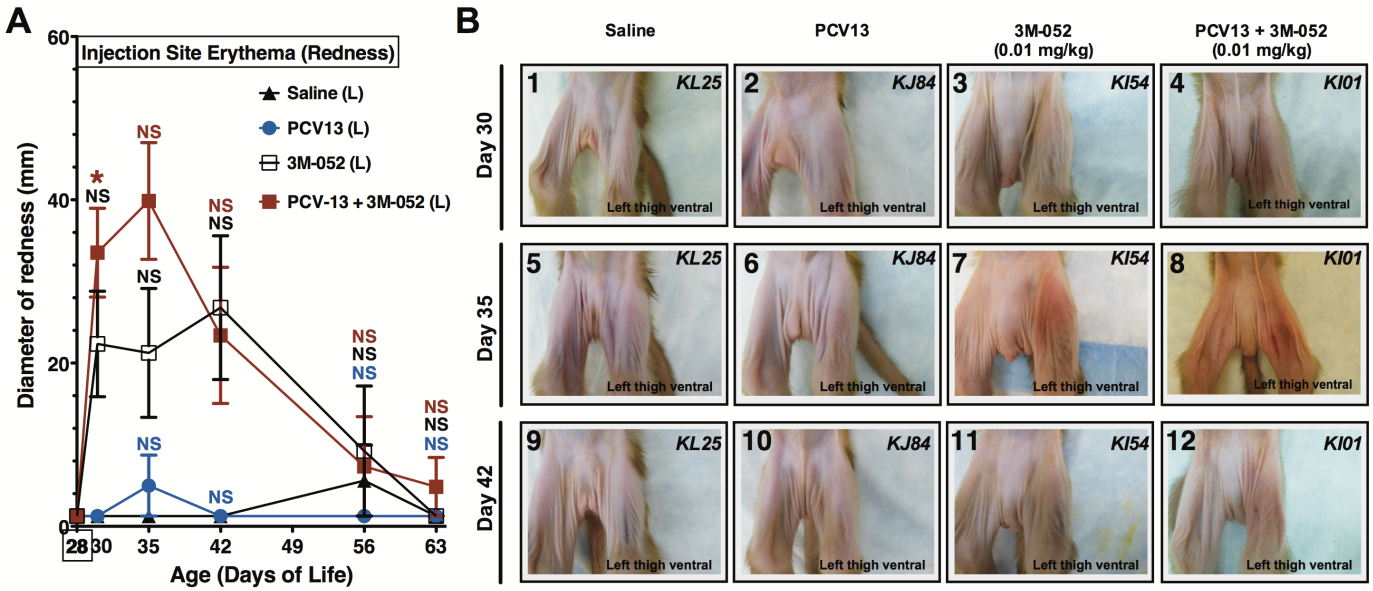
Supplemental Figure 13. Weight and body temperature of immunized neonatal and infant rhesus macaques. (A) Weight, a sensitive indicator of neonatal well-being, was measured regularly to DOL150 and are depicted as normalized values relative to birth weight (100%) for each treatment group. Red dotted lines indicate normal age-matched norms with standard deviations. (B) Body temperature was measured by rectal thermometer at regular intervals up to DOL150. (C) Body temperatures pre- and post- each immunization at DOL0, 28, and 56. For comparison at individual time-points, the unpaired Mann-Whitney test was applied, with statistical significance denoted as *p < 0.05. Results represent means ± SEM of 3-5 animal's per group.

Supplemental Table 3. Average complete blood count per study treatment group.

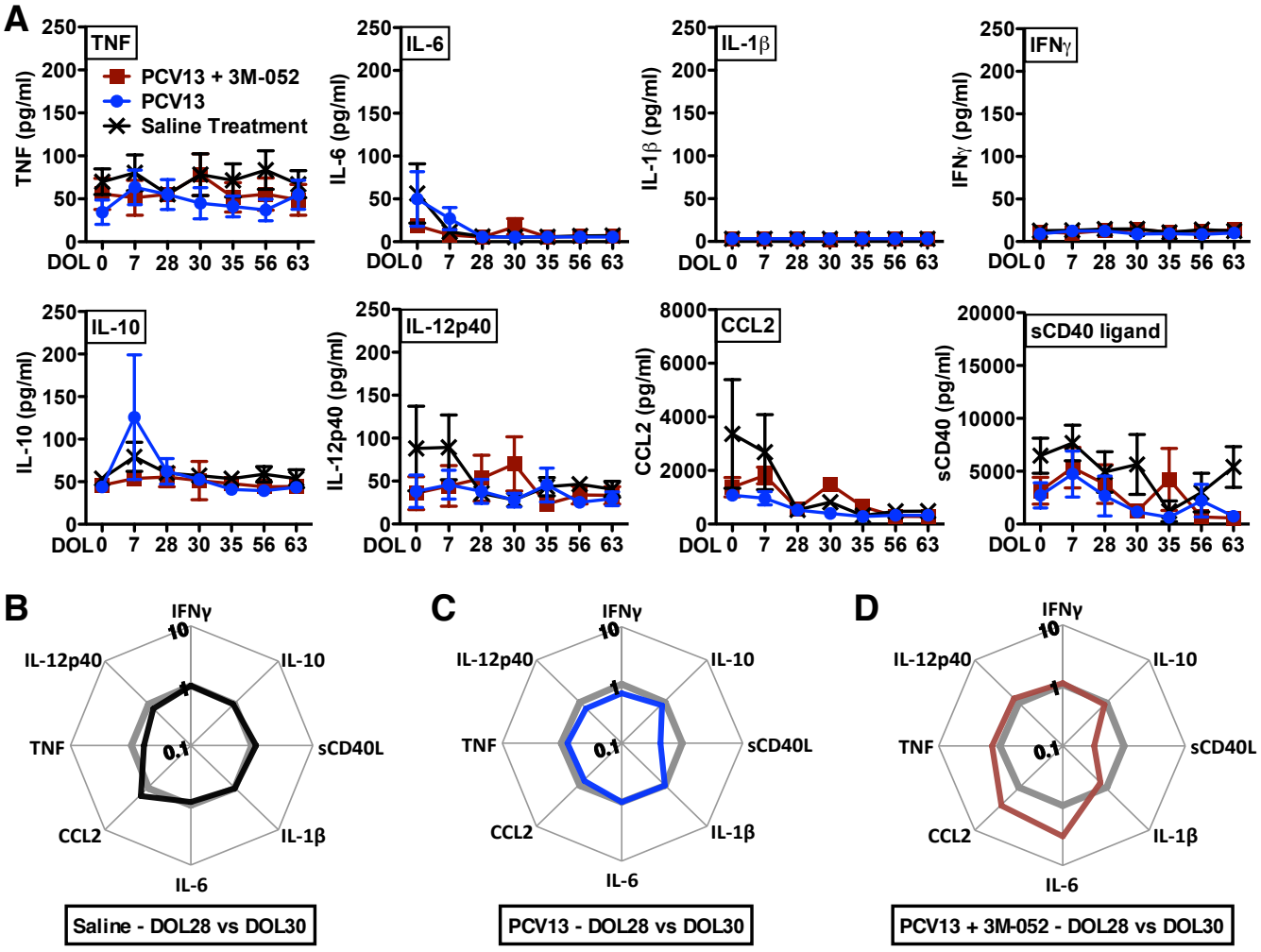
		Day Of Life 90								Day Of Life 120							
		Saline		PCV13		3M-052		PCV13 + 3M-052		Saline		PCV13		3M-052		PCV13 + 3M-052	
Measurement	**Unit**	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
White Blood Cell Count	per µl	8.42	1.89	11.59	2.37	9.05	0.78	11.60	4.24	8.65	1.77	10.11	2.96	10.27	1.34	12.12	5.92
Red Blood Cell Count	per µl	5.22	0.40	5.11	0.37	5.19	0.21	5.27	0.60	5.50	0.19	5.73	0.55	5.36	0.33	5.25	0.76
Hemoglobin	g/dl	11.48	1.04	9.18	2.86	10.60	1.21	10.70	0.88	11.45	1.33	8.38	3.06	10.80	1.25	9.98	2.04
Hematocrit	%	35.38	2.68	29.73	6.93	33.96	3.25	34.53	3.61	36.10	2.84	29.30	7.85	34.93	2.07	33.04	6.03
Corpusular Volume	fl	67.93	2.89	57.80	10.06	65.28	4.17	65.63	1.33	65.60	4.87	51.12	13.00	65.40	6.41	62.78	3.97
Corpuscular Hemoglobin	pg	22.03	1.40	17.75	4.48	20.36	1.70	20.35	1.03	20.80	2.38	14.62	5.26	20.25	3.16	18.88	1.61
Corpuscular Hemoglobin Conc.	g/dl	32.40	0.96	30.43	2.56	31.14	0.90	31.03	1.19	31.65	1.53	28.18	3.24	30.80	2.22	30.08	0.90
Red Blood Cell Distribution Width	%	12.93	0.43	14.75	2.06	13.72	0.68	13.70	1.28	13.23	1.47	***23.70***	***7.54***	13.57	6.80	14.46	1.83
Platelet Count	µl	550.50	128.28	732.00	428.78	703.80	93.89	465.75	256.20	661.75	119.75	739.60	219.59	532.25	295.28	657.20	220.97
Mean platelet volume	fl	10.63	2.11	12.43	4.45	10.38	1.13	11.25	0.90	10.33	1.20	9.00	5.03	11.45	2.68	10.40	1.14
% Reticuloyctes	%	0.00	0.00	1.38	1.63	0.26	0.58	0.00	0.00	0.00	0.00	1.60	1.37	0.00	0.00	1.12	1.55
% Neutrophils	%	22.05	5.74	26.13	15.28	18.98	3.79	22.83	3.01	19.50	5.53	24.38	8.07	25.03	4.82	***35.00***	***5.58***
% Lymphocytes	%	71.65	3.50	68.68	15.04	74.84	5.44	70.28	4.12	75.80	4.87	70.74	8.23	69.30	3.54	***58.78***	***5.54***
% Monocytes	%	2.78	0.45	3.20	0.55	3.02	0.65	4.05	2.25	2.88	1.06	3.22	1.22	3.50	0.63	4.30	1.25
% Eosinophils	%	2.80	1.97	1.35	1.34	2.42	1.66	2.00	1.33	1.28	0.39	1.04	0.84	1.80	1.89	1.40	0.70
% Basophils	%	0.73	0.48	0.65	0.13	0.74	0.34	0.88	0.35	0.55	0.13	0.62	0.50	0.40	0.12	0.52	0.13
# Neutrophils	per µl	1.91	0.86	2.90	1.40	1.71	0.36	2.56	0.69	1.69	0.60	2.33	0.56	2.56	0.57	4.40	2.72
# Lymphocytes	per µl	6.01	1.18	8.10	2.98	6.78	0.83	8.25	3.36	6.56	1.40	7.26	2.61	7.13	1.05	7.01	3.09
# Monocytes	per µl	0.23	0.04	***0.36***	***0.02***	0.27	0.07	0.50	0.32	0.24	0.08	0.32	0.17	0.36	0.08	0.50	0.18
# Eosinophils	per µl	0.22	0.15	0.15	0.13	0.21	0.14	0.20	0.06	0.11	0.03	0.11	0.11	0.19	0.21	0.15	0.06
# Basophils	per µl	0.06	0.04	0.07	0.01	0.07	0.03	0.10	0.05	0.05	0.02	0.07	0.07	0.04	0.02	0.06	0.01

		Day Of Life 150								Day Of Life 180							
		Saline		PCV13		3M-052		PCV13 + 3M-052		Saline		PCV13		3M-052		PCV13 + 3M-052	
Measurement	**Unit**	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
White Blood Cell Count	per µl	9.06	2.23	9.91	0.79	12.40	3.20	12.80	1.49	9.23	3.10	11.85	4.19	10.38	1.92	10.95	1.98
Red Blood Cell Count	per µl	6.05	0.62	5.53	1.01	5.47	0.33	5.05	1.09	6.07	0.69	5.78	0.69	5.72	0.67	5.23	1.36
Hemoglobin	g/dl	12.27	0.74	8.63	4.20	10.45	1.77	9.10	3.78	12.60	0.87	10.13	4.89	***9.63***	***0.90***	8.57	4.84
Hematocrit	%	39.10	1.61	29.48	11.10	34.05	3.71	29.83	11.21	39.30	2.62	34.50	11.44	***32.83***	***0.93***	28.20	13.98
Corpusular Volume	fl	65.07	6.84	52.20	13.94	62.75	9.50	57.70	10.21	64.97	3.79	58.90	15.65	57.97	6.98	52.23	15.48
Corpuscular Hemoglobin	pg	20.47	2.82	15.28	6.21	19.28	4.06	17.48	3.65	20.87	1.75	17.13	7.46	17.07	3.23	15.77	5.84
Corpuscular Hemoglobin Conc.	g/dl	31.37	1.10	28.60	4.83	30.48	2.20	30.18	1.23	32.07	0.83	28.07	6.06	29.30	2.14	29.87	2.14
Red Blood Cell Distribution Width	%	16.27	5.74	24.48	9.96	12.87	6.45	17.60	3.29	17.07	7.30	19.30	13.21	15.23	3.70	19.23	5.58
Platelet Count	µl	611.33	88.56	650.75	316.08	567.50	400.01	1256.75	1615.86	487.33	141.64	659.67	258.02	***852.00***	***109.78***	1364.67	1253.15
Mean platelet volume	fl	10.00	1.04	8.15	4.71	10.80	1.90	11.40	1.64	9.50	5.51	8.15	4.78	10.20	1.73	11.85	7.14
% Reticuloyctes	%	0.00	0.00	1.13	1.33	5.03	10.05	1.90	2.20	0.00	0.00	0.00	0.00	6.87	11.89	0.73	1.27
% Neutrophils	%	19.00	5.60	26.15	9.37	25.23	15.43	28.33	8.69	20.07	9.34	36.57	18.90	26.90	2.23	27.37	9.12
% Lymphocytes	%	73.37	5.13	68.95	9.76	69.73	14.04	65.55	7.42	73.60	11.84	57.77	18.25	67.17	2.76	68.63	10.34
% Monocytes	%	5.27	1.19	2.90	0.90	3.33	1.71	4.35	1.09	4.67	3.31	4.00	0.95	2.90	0.79	2.53	1.07
% Eosinophils	%	1.37	0.55	1.58	0.80	1.30	0.39	1.18	0.78	1.07	0.99	1.13	0.76	2.47	2.80	1.10	0.36
% Basophils	%	1.03	0.15	***0.43***	***0.17***	***0.45***	***0.10***	***0.60***	***0.22***	0.60	0.20	0.50	0.36	0.50	0.17	0.40	0.20
# Neutrophils	per µl	1.66	0.29	2.58	0.91	2.96	1.70	3.69	1.46	1.74	0.65	4.07	1.92	2.77	0.32	2.92	0.71
# Lymphocytes	per µl	6.70	1.96	6.84	1.22	8.76	3.28	8.35	1.04	6.89	3.13	7.10	4.36	7.01	1.59	7.58	2.25
# Monocytes	per µl	0.49	0.20	0.28	0.08	0.44	0.30	0.55	0.14	0.43	0.28	0.45	0.05	0.31	0.13	0.27	0.11
# Eosinophils	per µl	0.12	0.03	0.17	0.09	0.17	0.07	0.14	0.07	0.08	0.06	0.15	0.15	0.24	0.26	0.12	0.03
# Basophils	per µl	0.09	0.02	***0.04***	***0.01***	0.06	0.02	0.08	0.01	0.06	0.04	0.07	0.07	0.05	0.01	0.04	0.02

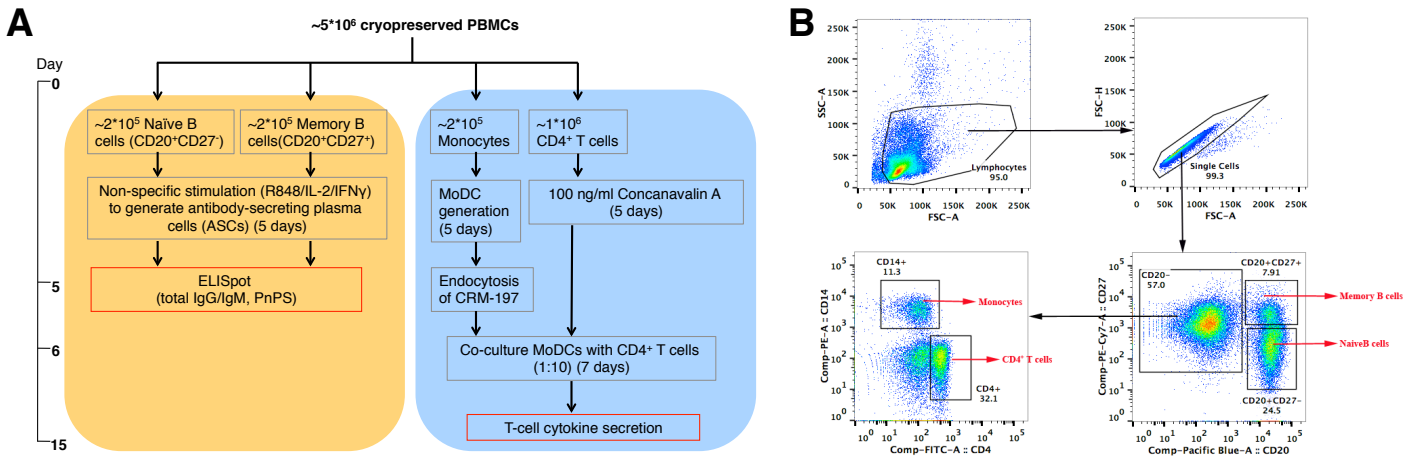
SD: standard deviation. Statistial difference as compared to saline treatment alone denoted by enbolded/italics. n = 3 - 5.



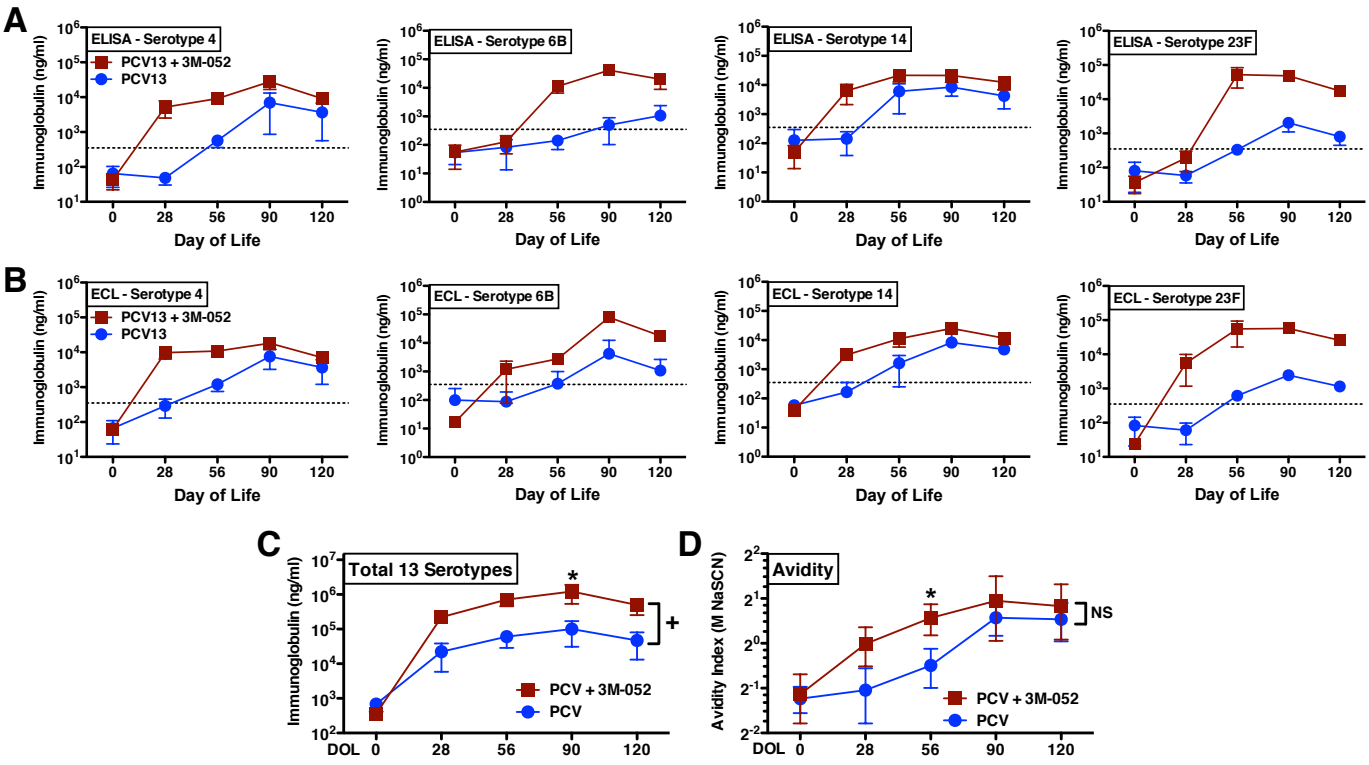
Supplemental Figure 14. Intramuscular injection of 3M-052 induces injection site erythema post-second immunization. Neonatal and infant rhesus macaques were immunized at DOL0, 28, and 56 with either PCV13 alone or (PCV13 + 3M-052). (A) Significant injection site erythema (diameter of redness in mm as measured using calipers), as compared to saline (n = 5), was only observed after the second of three immunizations with PCV13 co-administered with 3M-052 (n = 8, combining both 0.01 and 0.1 mg/kg treatment groups). (B) Photographs are labeled 1 – 12 in the top left corner and each individual animal study identification code is indicated in the top right corner of each image. Left thigh ventral photography of representative infant rhesus macaques on DOL30 (images 1 – 4), 35 (images 5 – 8), and 42 (images 9 – 12). While there was a trend towards increased erythema for some individual animals treated with 3M-052 or (PCV13 + 3M-052), no significant erythema at the site of injection was observed pre- or post-first or third immunization. As the maculopapular rash was only observed in a) the second of two birthing/enrollment seasons, b) co-housed animals and c) 3 of the total of 16 3M-052- (or (PCV13 + 3M-052))-treated infant animals, it was unclear whether it was adjuvant-related. For comparison at individual time-points, the unpaired Mann-Whitney test was applied, with with statistical significance denoted as *p < 0.05 or NS (not significant) as compared to saline treatment group. Results represent means ± SEM.



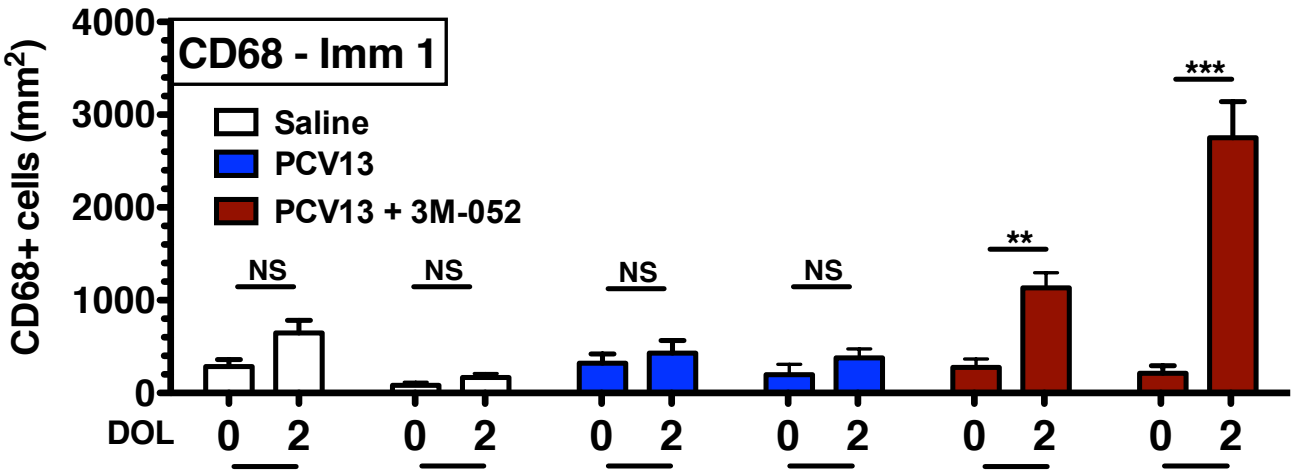
Supplemental Figure 15. 3M-052 administration with or without PCV13 does not induce systemic cytokines in neonatal/infant rhesus macaques. (A) Evaluation of rhesus plasma cytokine kinetics post- each dose of IM PCV13 or (PCV13 + 0.1 mg/kg 3M-052) formulated in O/W emulsion (vehicle) or Saline (n = 3 - 5). (B – D) Evaluation of rhesus plasma cytokine pre- (DOL28, gray) and post- (DOL30, black, blue and red) single dose of (PCV13 + 0.1 mg/kg 3M-052) (n = 3 - 4).



Supplemental Figure 16. Experimental approach used for mononuclear cell sorting and ex vivo assessment of vaccine-specific B and T cells in infant rhesus macaques. (A) Sorted leukocytes were incubated as depicted. B cell subsets (left) were non-specifically stimulated with R848/IL-2/IFNγ to induce differentiation to Ab-secreting plasma cells. Plasma cells were subsequently plated on ELISpot plates for detection of pneumococcal polysaccharide (PnPS)-specific B cells. Monocytes were differentiated to monocyte-derived dendritic cells (MoDCs) by the addition of GM-CSF and IL-4. After treatment of MoDCs with CRM197 (the protein component of PCV13), cells were co-cultured with CD4+ and CD8+ T cells and activation of vaccine-specific T cells was measured. (B) Frozen PBMCs were thawed and stained with CD20-Pacific Blue, CD27-PE.Cy7, CD14-PE, CD4-FITC, and CD8-APC.Cy7. Cells were subsequently sorted according to the gating strategy depicted on a FACSAria II cytometer.



Supplemental Figure 17. 3M-052 accelerated and enhanced the magnitude of neonatal and infant anti-PnPS antibody (IgG) responses and may enhance antibody avidity. Ab titer to pneumococcal conjugate vaccine serotypes 4, 6B, 14, and 23F were compared and confirmed using (A) WHO recommended ELISA total (n = 3), (B) 96-well electrochemiluminescence (ECL) multiplex assay (n = 5). Ab titer to all 13 pneumococcal conjugate vaccine serotypes were compared and confirmed using (C) ELISA and (D) avidity assay (n = 3). For comparisons between overall groups (i.e., PCV13 vs. (PCV13 + 3M-052)), two-way repeated measures ANOVA for non-parametric sample populations were applied and statistical significance denoted as +$p < 0.05$, or NS (not significant). For comparison at individual time-points (i.e. PCV13 vs. (PCV13 + 3M-052) at DOL56), unpaired Mann-Whitney test was applied at each time-point. Results represent means ± SEM, with statistical significance denoted as *$p < 0.05$.



Supplemental Figure 18. Co-administration of 3M-052 with PCV13 increased infiltration of CD68+ cells at the vaccine injection site. Immunization with (PCV13 + 3M-052) accelerates injection site infiltration by monocytes/macrophages. 2 mm cube muscle biopsies were obtained from the injection site (quadriceps muscle) prior to and 48 hours after each immunization (obtained in an alternating pattern (e.g. DOL0 left leg, DOL2 right leg)). Frequencies of CD68+ cells in muscle were determined by immunofluorescence. For comparison at individual time-points, the unpaired Mann-Whitney test was applied, with statistical significance denoted as *$p < 0.05$, **$p < 0.01$, or NS (not significant). Data are representative of two animals per treatment group.