SUPPORTING INFORMATION

Shaping neonatal immunization by tuning delivery of synergistic adjuvants via nanocarriers

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Supplementary Figures

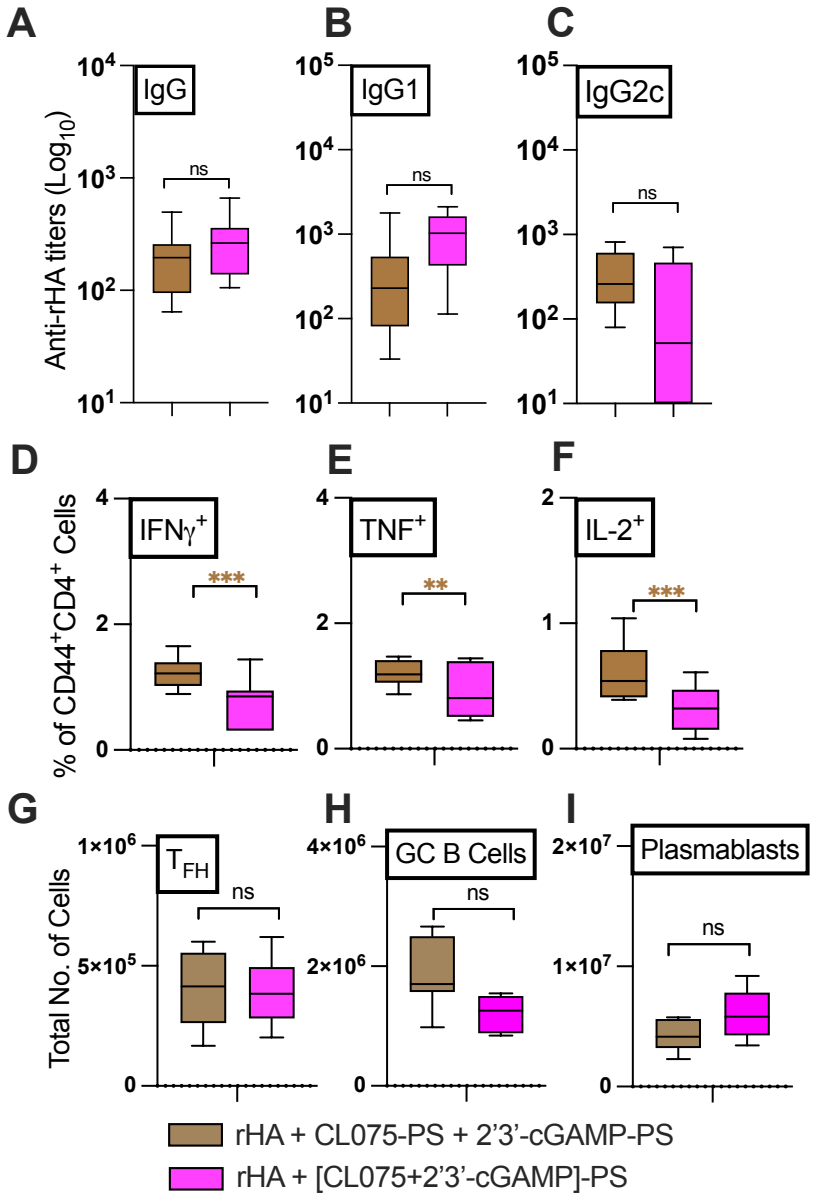

Figure S1. Comparison of rHA specific humoral and cell-mediated responses in between co-encapsulation and individual encapsulation of cGAMP and CL075 in PEG-*b*-PPS nanocarriers. After immunization of infant C57BL/6 mice i.m. on DOL (day of life) 7 and 14, antibody titers for rHA-specific IgG (A), IgG1(B) and IgG2c (C) were determined by ELISA in serum samples collected at DOL 21. (D-F) Splenic $CD4^+$ T cell responses after rHA stimulation. (G-I) Total number of T_{FH} cells ($CD3^+CD4^+PD\text{-}1^+CXCR5^+$), GC B cells ($CD3^-CD19^+CD95^+GL7^+$), plasmablasts ($CD3^-CD19^+CD138^+$) in DLN. Statistical comparison was performed either using one-way ANOVA or nonparametric Kruskal-Wallis test corrected for multiple comparisons; ns denoted non-significant, $^{**}p < 0.002$, $^{***}p < 0.001$ (n = 5 - 7 per group), with comparison to PBS, rHA and/or mock loaded PS nanocarriers control or test groups. Study was inclusive of two independent repeats.

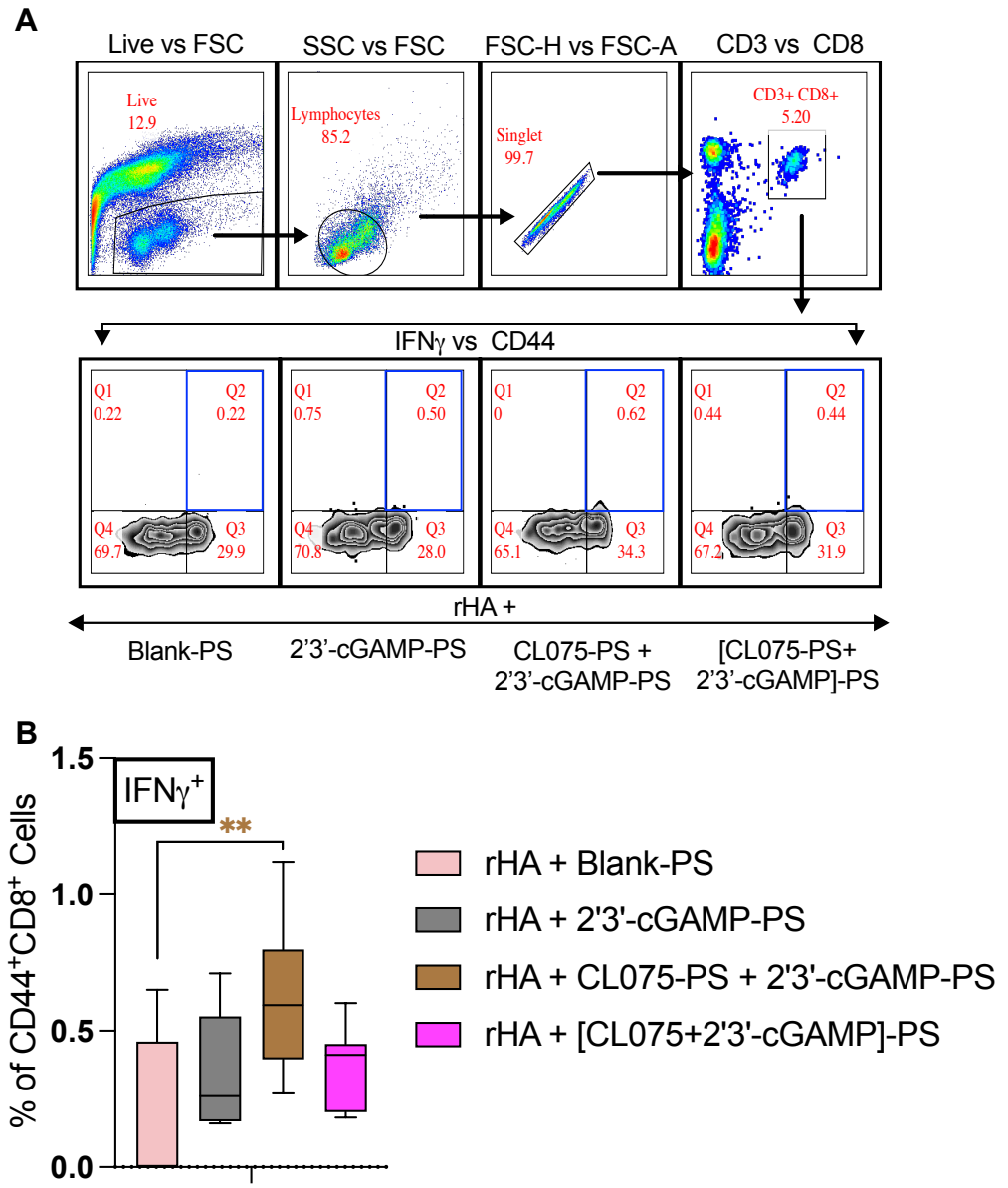

Figure S2. Gating strategy to identify rHA-specific CD8$^+$ T cells. (A) Splenocytes were isolated from immunized mice following 12 days booster. Antigen-specific T cell responses following rHA stimulation were defined as CD3$^+$CD8$^+$CD44^{high} IFNγ^+ using FlowJo software, v.10.8.1. Shown is an example of the hierarchical gating strategy leading to the identification of live, singlet, CD3$^+$, CD4$^+$, CD44^{high} and IFNγ^+ T cells in different immunize groups. (B) Splenic CD8$^+$ IFNγ^+ T cell signature was analyzed from indicated immunized groups after 12 days booster. Statistical comparison was performed using nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons; **p < 0.002 (n = 5 - 12 per group). Study was inclusive of two independent repeats.

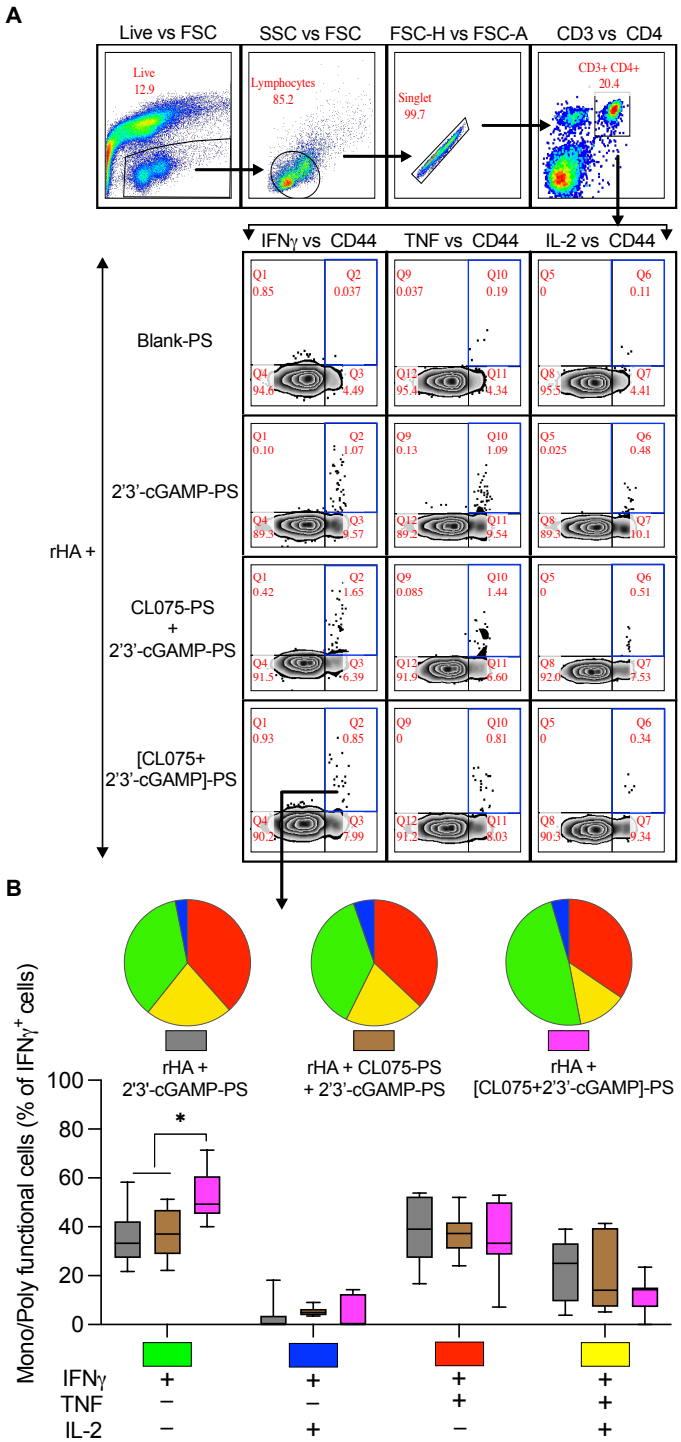

Figure S3. Gating strategy to identify rHA-specific CD4$^+$ T cells and it's multifunctionality. (A) Splenocytes were isolated from immunized mice following 12 days booster. Antigen-specific T cell responses following rHA stimulation were defined as CD3$^+$CD4$^+$CD44^{high} cytokine$^+$ using FlowJo software, v.10.8.1. Shown is an example of the hierarchical gating strategy leading to the identification of live, singlet, CD3$^+$, CD4$^+$, CD44^{high} and cytokine$^+$ T cells with an example for IFNγ, TNF and IL-2 responses in different immunized groups. (B) Multifunctionality of the CD4$^+$ IFNγ$^+$ T cells cytokine responses was analyzed from indicated immunized groups after 12 days booster. Pie chart represents the fraction of the total CD4$^+$ IFNγ$^+$ cytokines response comprising any combination of TNF and IL-2 production after Flublok stimulation in different immunized groups. Beneath pie chart, bar graph represents the frequencies of multifunctional T cells in CD4$^+$ IFNγ$^+$ T cell compartment. Statistical comparison was performed either using one-way ANOVA or nonparametric Kruskal-Wallis test corrected for multiple comparisons; *p < 0.033 (n = 5 - 12 per group). Study was inclusive of two independent repeats.

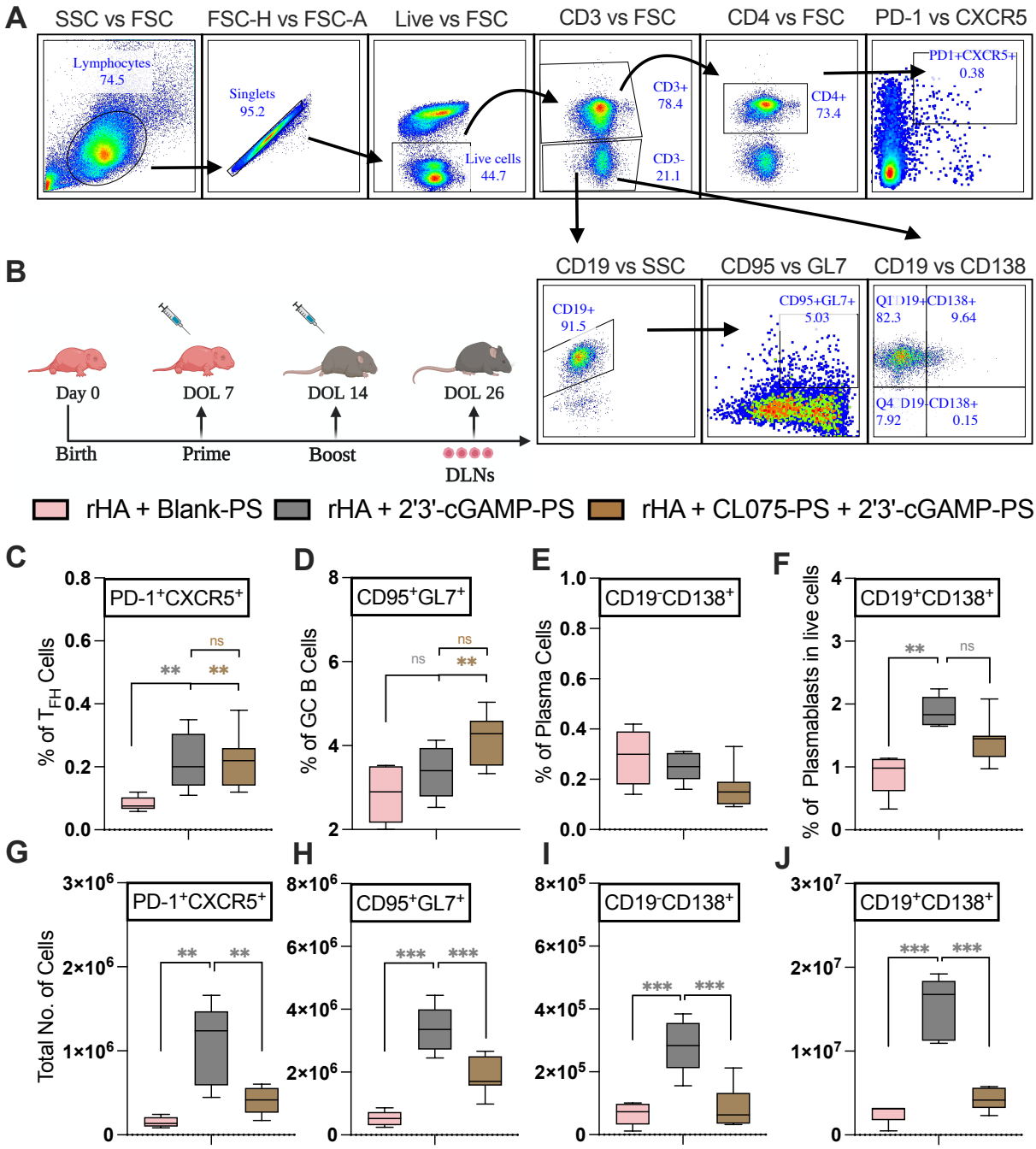

Figure S4. cGAMP and CL075 encapsulating PS promotes T_{FH} and B cell responses in draining lymph nodes (DLN). (A) Shown is an example of the hierarchical gating strategy leading to the identification of T_{FH} cells ($CD3^+CD4^+PD\text{-}1^+CXCR5^+$), GC B cells ($CD3^-CD19^+CD95^+GL7^+$), plasmablasts ($CD3^-CD19^+$ $CD138^+$) and plasma cells ($CD3^-$ $CD19^-CD138^+$). (B) Infant C57BL/6 were immunized i.m. as described in Figure 4. Lymphocytes from DLN (both popliteal and inguinal) of immunized mice were harvested at DOL 26 for FACS analysis. (C) % of T_{FH} cells, (D) % of GC B cells, (E) % of Plasma cells and (F) % of plasmablasts among live cells. (G-J) Total number of immune subsets in DLN. Statistical comparison was performed either using one-way ANOVA or nonparametric Kruskal-Wallis test corrected for multiple comparisons; $^{**}p < 0.002$, $^{***}p < 0.001$ (n = 5 - 7 per group). Study was inclusive of two independent repeats.

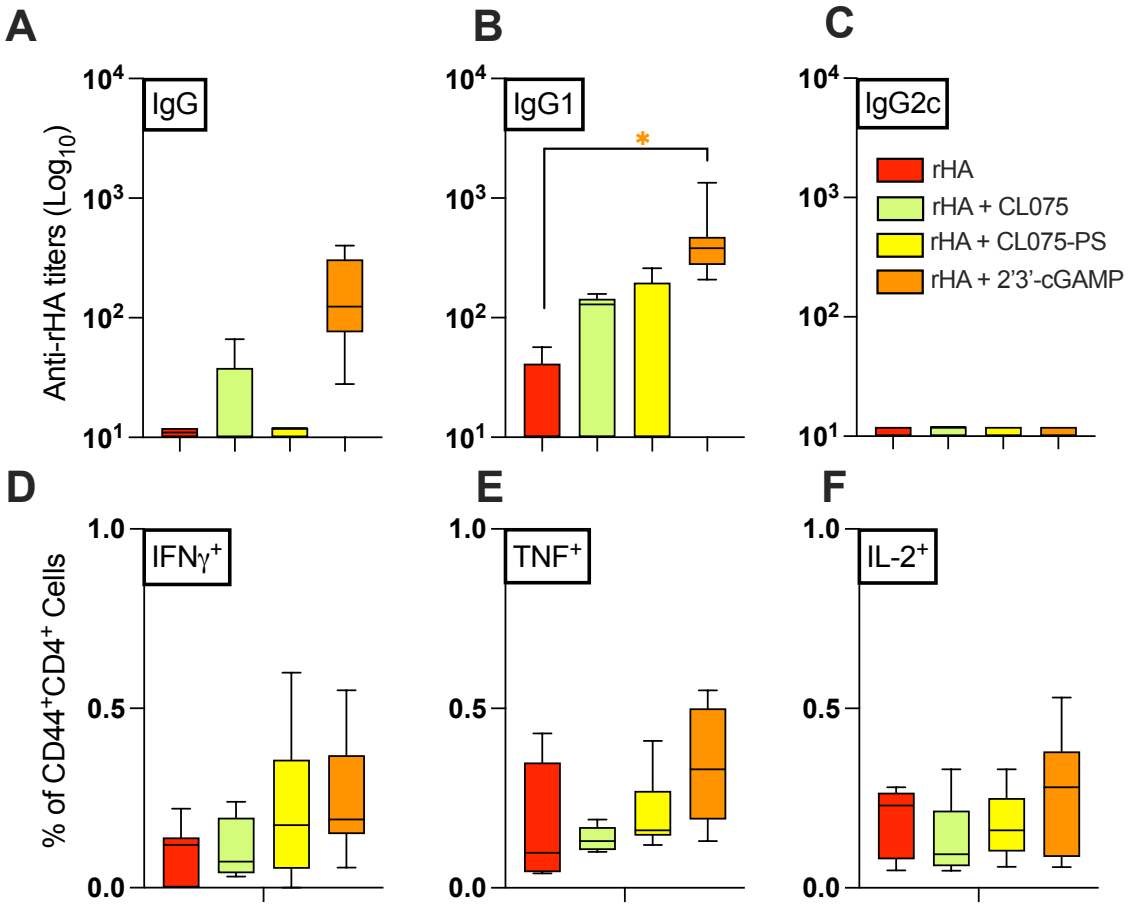

Figure S5. Comparison of rHA specific humoral and cell mediated responses in between different formulations. After immunization of infant C57BL/6 mice i.m. on DOL 7 and 14, antibody titers for rHA-specific IgG (A), IgG1(B) and IgG2c (C) were determined by ELISA in serum samples collected at DOL 21. (D-F) Splenic $CD4^+$ T cell responses after rHA stimulation was determined by flow cytometry. Statistical comparison was performed either using one-way ANOVA or nonparametric Kruskal-Wallis test corrected for multiple comparisons; *$p < 0.033$ ($n = 5$ - 12 per group). Study was inclusive of two independent repeats.

Supplementary Table 1: Vaccine formulations for *in vivo* study.

For the (rHA+Blank-PS) group, PS content was maintained at 400 µg to match the PS content of admixture group, i.e. rHA+CL075-PS+2'3'-cGAMP-PS.

Group	rHA (Flublok® Quadrivalent 2020-2021)	2'3'-cGAMP (µg)	CL075 (µM)	PS (µg)
PBS	--	--	--	--
Blank-PS	4µg (1µg of each variant)	--	--	400 µg
CL075	4µg (1µg of each variant)	--	164 µM	--
2'3'-cGAMP	4µg (1µg of each variant)	1µg	--	--
CL075+2'3'-cGAMP	4µg (1µg of each variant)	1µg	164 µM	--
CL075-PS	4µg (1µg of each variant)	--	164 µM	200 µg
2'3'-cGAMP-PS	4µg (1µg of each variant)	1µg	--	200 µg
CL075-PS + 2'3'-cGAMP-PS	4µg (1µg of each variant)	1µg	164 µM	400 µg
[CL075+2'3'-cGAMP]-PS	4µg (1µg of each variant)	1µg	164 µM	200 µg

Supplementary Table 2:

Panels and regents for flow cytometry assay.

Assay	Target	Clone	Fluorochrome	Vendor	Identifier	Titer
T-Cell Cytokines (Human)	Viability	---	eFlour780	eBioscience	65-0865	1:1000
	CD3	UCHT1	PerCP-Cy5.5	BioLegend	300430	1:40
	CD4	RPA-T4	PE	BioLegend	300508	1:40
	CD8	RPA-T8	PE-Dazzle 594	BioLegend	301057	1:40
	IL-4	MP4-25D2	BV421	BioLegend	500826	1:40
	IL-17A	N49-653	Alexa Fluor 647	BD Biosciences	560491	1:10
	IFN-γ	B27	Alexa Fluor 488	BD Biosciences	557718	1:40

Assay	Target	Clone	Fluorochrome	Vendor	Identifier	Titer
T-Cell Cytokines (Mice)	Viability	---	Brilliant Violet 510	Invitrogen	L34966	1:500
	CD3	17A2	Brilliant Violet 785	BioLegend	100232	1:40
	CD4	RM4-5	APC/Fire 750	BioLegend	100568	1:160
	CD8	53-6.7	Brilliant UltraViolet 395 (BUV395)	BD Biosciences	563786	1:80
	CD44	IM7	PerCP-Cy5.5	BioLegend	103032	1:160
	IFN-γ	XMG1.2	Alexa Fluor 488	BioLegend	505813	1:160
	TNF	MP6-XT22	PE Cy7	BioLegend	506324	1:160
	IL-2	JES6-5H4	PE	BioLegend	503808	1:40

Assay	Target	Clone	Fluorochrome	Vendor	Identifier	Titer
DLNs immunophenotyping (Mice)	CD3	17A2	BUV395	BD Biosciences	612803	1:80
	PD-1	29F.1A12	PE	BioLegend	135206	1:40
	CXCR5	L138D7	Brilliant Violet 421	BioLegend	145512	1:40
	CD19	1D3/CD19	FITC	BioLegend	152404	1:320
	CD95	Jo2	PE Cy7	BD Biosciences	557653	1:160
	GL7	GL7	Alexa Fluor 647	BioLegend	144606	1:80
	CD138	281-2	Brilliant Violet 785	BioLegend	142534	1:80