

List of Supplementary Figures

- Fig. 1** | Flow cytometry in transfected mammalian cells.
- Fig. 2** | Example lymph node and muscle ROIs for mouse image analysis.
- Fig. 3** | Biodistribution of [^{18}F]FP-TMP in vaccinated mice.
- Fig. 4** | Mouse lymph node flow gating strategy and in vivo expression of eDHFR.
- Fig. 5** | In vivo expression of S2P in mouse lymph nodes.
- Fig. 6** | Antibody response against spike and eDHFR in mice.
- Fig. 7** | Representative gating strategy for mouse splenocytes for T cell polyfunctionality.
- Fig. 8** | Mouse T cell reactivity toward spike and eDHFR peptide pools following boost.
- Fig. 9** | Mouse CD8⁺ T cell reactivity at day 35 following vaccination.
- Fig. 10** | PET imaging of S2P^{Δf}-eDHFR vaccinated NHP 2.
- Fig. 11** | Time activity curves of [^{18}F]FP-TMP uptake in S2P^{Δf}-eDHFR vaccinated NHP 1.
- Fig. 12** | Time activity curves of [^{18}F]FP-TMP uptake in S2P^{Δf}-eDHFR vaccinated NHP 2.
- Fig. 13** | Time activity curves of [^{18}F]FP-TMP uptake in *C. diff* vaccinated NHP on day 1.
- Fig. 14** | Time activity curves in the brain and gonads in all vaccinated NHPs.
- Fig. 15** | Protein sequence alignment of DHFR homologues across species.

List of Supplementary Tables

- Table 1** | eDHFR peptide pools used in polyfunctionality and cytotoxicity assays.
- Table 2** | In vivo cytotoxicity individual mouse data.
- Table 3** | Summary of antibodies used for T cell polyfunctional assay.
- Table 4** | Summary of antibodies and stains used for mouse lymph node flow.

List of Supplementary Methods

- [^{18}F]FP-TMP biodistribution in mice
- Flow cytometry of vaccinated mouse lymphocytes

Supplementary Figures

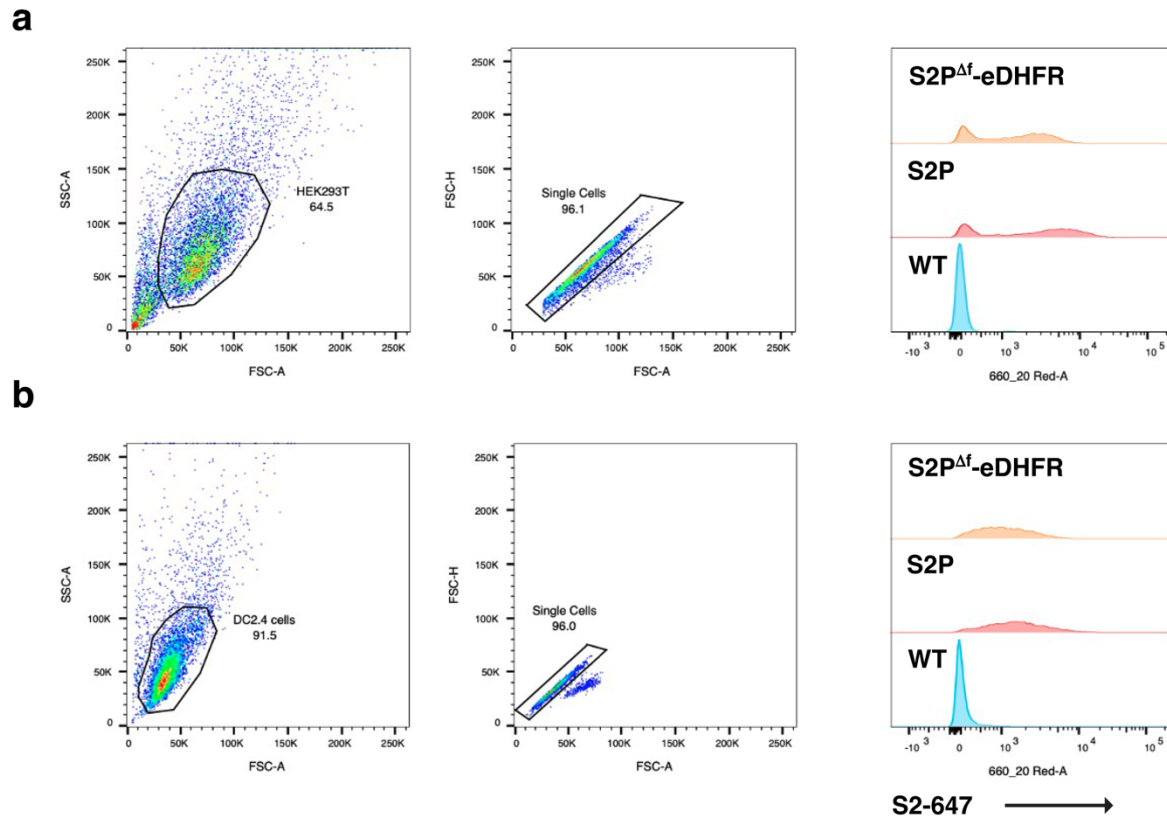


Fig. 1 | Flow cytometry in transfected mammalian cells. a Gating strategy and representative histograms for HEK293T and **b** DC2.4 cells transfected with S2P Δ f-eDHFR or S2P and stained with an AF647 conjugated antibody against spike.

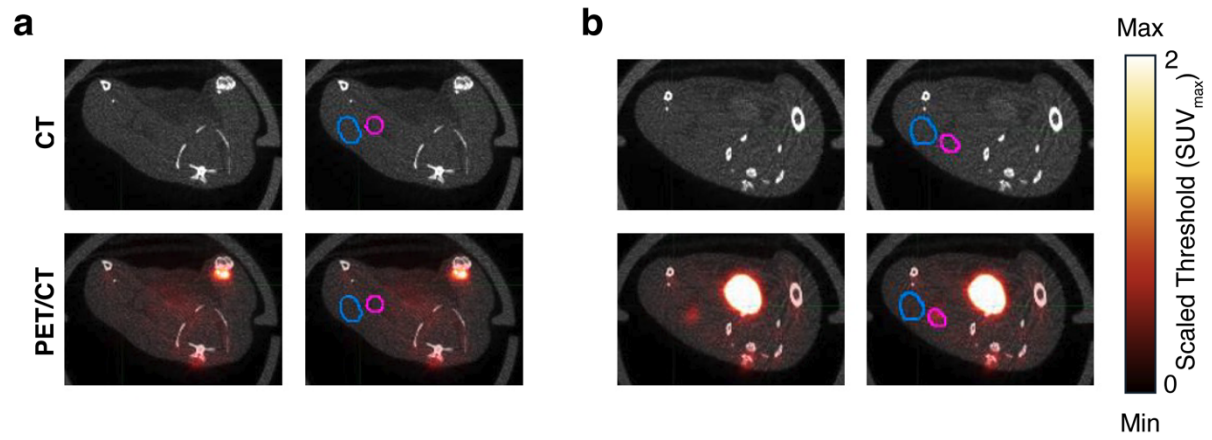


Fig. 2 | Example lymph node and muscle ROIs for mouse image analysis. a eLNP and **b** S2P^{Δf}-eDHFR vaccinated mice on day 1. Muscle ROI is shown in blue and LN in pink.

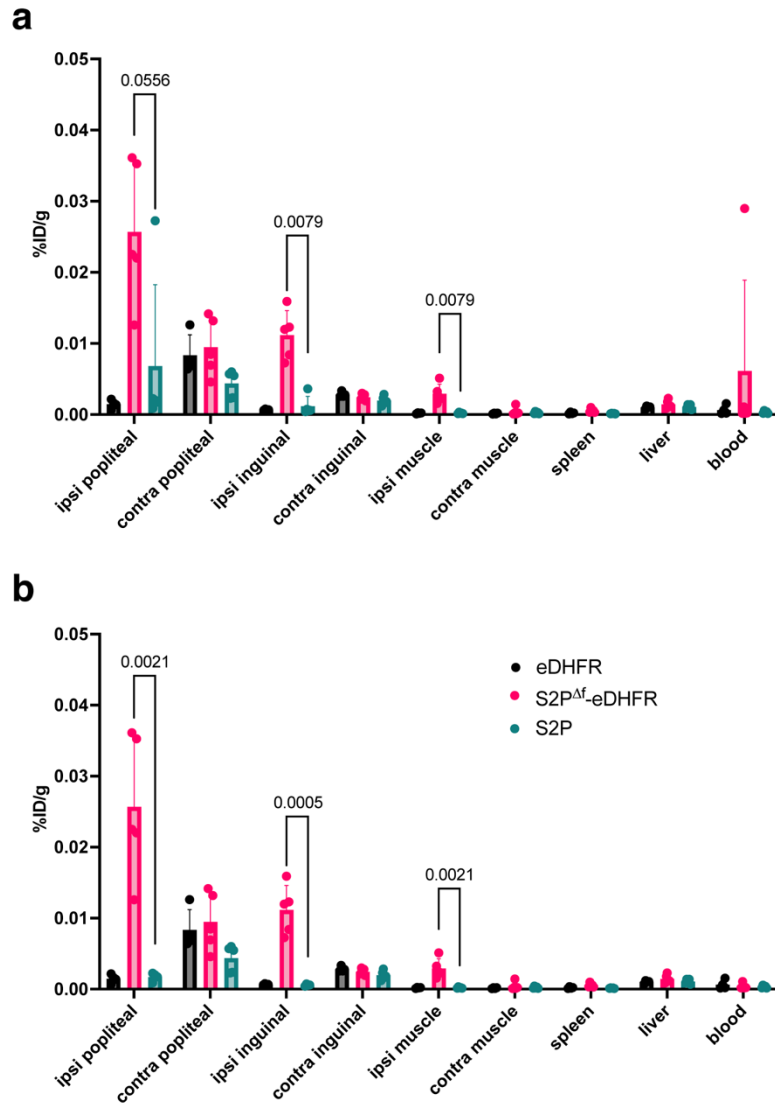


Fig. 3 | Biodistribution of [¹⁸F]FP-TMP in vaccinated mice. a All data is shown. Uptake in the ipsilateral popliteal ($p = 0.0556$), ipsilateral inguinal ($p = 0.0079$), and ipsilateral muscle ($p = 0.0079$) of the S2P^{Δf}-eDHFR and S2P vaccinated mice were compared using a Mann-Whitney test. **b** Outlier data points in the ipsilateral popliteal, ipsilateral inguinal, and blood were excluded using a ROUT test with $Q = 0.1\%$. The ipsilateral popliteal ($p = 0.0021$), ipsilateral inguinal ($p = 0.0005$), and ipsilateral muscle ($p = 0.0021$) of the S2P^{Δf}-eDHFR and S2P vaccinated mice were compared using an unpaired, two-tailed t test. Data points are mean $\pm$ SD. eDHFR, $n = 4$; S2P^{Δf}-eDHFR, $n = 5$; S2P, $n = 5$ mice per group. Source data are provided as a Source Data file.

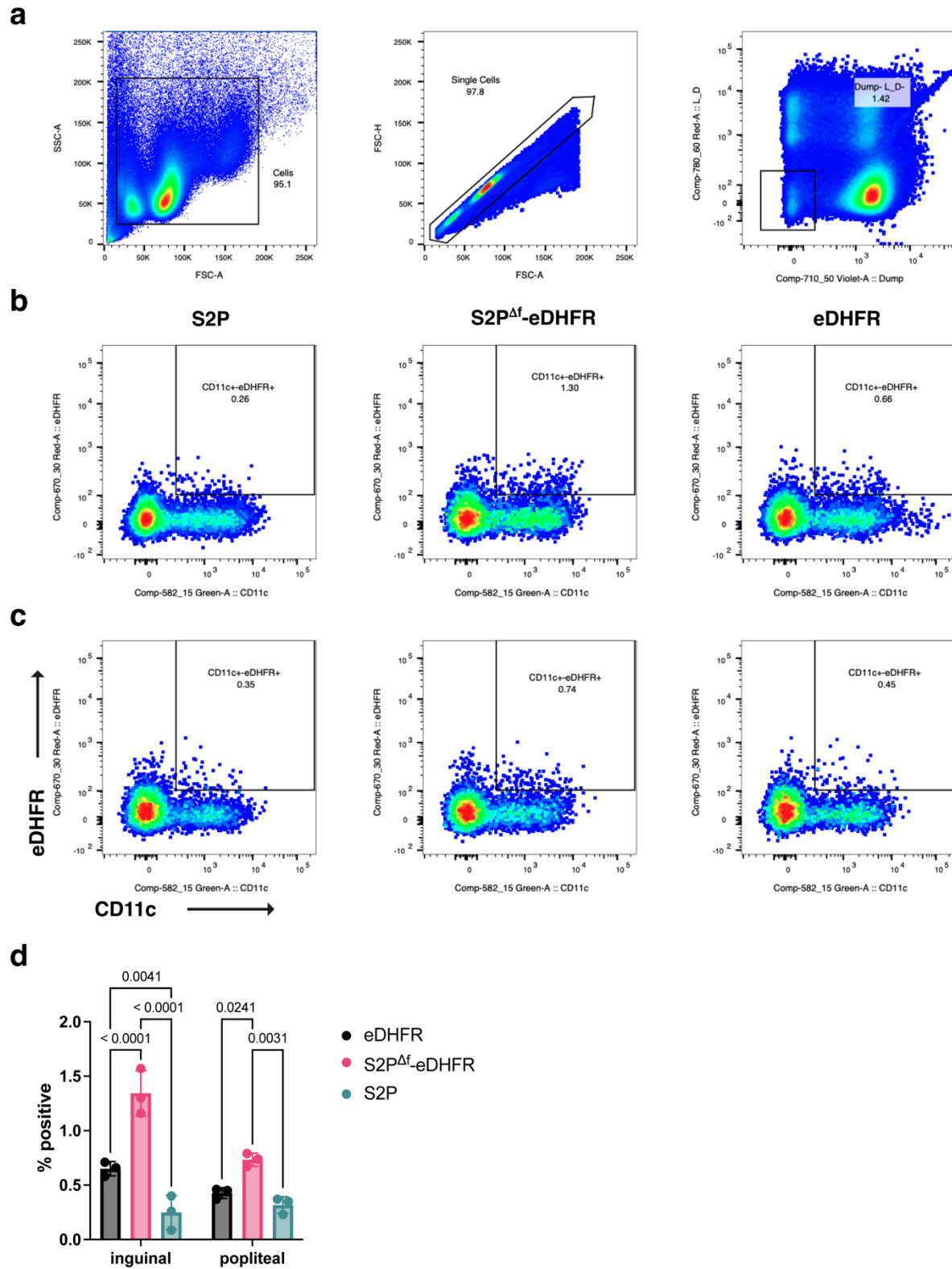


Fig. 4 | Mouse lymph node flow gating strategy and in vivo expression of eDHFR. a Gating strategy for lymphocytes. Live cells were identified using a Live/Dead stain and B, T, and NK cells

were dumped using a CD19, CD3, and NK1.1 antibody, respectively. Lymphocytes were incubated with all antibodies and stains for 1 hour. **b** Flow plots of dendritic cells with CD11c-eDHFR positivity in the inguinal and **c** popliteal LN. Median flow plot is shown. **d** Quantification of the % positivity for TMP-JF646 staining. Lymph nodes from $n = 5$ mice per group were pooled into one sample and data points represent 3 technical repeats. Groups were compared using a two-way ANOVA with Sidak's multiple comparisons test. Only significant pairwise comparisons are shown. Data points are mean $\pm$ SD. (**d**) $p < 0.0001$ unless otherwise stated. Inguinal: eDHFR vs. S2P, $p = 0.0041$; popliteal: eDHFR vs. S2P^{Δf}-eDHFR, $p = 0.0241$ and S2P^{Δf}-eDHFR vs. S2P, $p = 0.0031$. Source data for panel **d** are provided as a Source Data file.

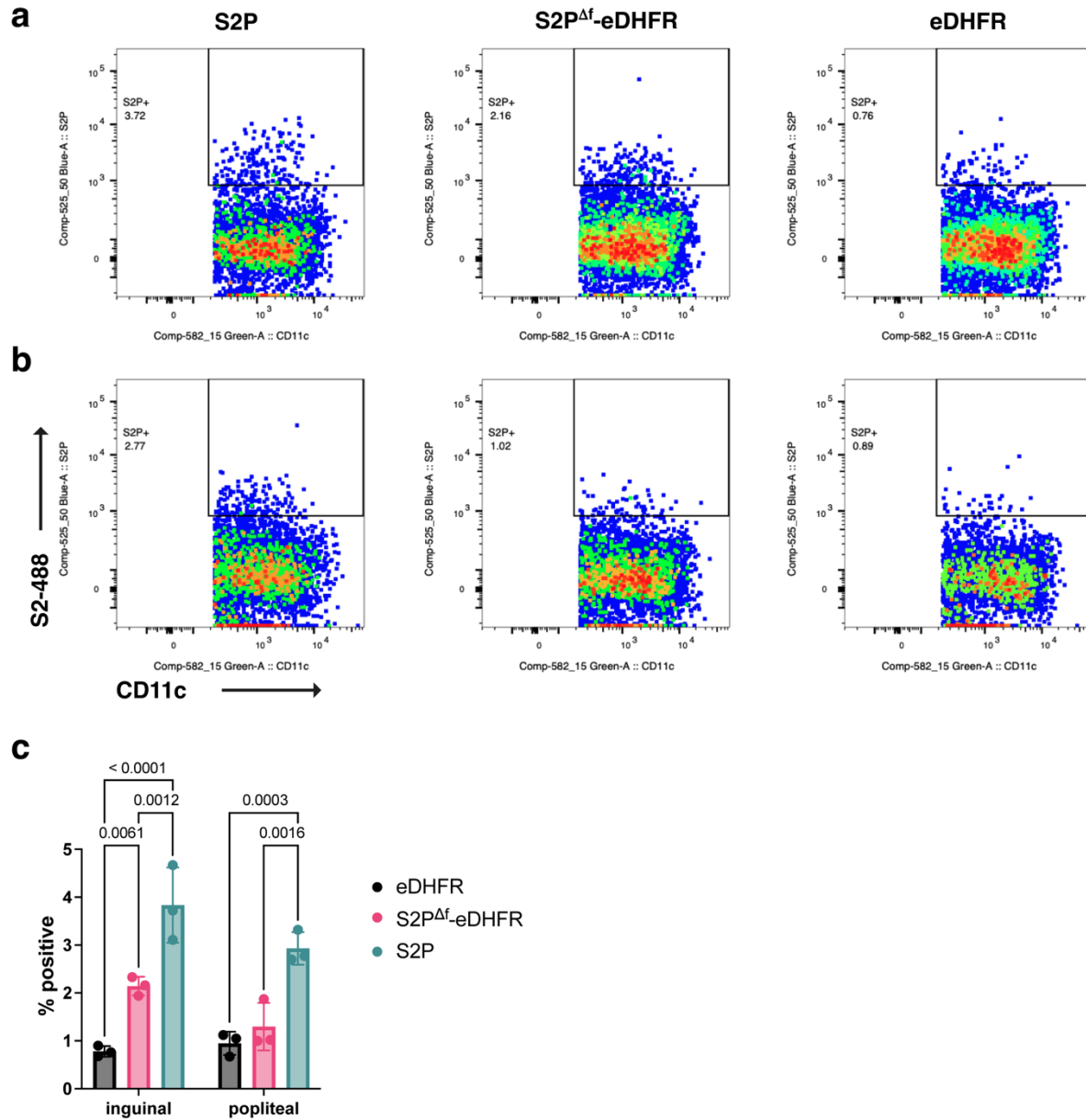


Fig. 5 | In vivo expression of S2P in mouse lymph nodes. **a** Flow plots of dendritic cells showing CD11c-S2P positivity in the inguinal and **b** popliteal LN. Median flow plot is shown. **c** Quantification of the % positivity for S2-488 staining. Two lymph nodes from $n = 6$ mice per group were pooled into 3 total samples represented by the data points. Lymphocytes were incubated with all antibodies and stains for 2 hours. Groups were compared using a two-way ANOVA with Sidak's multiple comparisons test. Only significant pairwise comparisons are shown. Data points are mean $\pm$ SD. (c) $p < 0.0001$ unless otherwise stated. Inguinal: eDHFR vs. S2P Δ f-eDHFR, $p = 0.0061$ and

S2P^{Δf}-eDHFR vs. S2P, $p = 0.0012$; popliteal: eDHFR vs. S2P, $p = 0.0003$ and S2P^{Δf}-eDHFR vs. S2P, $p = 0.0016$. Source data for panel **c** are provided as a Source Data file.

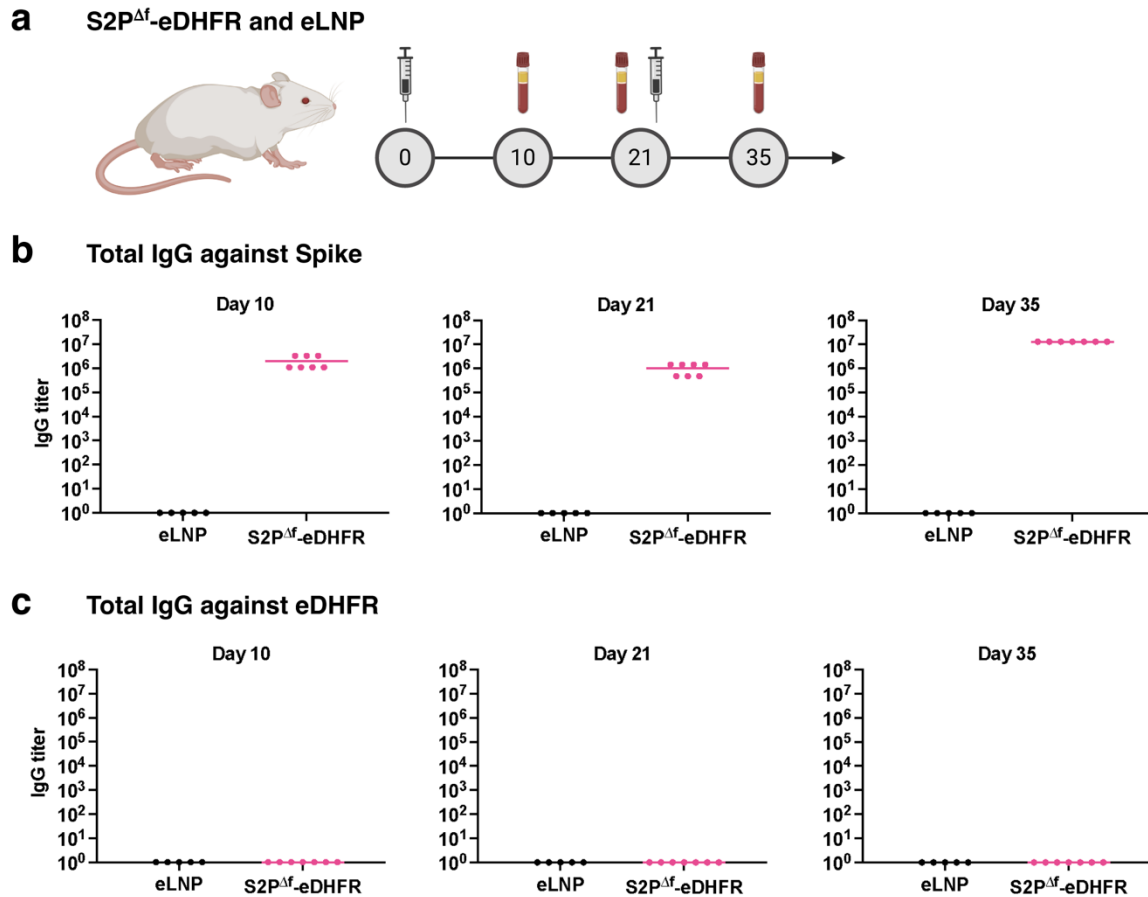


Fig. 6 | Antibody response against spike and eDHFR in mice. **a** Experimental timeline. Balb/c mice were IM injected with 10 μ g of eLNP or S2P^{Δf}-eDHFR mRNA-LNPs in the right hindlimb on day 0 and 21. On day 10, 21, and 35, serum was collected for analysis. The ALC-307 lipid was used in this experiment. **b** Total IgG antibodies against spike and **c** eDHFR in serum of vaccinated mice at days 10 and 21 post prime and day 14 post boost (day 35). eLNP, $n = 5$; S2P^{Δf}-eDHFR, $n = 7$ mice per group. Data points represent mean. **(a)** Created in BioRender. Sellmyer, M. (2025) <https://BioRender.com/o41w650>. Source data for panel **b**, **c** are provided as a Source Data file.

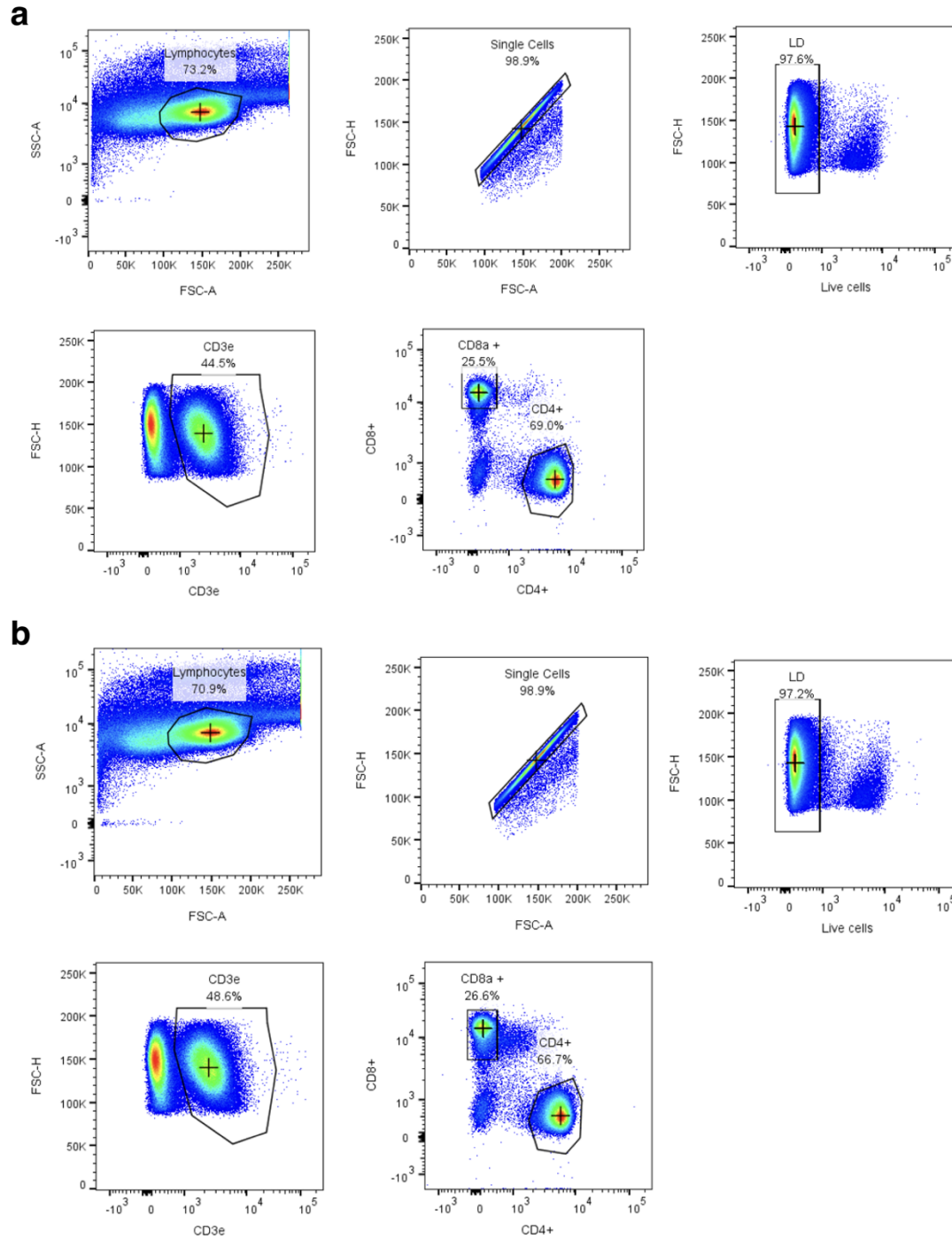


Fig. 7 | Representative gating strategy for mouse splenocytes for T cell polyfunctionality. a Identification of $CD4^+$ and $CD8^+$ T cells in unstimulated and **b** pooled spike peptides-stimulated mouse splenocytes.

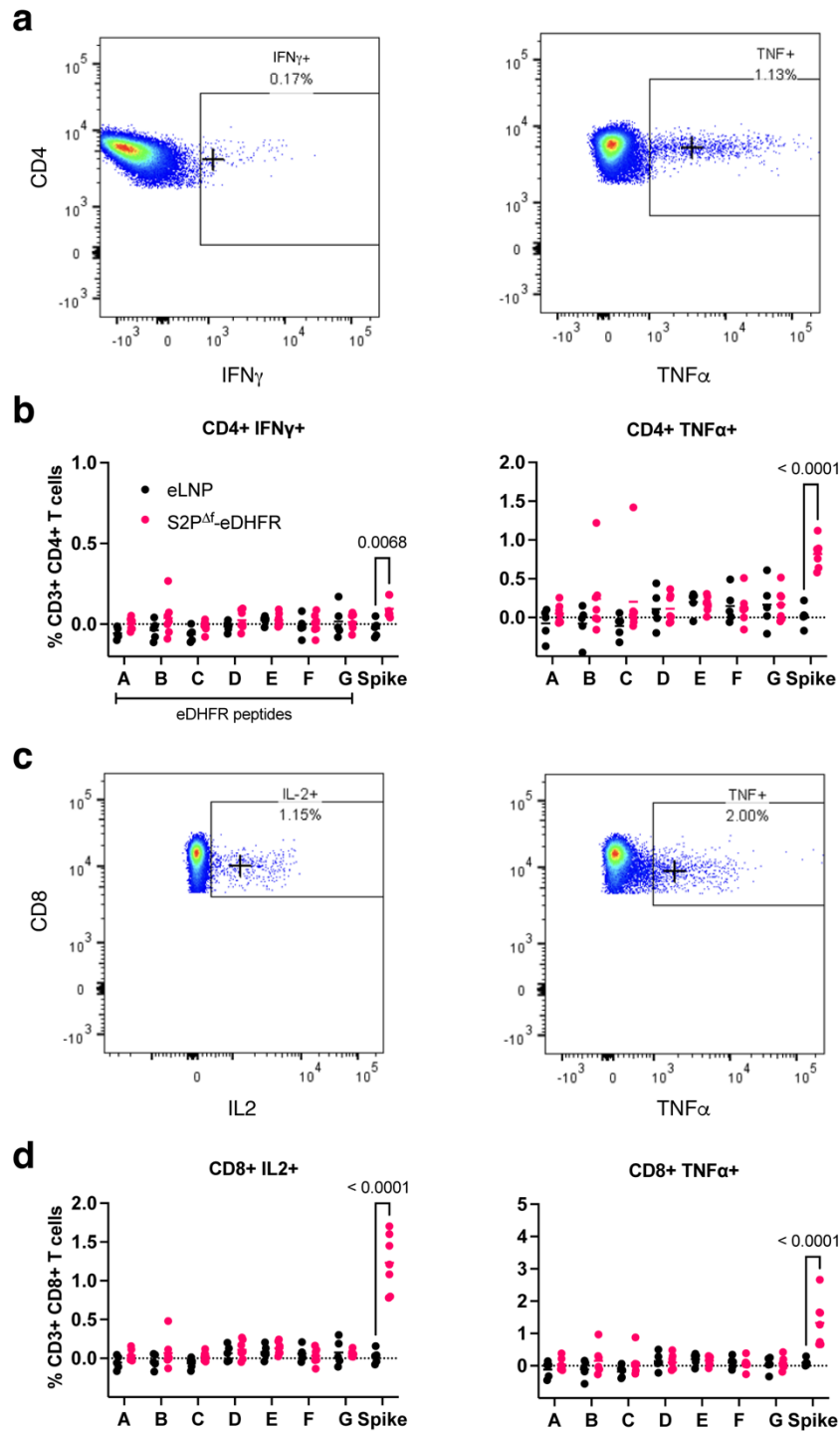


Fig. 8 | Mouse T cell reactivity toward spike and eDHFR peptide pools following boost. a Representative flow plots of cytokine expressing CD4⁺ T cells after spike stimulation. **b** Quantification of antigen-specific CD4⁺ T cells expressing INF- γ and TNF- α following stimulation with eDHFR and spike peptide pools. **c** Representative flow plots of cytokine

expressing CD8⁺ T cells after spike stimulation. **d** Quantification of antigen-specific CD8⁺ T cells expressing IL-2 and TNF- α following stimulation with eDHFR and spike peptide pools. eLNP, $n = 5$; S2P ^{Δ f}-eDHFR, $n = 7$ mice per group. The ALC-307 lipid was used in this experiment. Groups were compared using a two-way ANOVA with Sidak's multiple comparisons test. Only significant pairwise comparisons are shown. Data points are mean. (**b**, **d**) $p < 0.0001$ unless otherwise stated. CD4⁺ INF- γ ⁺: $p = 0.0068$. Source data for panel **b**, **d** are provided as a Source Data file.

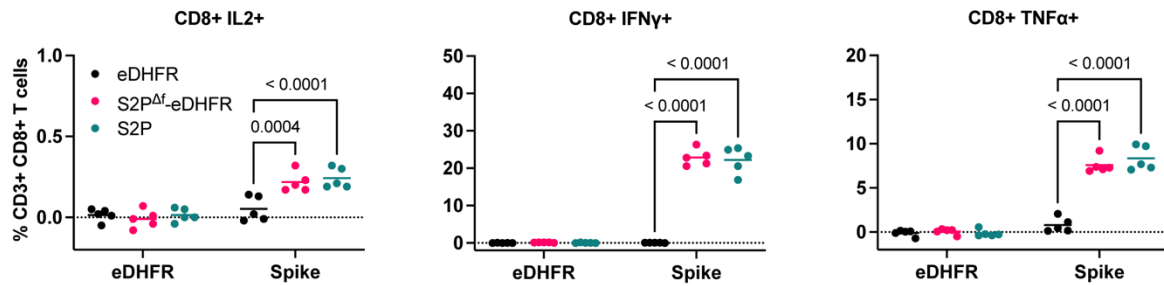


Fig. 9 | Mouse CD8⁺ T cell reactivity data at day 35 following vaccination. $n = 5$ mice per group. The ALC-0315 lipid was used in this experiment. Groups were compared using a two-way ANOVA with Tukey's multiple comparisons test. Only significant pairwise comparisons are shown. Data points are mean. $p < 0.0001$ unless otherwise stated. CD8⁺ IL2⁺: eDHFR vs. S2P^{Δf}-eDHFR, $p = 0.0004$. Source data are provided as a Source Data file.

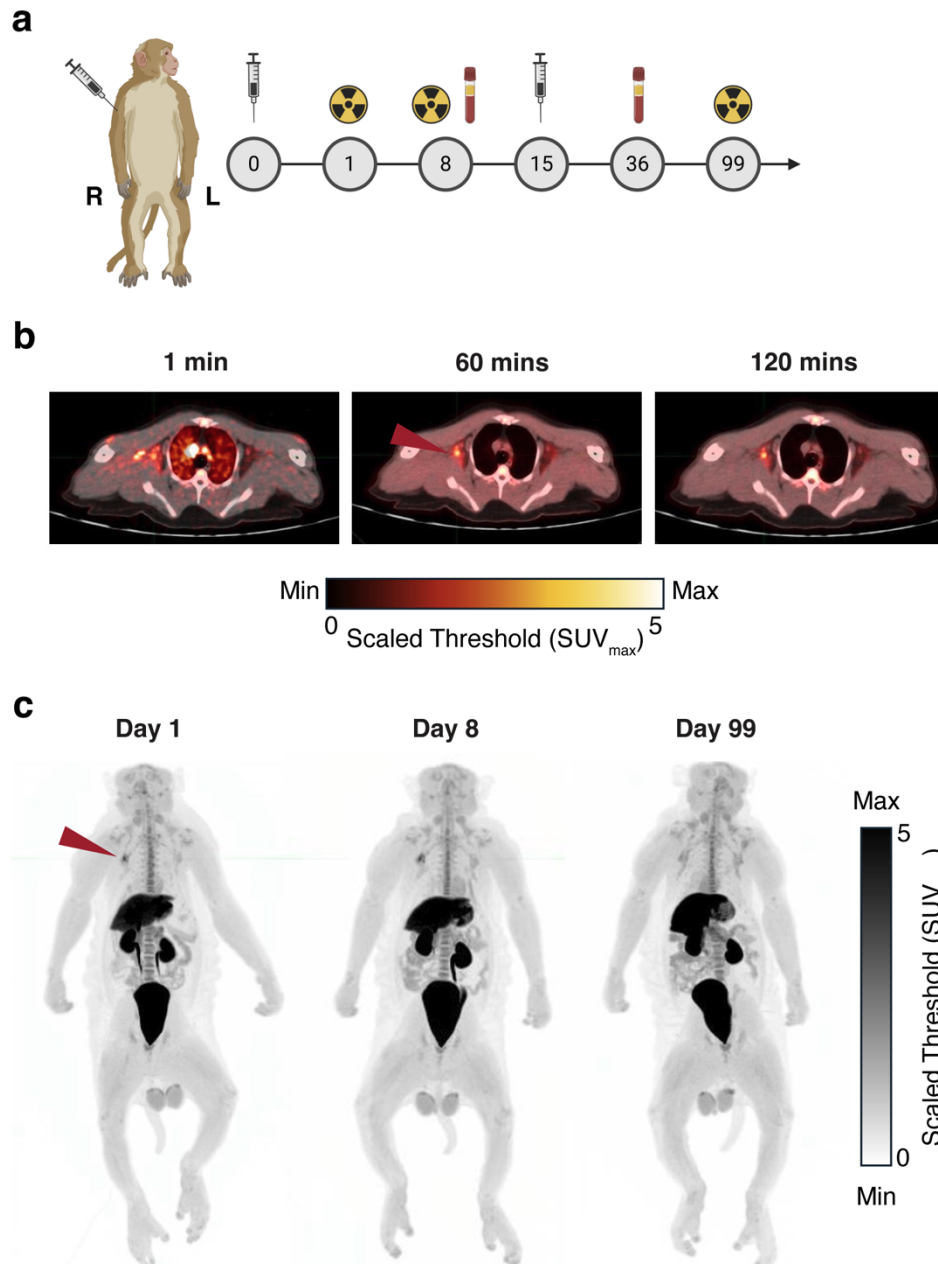


Fig. 10 | PET imaging of S2P Δ f-eDHFR vaccinated NHP 2. **a** Experimental timeline. An NHP was vaccinated in the right deltoid with S2P Δ f-eDHFR on day 0 and boosted on day 15. Dynamic PET/CT images were acquired on day 1, 8, and 99. Blood samples were taken on day 8 and 36 for analysis. The ALC-307 lipid was used in this experiment. **b** Axial PET/CT slices through the axillary LNs at 1, 60, and 120 minutes post [^{18} F]FP-TMP administration on day 1. **c** Whole body PET/CT images at 60 minutes post [^{18} F]FP-TMP administration day 1, 8, and 99. Red arrow

indicates ipsilateral axillary LN. (a) Created in BioRender. Sellmyer, M. (2025)
<https://BioRender.com/x90a119>.

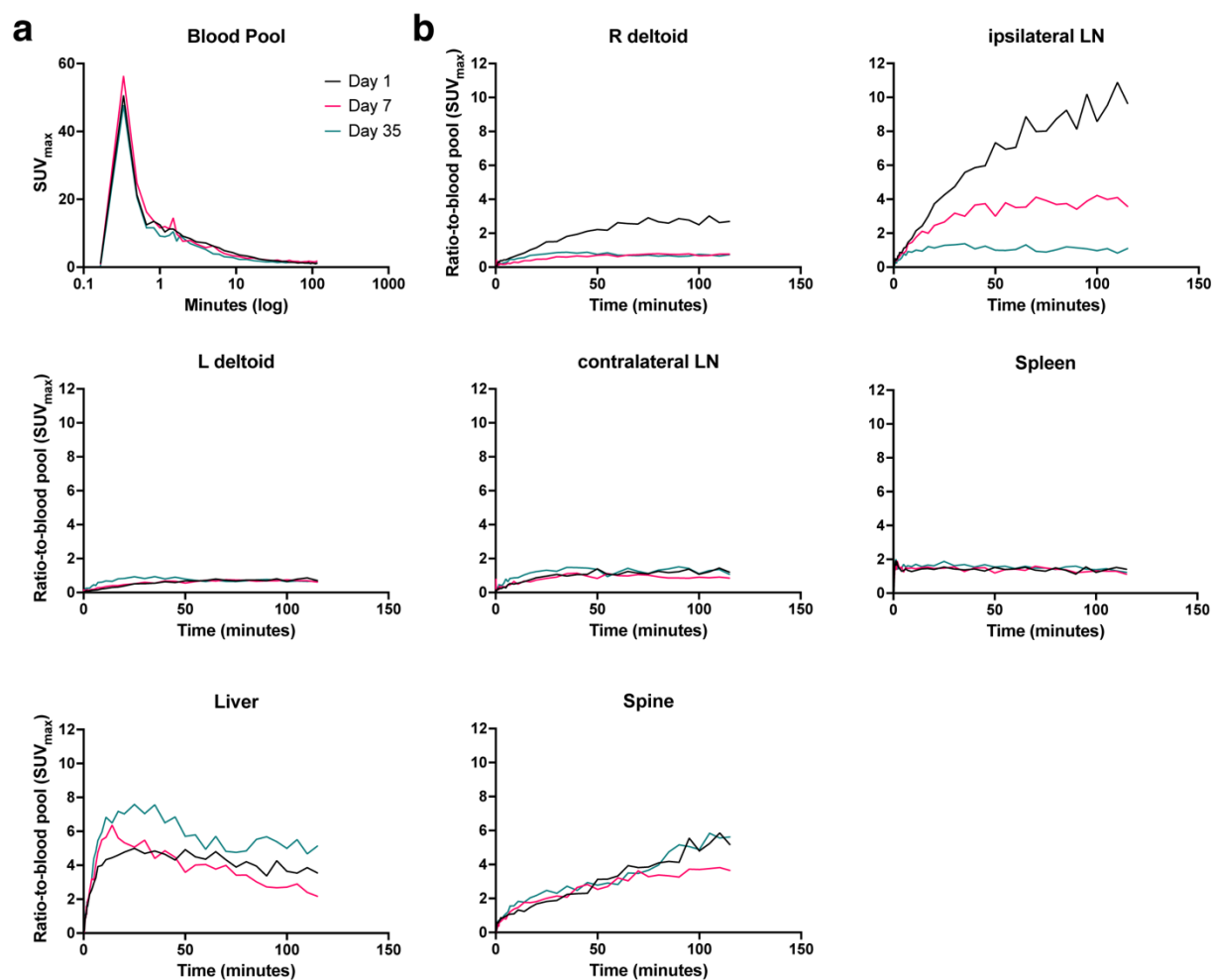


Fig. 11 | Time activity curves of [^{18}F]FP-TMP uptake in S2P Δf -eDHFR vaccinated NHP 1. **a Blood pool SUV_{max} over the 120 minute dynamic imaging period with time shown in log scale. **b** All remaining tissues SUV_r over the 120 minute dynamic imaging period with time shown in linear scale. Source data are provided as a Source Data file.**

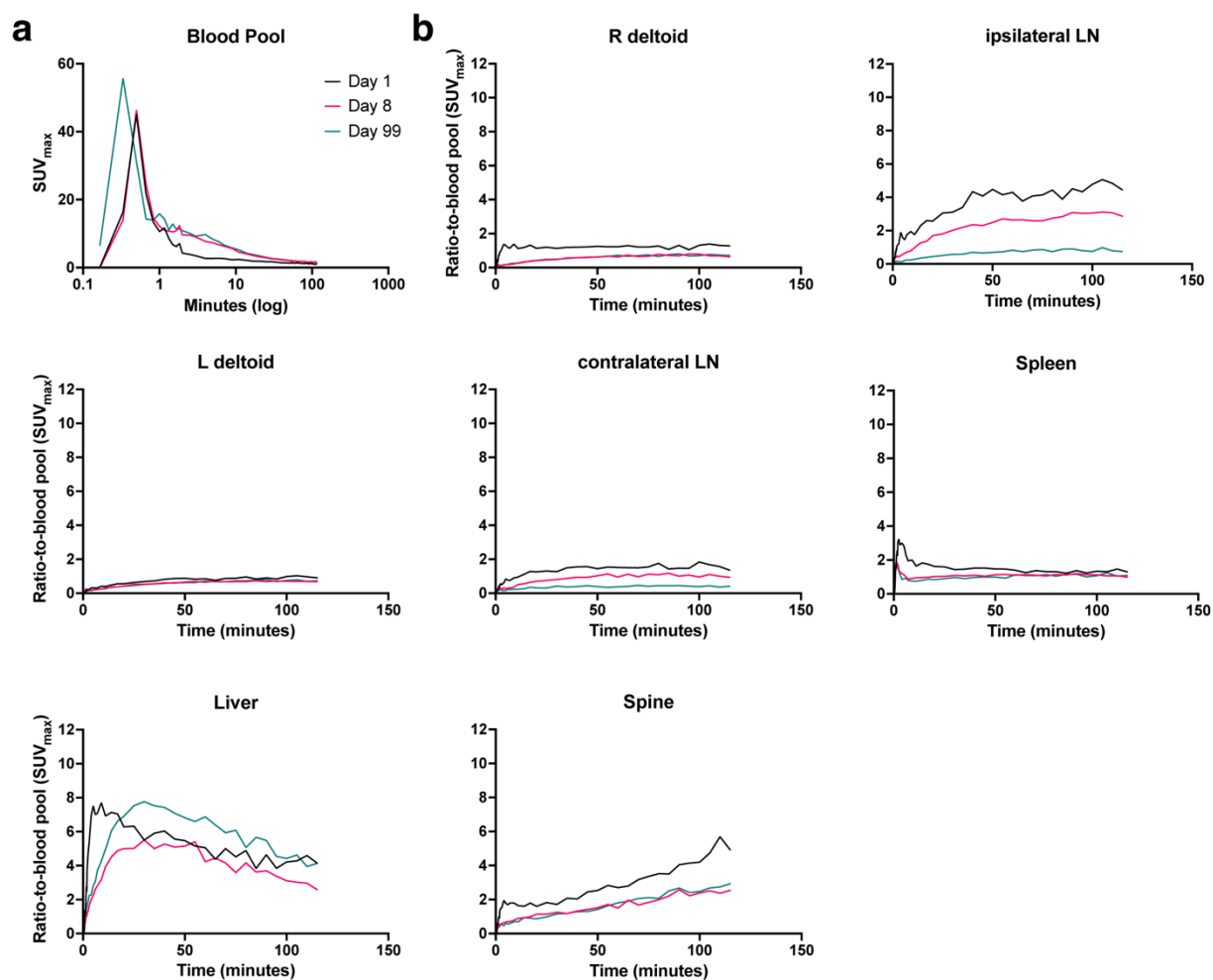


Fig. 12 | Time activity curves of [¹⁸F]FP-TMP uptake in S2P^{Δf}-eDHFR vaccinated NHP 2. **a Blood pool SUV_{max} over the 120 minute dynamic imaging period with time shown in log scale. **b** All remaining tissues SUV_r over the 120 minute dynamic imaging period with time shown in linear scale. Source data are provided as a Source Data file.**

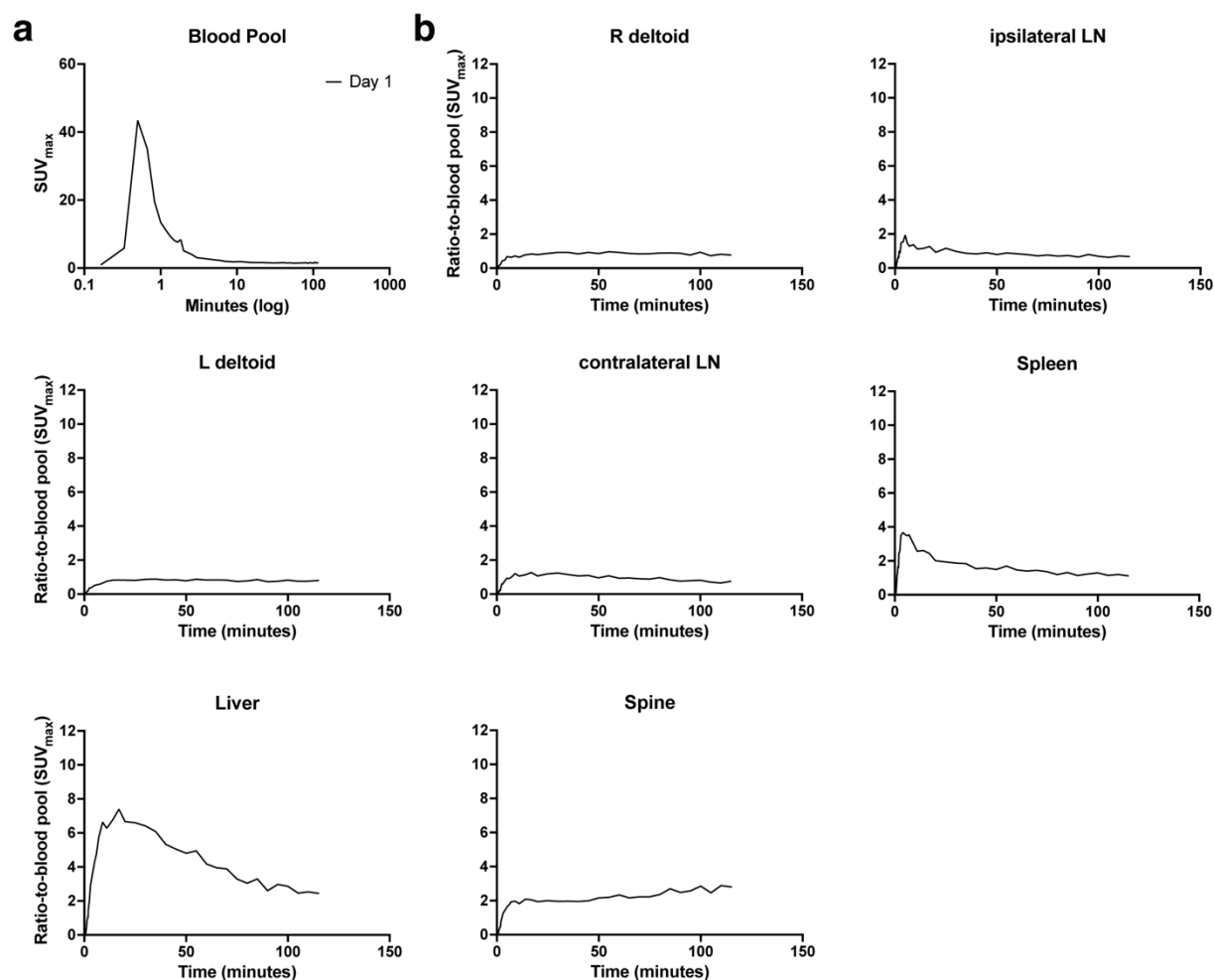


Fig. 13 | Time activity curves of $[^{18}\text{F}]$ FP-TMP uptake in *C. diff* vaccinated NHP on day 1. **a Blood pool SUV_{max} over the 120 minute dynamic imaging period with time shown in log scale. **b** All remaining tissues SUV_r over the 120 minute dynamic imaging period with time shown in linear scale. Source data are provided as a Source Data file.**

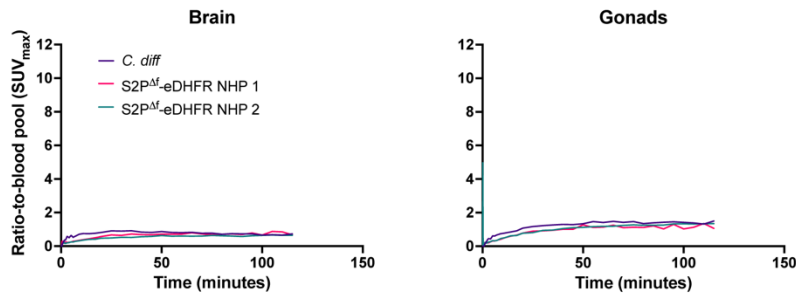


Fig. 14 | Comparison of $[^{18}\text{F}]$ FP-TMP uptake in the brain and gonads in all vaccinated NHPs.

Time activity curve of $[^{18}\text{F}]$ FP-TMP perfusion in the brain and gonads of S2P Δ^f -eDHFR and *C. diff* vaccinated NHPs on day 1. Brain and gonad measurements for S2P Δ^f -eDHFR NHP 1 were analyzed on 2 mm voxel reconstructions and S2P Δ^f -eDHFR NHP 2 and *C. diff* NHP on 4 mm voxel reconstructions.

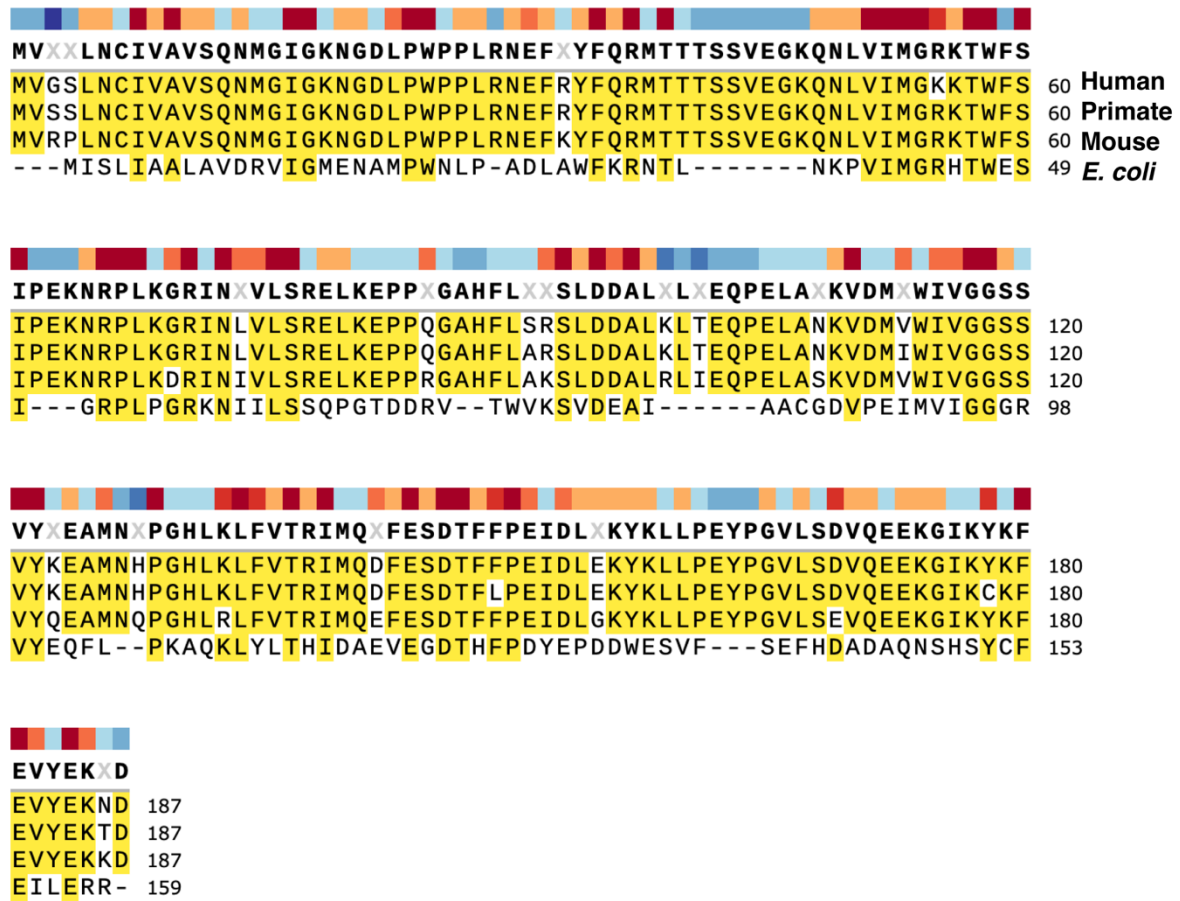


Fig. 15 | Protein sequence alignment of DHFR homologues across species.

Supplementary Tables

Table 1 | eDHFR peptide pools used in polyfunctionality and cytotoxicity assays.

Full sequence: MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWESI
 GRPLPGRKNIILSSQPGTDDRVTWVKSVD E A I A A C G D V P E I M V I G G G R V Y E Q F L P K A Q K Y
 L T H I D A E V E G D T H F P D Y E P D D W E S V F S E F H D A D A Q N S H S Y C F E I L E R R

Peptide	Peptide Sequence	Pool	Peptide	Peptide Sequence	Pool
1	MISLIAALAVDRVIG	A	20	SVDEAIAACGDVPEI	D
2	IAALAVDRVIGMENA	A	21	AIAACGDVPEIMVIG	E
3	AVDRVIGMENAMPWN	A	22	CGDVPEIMVIGGGRV	E
4	VIGMENAMPWNLPAD	A	23	PEIMVIGGGRVYEQF	E
5	ENAMPWNLPADLAWF	A	24	VIGGGRVYEQFLPKA	E
6	PWNLPADLAWFKRNT	B	25	GRVYEQFLPKAQKLY	E
7	PADLAWFKRNTLNKP	B	26	EQFLPKAQKLYLTHI	F
8	AWFKRNTLNKPVIMG	B	27	PKAQKLYLTHIDAEV	F
9	RNTLNKPVIMGRHTW	B	28	KLYLTHIDAEVEGDT	F
10	NKPVIMGRHTWESIG	B	29	THIDAEVEGDTHFPD	F
11	IMGRHTWESIGRPLP	C	30	AEVEGDTHFPDYEPD	F
12	HTWESIGRPLPGRKN	C	31	GDTHFPDYEPDDWES	F
13	SIGRPLPGRKNIILS	C	32	FPDYEPDDWESVFSE	G
14	PLPGRKNIILSSQPG	C	33	EPDDWESVFSEFHDA	G
15	RKNIILSSQPGTDDR	C	34	WESVFSEFHDAQNA	G
16	ILSSQPGTDDRVTWV	D	35	FSEFHDAQNSHSY	G
17	QPGTDDRVTWVKSVD	D	36	HDADAQNSHSYCFEI	G
18	DDRVTWVKSVD E A I A	D	37	AQNSHSYCFEILERR	G
19	TWVKSVD E A I A A C G D	D			

Table 2 | In vivo cytotoxicity individual mouse data.

Mouse	Immunization	Stimulation	CFSE^{lo} % cell population	CFSE^{hi} % cell population	Ratio (hi:lo)
1	S2P ^{Δf} -eDHFR	spike	11.1	88.9	8.0
2	S2P ^{Δf} -eDHFR	spike	9.29	90.5	9.7
3	S2P ^{Δf} -eDHFR	spike	12.4	87.5	7.1
4	S2P ^{Δf} -eDHFR	spike	9.59	90.1	9.4
5	S2P ^{Δf} -eDHFR	spike	9.45	90.2	9.5
6	S2P ^{Δf} -eDHFR	eDHFR	46.9	53.1	1.1
7	S2P ^{Δf} -eDHFR	eDHFR	46.8	53.1	1.1
8	S2P ^{Δf} -eDHFR	eDHFR	47.2	52.8	1.1
9	S2P ^{Δf} -eDHFR	eDHFR	47.5	52.5	1.1
10	S2P ^{Δf} -eDHFR	eDHFR	46	53.4	1.2
11	eLNP	spike	48.6	50.3	1.0
12	eLNP	spike	49.4	50.5	1.0
13	eLNP	spike	48.5	50.9	1.0
14	eLNP	eDHFR	47.4	52.4	1.1
15	eLNP	eDHFR	45.5	54.0	1.2
16	eLNP	eDHFR	46.8	53.0	1.1

Table 3 | Summary of antibodies used for T cell polyfunctional assay.

Brand	Cat#	F-Tag	Antigen	Clone	Laser	[mg/mL]
BD	553793	FITC	CD107a	1D4B	Blue (515)	0.5
Biolegend	100434	PerCP-Cy5.5	CD4	GK1.5	Blue (569)	0.2
Biolegend	100725	Pac Blue	CD8a	53-6.7	Violet (450)	0.5
ThermoFisher	L34966	Aquablue	L/D	N/A	Violet (515)	N/A
Biolegend	100351	BV605	CD3e	145-2C11	Violet (605)	0.2
BD	564006	BV786	IL-4	11B11	Violet (780)	0.2
BD	554429	APC	IL-2	JES6-5H4	Red (670)	0.2
BD	557998	AF700	IFN- γ	XMG1.2	Red (710)	0.2
Biolegend	506940	APC/Cy7	IL-17	TC11-18H10.1	Red (780)	0.2
Biolegend	504304	PE	IL-5	TRFK5	Green (575)	0.2
BD	557644	PE/Cy7	TNF- α	MP6-XT22	Green (780)	0.2

Table 4 | Summary of antibodies and stains used for mouse LN flow.

Brand	Cat#	F-Tag	Antigen	Clone	Laser	[mg/mL]
Invitrogen	L34976A	Near IR	L/D	N/A	Red (780)	N/A
Biolegend	108745	BV711	NK1.1	PK136	Violet (710)	0.2
Biolegend	115555	BV711	CD19	6D5	Violet (710)	0.2
Biolegend	100241	BV711	CD3	17A2	Violet (710)	0.2
Biolegend	123114	PE/Cy7	F4/80	BM8	Green (780)	0.2
Biolegend	117308	PE	CD11c	N418	Green (582)	0.2
Sino Biological	40590-MM05	AF488	Spike S2	05	Blue (525)	N/A
N/A	N/A	JF646	eDHFR	N/A	Red (670)	N/A

Supplementary Methods

[¹⁸F]FP-TMP biodistribution in mice

Adult female Balb/c mice were vaccinated IM in the right hindlimb with 10 µg of either eDHFR, S2P^{Δf}-eDHFR, or S2P mRNA-LNPs. The ALC-0315 lipid was used in these experiments. On day 1, mice were injected IV with ~70 µCi (2.59 MBq) of [¹⁸F]FP-TMP. Following a 2 hour uptake, mice were sacrificed and blood and tissue samples were collected. [¹⁸F]FP-TMP uptake per organ was quantified on a gamma counter (Perkin Elmer) and analyzed by ratioing the [¹⁸F] counts in each tissue to the injected dose and dividing by the weight of the sample (%ID/g). The weight of the LNs in each group were pooled and averaged.

Flow cytometry of vaccinated mouse lymphocytes

Adult female Balb/c mice were vaccinated IM in the right hindlimb with 10 µg of either eDHFR, S2P^{Δf}-eDHFR, or S2P mRNA-LNPs. The ALC-0315 lipid was used in these experiments. On day 1, the ipsilateral popliteal and inguinal LN were harvested and lymphocytes were prepared in a single cell suspension as previously described. Lymphocytes were stained with various immune cell markers and incubated with an Alexa Fluor 488 conjugated (Invitrogen, A20181) SARS-CoV-2 spike S2 mouse monoclonal antibody and 500 nM TMP-JF646 for 1 or 2 hours at 4°C (Supplementary Table 4). Stained lymphocytes were washed 3 times in FACS buffer (5% FBS in PBS) and filtered prior to analysis on a BD LSR II flow cytometer until at least 1x10⁶ events were recorded. Data were analyzed on FlowJo.