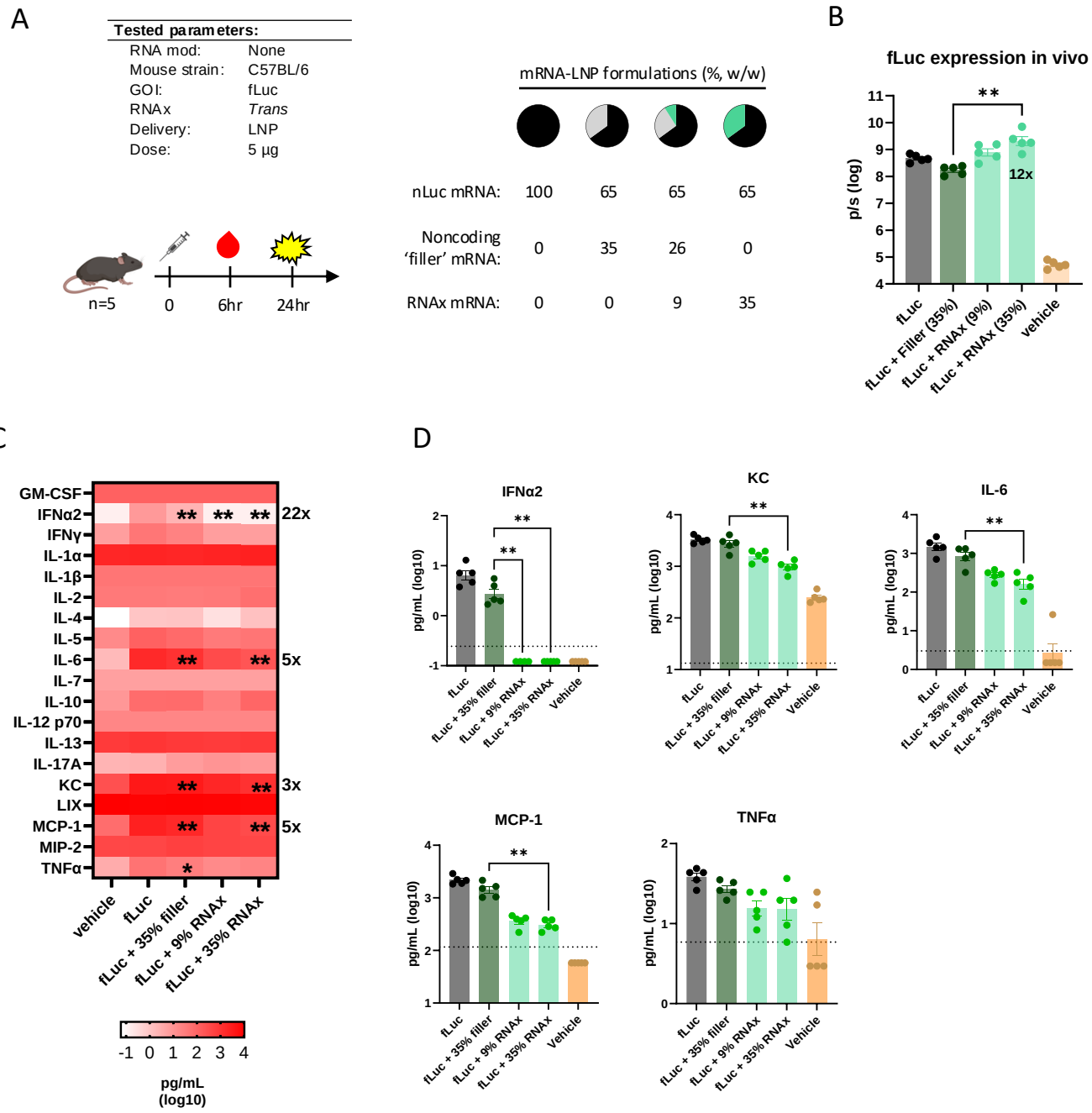
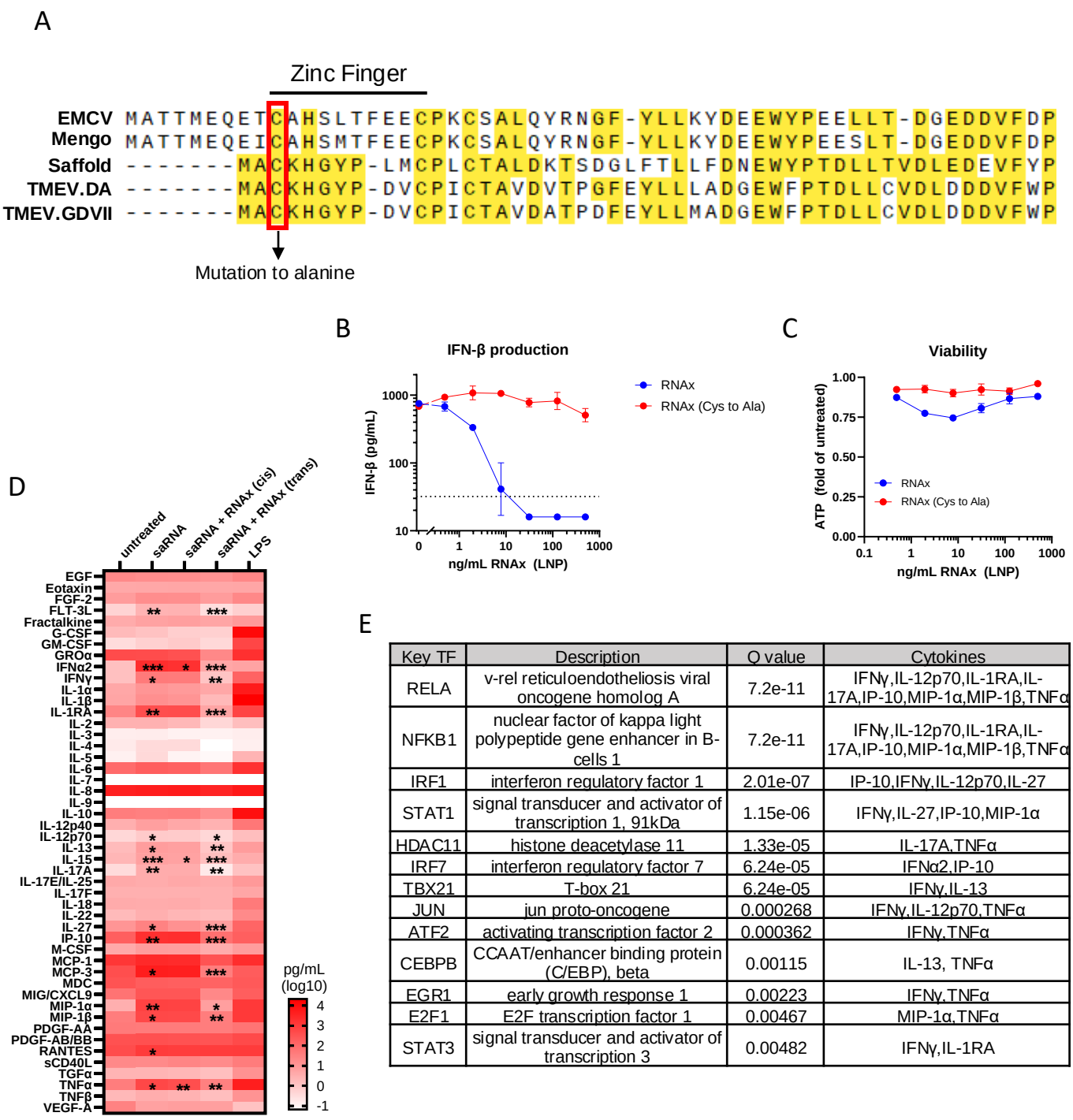


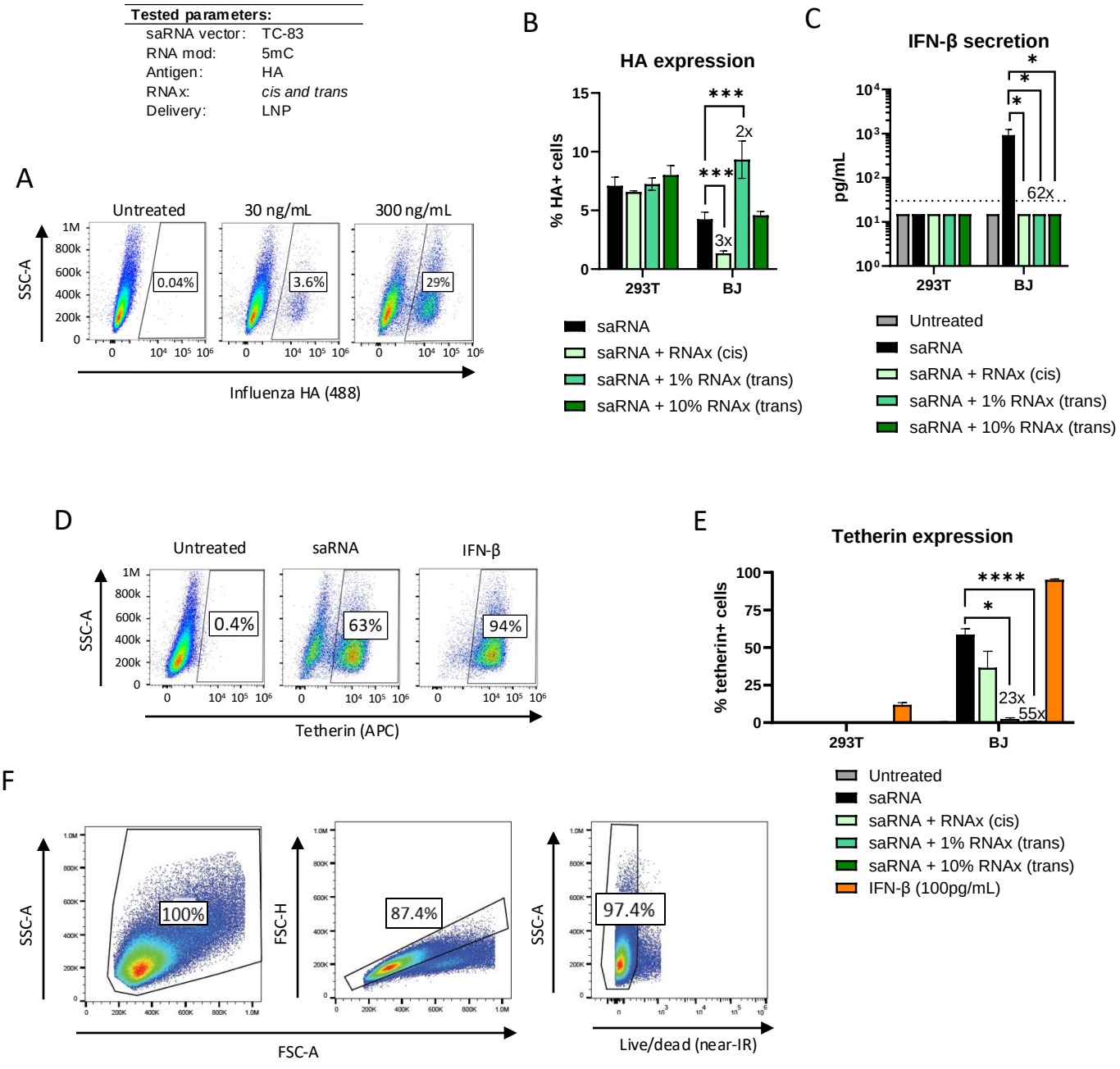
Supplementary figure 1. RNAx enhances GOI expression and suppresses proinflammatory cytokines from unmodified RNA *in vivo*. A) Unmodified fLuc mRNA was formulated into LNPs with or without RNAx in trans or a size-matched non-coding mRNA ('filler'). 5 µg mRNA-LNP was injected intramuscularly into C57BL/6 mice and 6 h later, serum was drawn for quantifying proinflammatory cytokines and 24 h later, mice were imaged for Luc activity measured at the injection site with IVIS. B) Total flux of Luc signal is represented by photons per sec (p/s). Geometric mean ± geometric SD, n=5 mice per group, **p<0.01 Kruskal-Wallis test with Dunn's multiple comparisons (RNAx groups compared to fLuc + filler). Numbers on bar indicate average fold change enhancement of RNAx containing groups vs. filler. C) Serum cytokine levels quantified with a multiplex assay and ELISA (IFNα2). Geometric mean. Asterisks in the 'fLuc + filler' column indicate cytokines that were significantly induced by mRNA vs. vehicle (*p<0.05, **p<0.01, Mann-Whitney Test). Asterisks in the '+ RNAx' columns indicate cytokines that were significantly inhibited by RNAx vs. mRNA + filler (*p<0.05, **p<0.01, Kruskal-Wallis test with Dunn's multiple comparisons) with the magnitude of the fold inhibition indicated to the right of the histogram. D) Full data for cytokines that were induced in the fLuc mRNA only group relative to vehicle. Created with BioRender.com



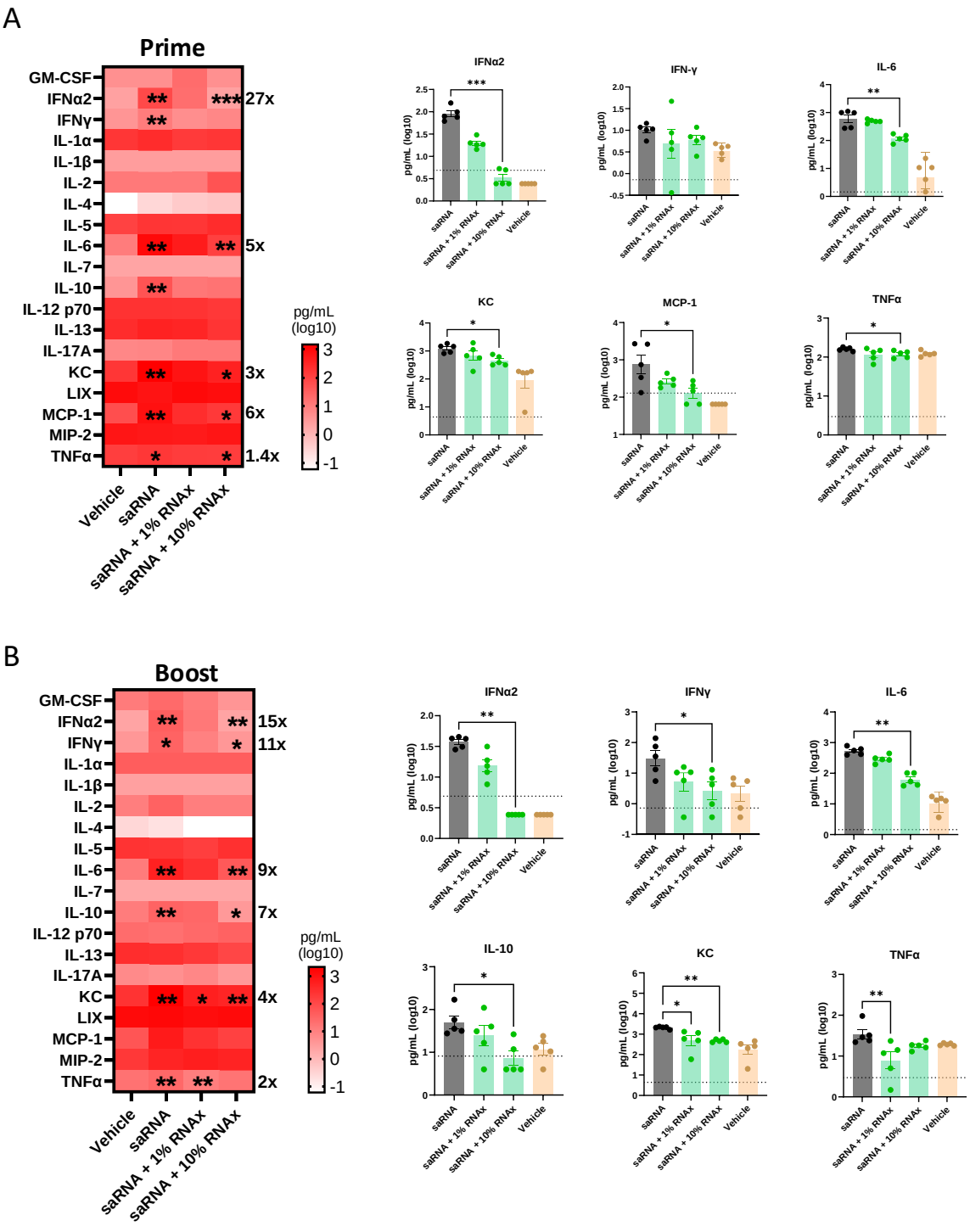
E

Key TF	Description	Q value	Cytokines
RELA	v-rel reticuloendotheliosis viral oncogene homolog A	7.2e-11	IFNγ,IL-12p70,IL-1RA,IL-17A,IP-10,MIP-1α,MIP-1β,TNFα
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	7.2e-11	IFNγ,IL-12p70,IL-1RA,IL-17A,IP-10,MIP-1α,MIP-1β,TNFα
IRF1	interferon regulatory factor 1	2.01e-07	IP-10,IFNγ,IL-12p70,IL-27
STAT1	signal transducer and activator of transcription 1, 91kDa	1.15e-06	IFNγ,IL-27,IP-10,MIP-1α
HDAC11	histone deacetylase 11	1.33e-05	IL-17A,TNFα
IRF7	interferon regulatory factor 7	6.24e-05	IFNα2,IP-10
TBX21	T-box 21	6.24e-05	IFNγ,IL-13
JUN	jun proto-oncogene	0.000268	IFNγ,IL-12p70,TNFα
ATF2	activating transcription factor 2	0.000362	IFNγ,TNFα
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	0.00115	IL-13, TNFα
EGR1	early growth response 1	0.00223	IFNγ,TNFα
E2F1	E2F transcription factor 1	0.00467	MIP-1α,TNFα
STAT3	signal transducer and activator of transcription 3	0.00482	IFNγ,IL-1RA

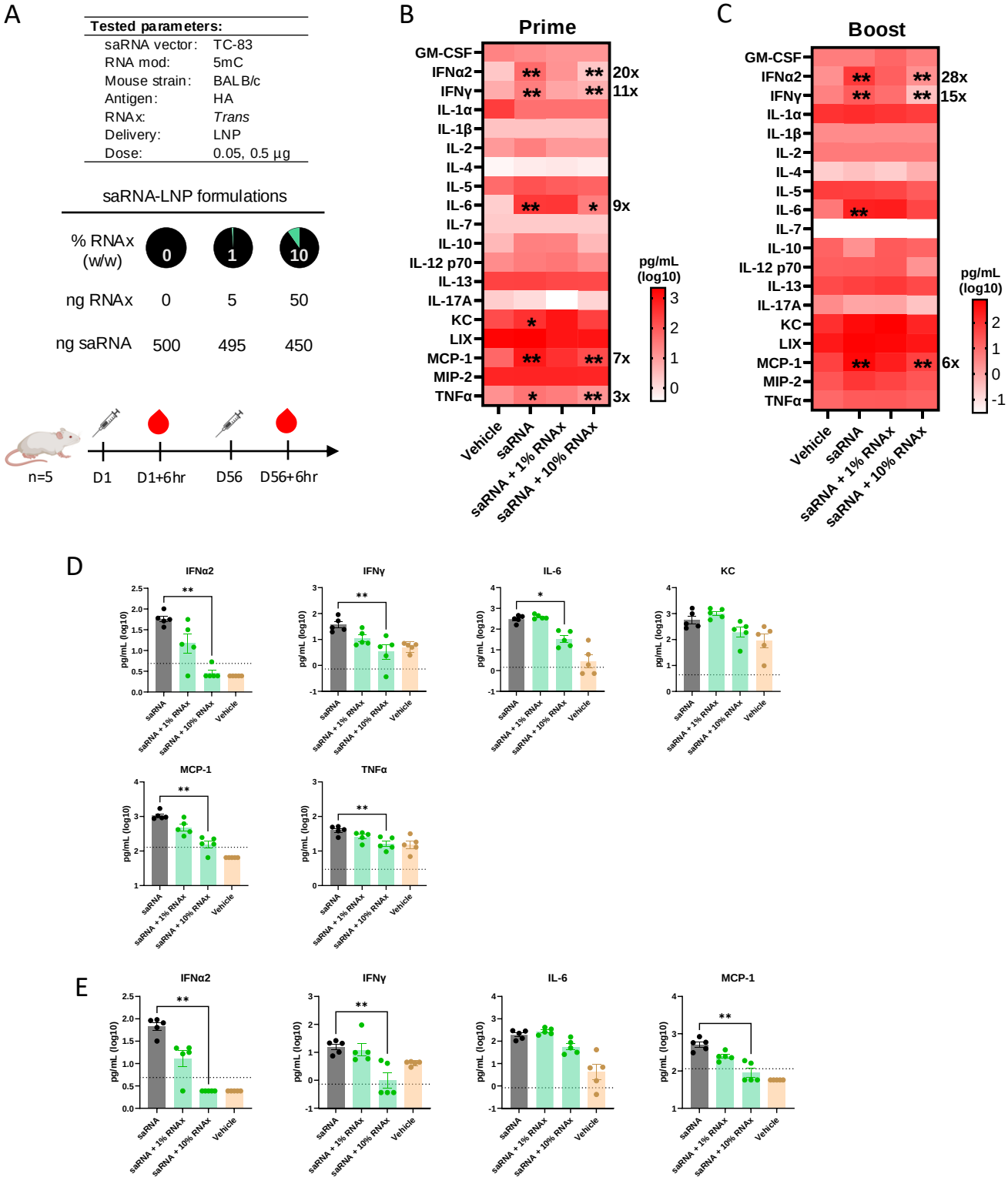
Supplementary figure 2: Functional domain and estimated transcription factor determinants of RNAx-mediated suppression of proinflammatory cytokine secretion. A) Amino acid alignment of leader proteins from major Cardioviruses EMCV (AHF20227.1), Mengo (AAA46547.1), Saffold (YP_001949875.1), TMEV.DA (AGM61326.1), TMEV.GDVII (NP_040350.1). Cysteine residue from the conserved zinc finger domain was mutated to Alanine, then mRNA formulated into LNPs and added to BJ cells at various concentration for 24 hin the presence of pre-formulated HA-nLuc saRNA-LNPs (See Fig. 2). B) IFN-β was quantified by Lumit assay (Promega) and C) viability by cell titer glo (Promega). Mean ± SEM, n=2. D) PBMCs treated with 5 µg/mL saRNA-LNPs were analyzed for the abundance of a panel of human cytokines. Geometric mean. Asterisks in the 'saRNA' column indicate cytokines that were induced 2-fold on average by saRNA only vs. untreated cells (*p<0.05, **p<0.01, ***p<0.001 paired t-test). Asterisks in the 'saRNA + RNAx' columns indicate cytokines that were suppressed on average by RNAx relative to saRNA only (*p<0.05, **p<0.01, ***p<0.001 paired t-test). E) Transcription factors (TF) linked to each of the RNAx-suppressed cytokines was extracted using the TRRUST tool (version 2, https://www.gmpedia.org/trrust/, accessed Jan 2025)



Supplementary figure 3: *In vitro* expression of Influenza HA, tetherin, and IFN-β from HA saRNA-LNPs. A) Representative flow cytometry plots for Influenza HA surface expression from HA saRNA-LNP treated BJ cells (no RNAx, 24 h post treatment). B) 293T and BJ cells were treated with 30 ng/mL saRNA-LNPs with or with co-formulation with RNAx for 24 h and Influenza HA surface expression quantified by flow cytometry and C) IFN-β secretion was quantified from the supernatants with ELISA. Dotted line = lower limit of quantification. D) Representative flow plots of surface tetherin staining by flow cytometry in BJ cell treated with 300 ng/mL HA saRNA-LNPs for 24 h. 100 pg/mL of IFN-β served as a positive control. E) BJ cells were treated with 300 ng/mL saRNA-LNPs with or with co-formulation with RNAx for 24 h and tetherin surface expression quantified by flow cytometry. Mean ± SEM, n=3, *p<0.05, ***p<0.001, ****p<0.0001 Ratio paired t-test. F) Representative plots of gating strategy for 293T (shown) and BJ cells. Numbers above bars indicate average fold change enhancement or inhibition of saRNA vs. saRNA.



Supplementary figure 4. C57BL/6 mice (n=5 per group) were intramuscularly injected with 0.5 µg of Influenza HA saRNA-LNPs (See Fig. 4) and were analyzed for serum cytokines 6 h post injection following A) the prime on day 1 or B) the boost on day 28. Geometric mean. Asterisks in the 'saRNA' column indicate cytokines that were significantly induced by saRNA alone vs. vehicle (*p<0.05, **p<0.01, Mann-Whitney Test). Asterisks in the '+ RNAx' columns indicate cytokines that were significantly inhibited by RNAx vs. saRNA alone (*p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's multiple comparisons) with the magnitude of the fold inhibition indicated to the right of each histogram. Complete dataset of individual animals for saRNA-LNP-induced cytokines are shown to the right of each respective histogram.

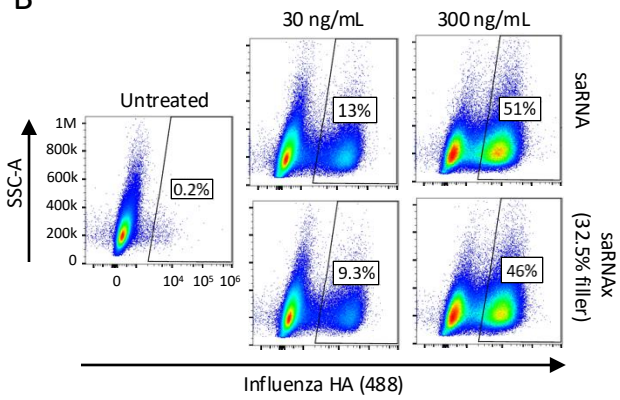


Supplementary figure 5: RNAx suppresses proinflammatory cytokines in BALB/c mice following vaccination. A) 5mC-modified Influenza HA expressing saRNA was co-formulated with modified nucleoside RNAx mRNA in trans at 1% and 10% (weight/weight) into LNPs. BALB/c mice (n=5 per group) were intramuscularly injected with 0.5 µg of Influenza HA saRNA-LNPs and were analyzed for serum cytokines 6 h post injection following B) the prime on day 1 or C) the boost on day 56. Geometric mean. Asterisks in the 'saRNA' column indicate cytokines that were significantly induced by saRNA vs. vehicle (*p<0.05, **p<0.01, Mann-Whitney Test). Asterisks in the '+ RNAx' columns columns indicate cytokines that were significantly inhibited by RNAx vs. saRNA alone (*p<0.05, **p<0.01, Kruskal-Wallis test with Dunn's multiple comparisons) with the magnitude of the fold inhibition indicated to the right of each histogram. Complete dataset of individual animals for saRNA-LNP-induced cytokines are shown for D) the prime or E) the boost. Created with BioRender.com

A

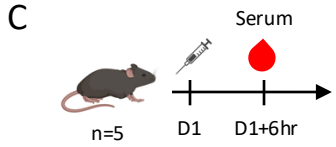
	saRNAx-LNP formulations (% w/w)				
					
RNAx mRNA	0	0	2	8.1	32.5
Noncoding 'filler' mRNA	0	32.5	30.5	24.4	0
HA-encoding saRNA	100	67.5	67.5	67.5	67.5

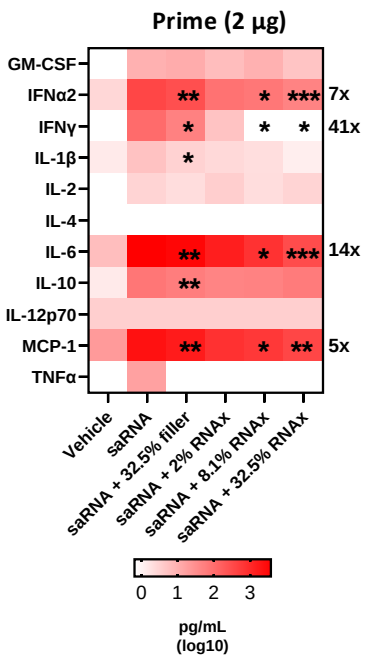
B



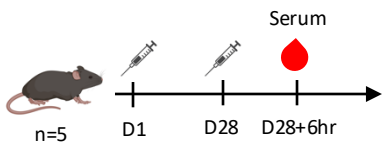
Tested parameters:	
saRNA vector:	Simplicon
RNA mod:	None
Mouse strain:	C57BL/6
Antigen:	HA
RNAx:	*Trans*
Delivery:	LNP
Dose:	2, 0.5 µg

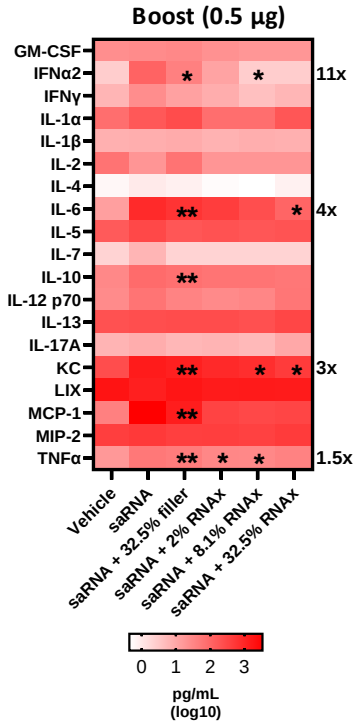
C



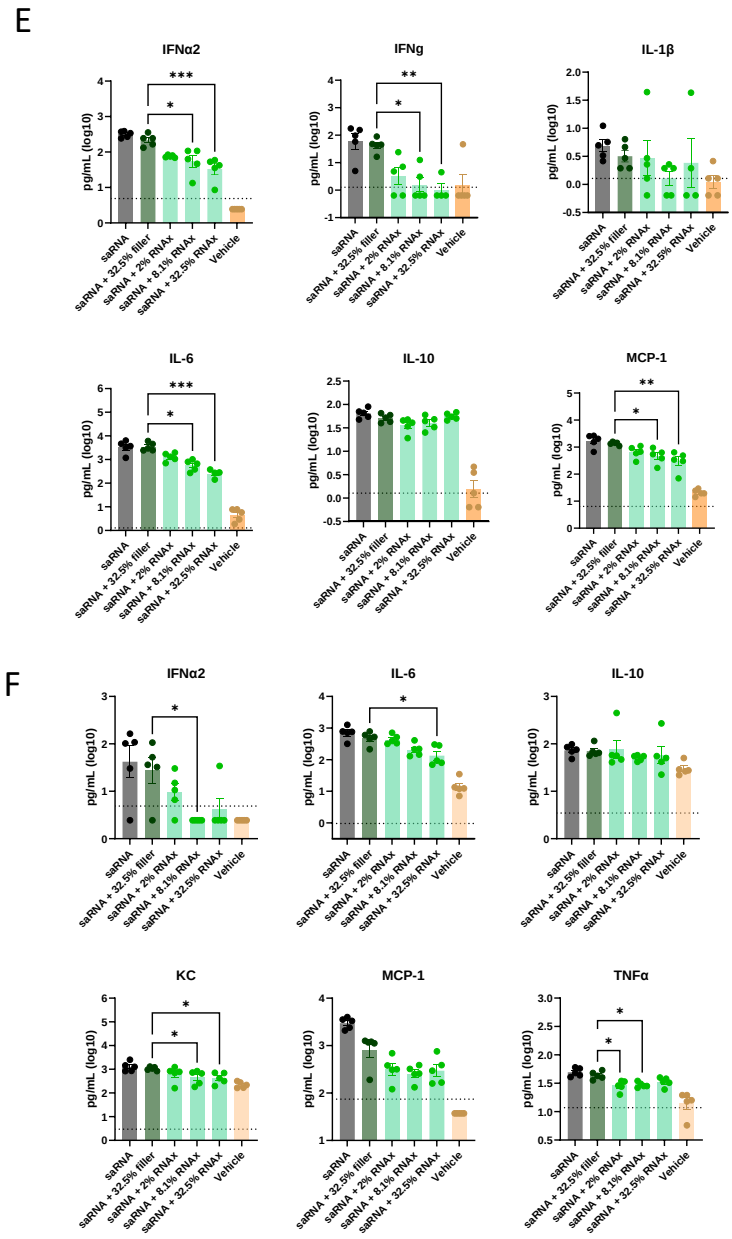


D

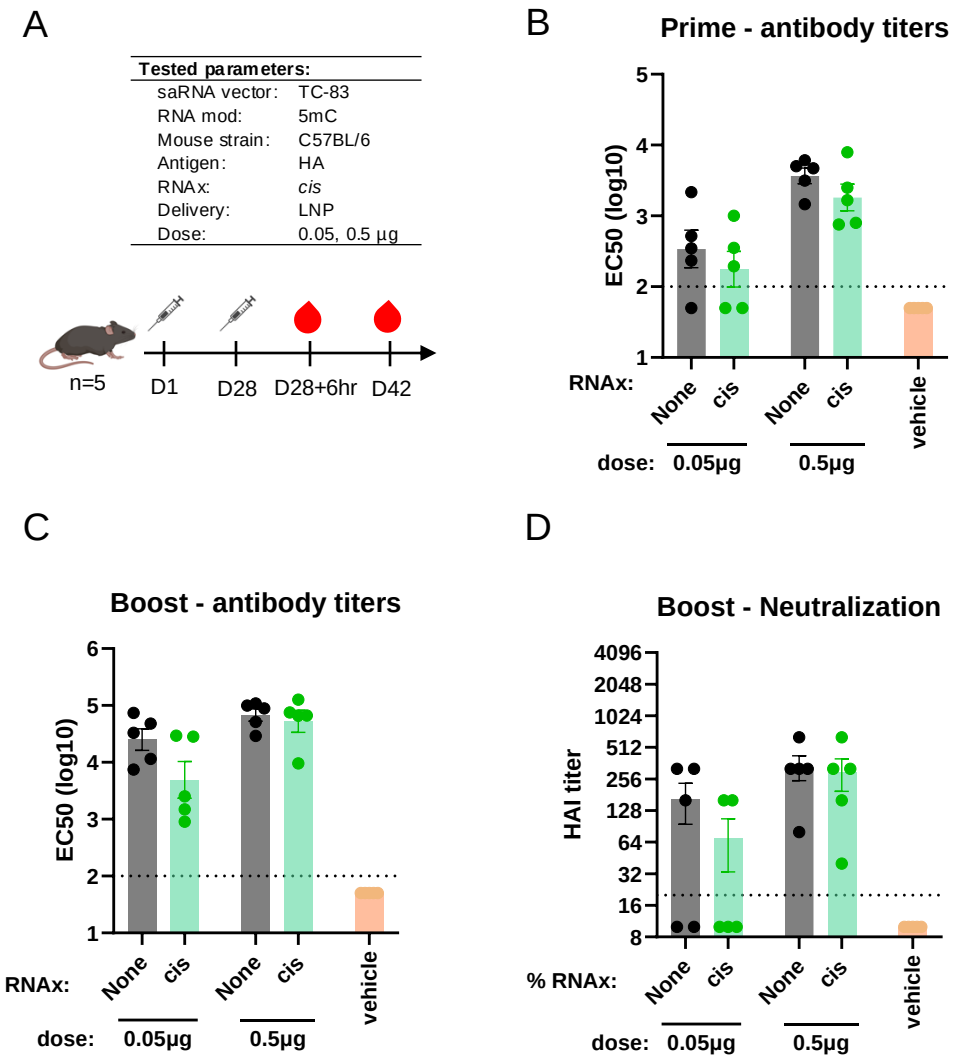




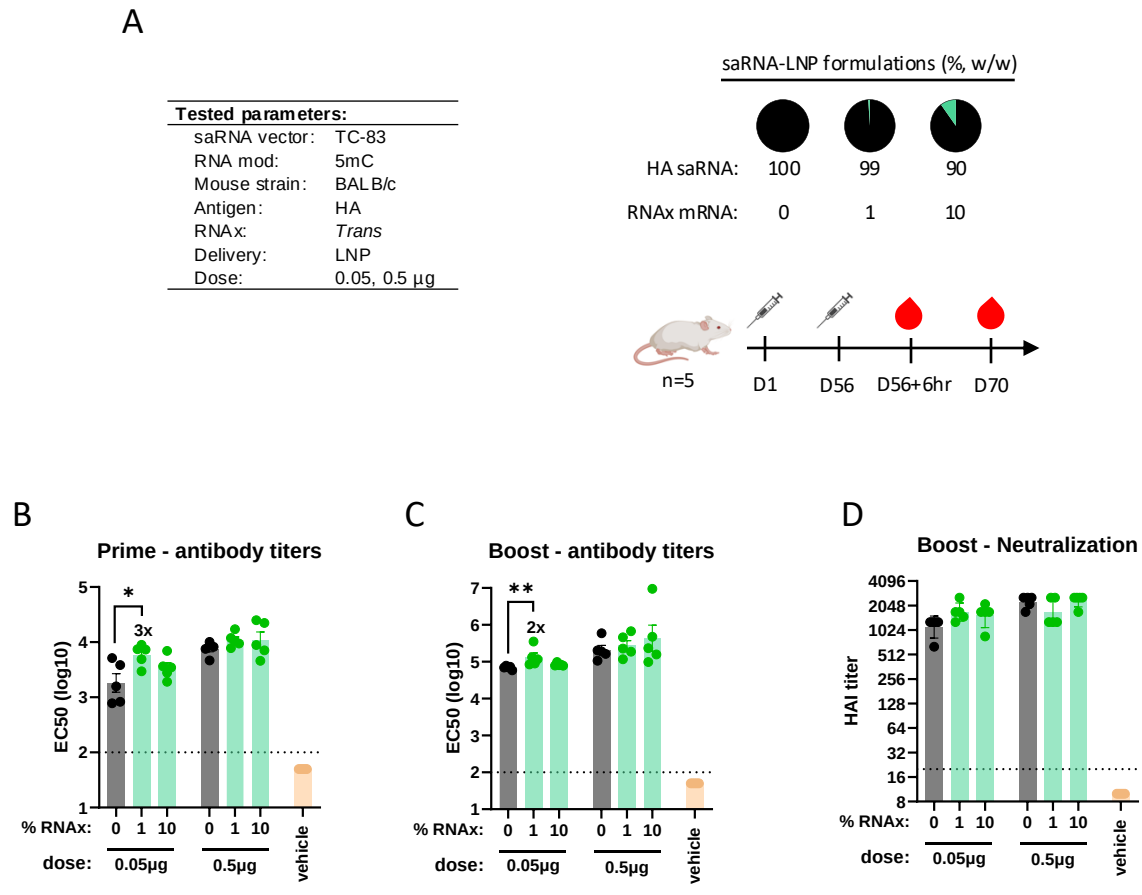
Supplementary figure 6: RNAx suppresses proinflammatory cytokines in mice following vaccination with Simplicon saRNA vector. Continued on next page



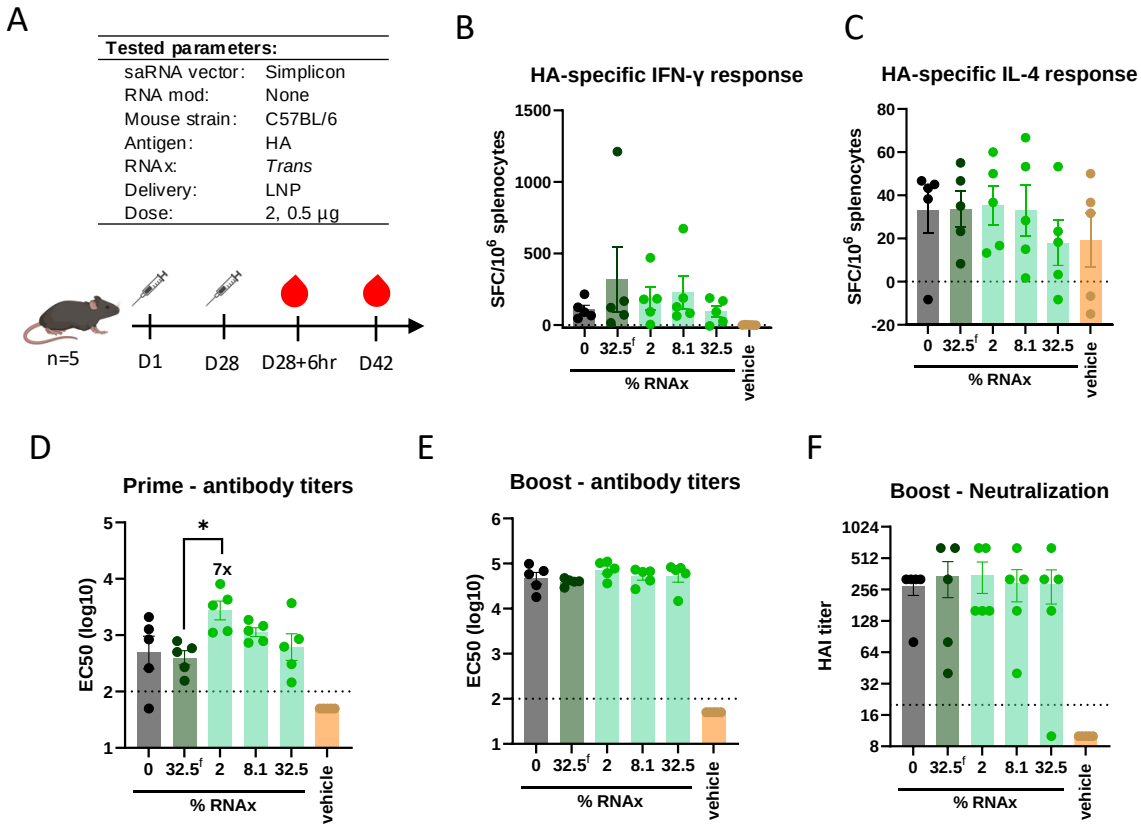
Supplementary figure 6: RNAx suppresses proinflammatory cytokines in mice following vaccination with Simplicon saRNA vector. A) Unmodified Influenza HA expressing saRNA was co-formulated into LNPs with modified nucleoside RNAx mRNA in trans at 2%, 8.1% and 32.5% (weight/weight) alongside a non-coding, sized matched 'filler' mRNA to control for potential changes when formulating differently sized RNAs into LNPs. This also results in identical doses of antigen-encoding saRNA across different amounts of RNAx. Total dose of RNA-LNP remained constant across groups. B) Flow cytometry of Influenza HA surface expression in 293T cells treated for 24 h with HA saRNA-LNPs (no RNAx) at the indicated concentrations. C57BL/6 mice (n=5 per group) were intramuscularly injected with Influenza HA expressing saRNA-LNPs and were analyzed for serum cytokines 6 h post injection following A) the prime on day 1 or B) the boost on day 28. Samples for the prime and boost were generated from independent studies from incidental lack of sample collections, but from the same lot of LNPs. Animals from the prime samples were injected with 2 µg of saRNA-LNP while for the study where boost samples are analyzed, animals were primed and boosted with 0.5 µg of saRNA-LNP. Geometric mean. Asterisks in the 'saRNA + filler' column indicate cytokines that were significantly induced vs. vehicle (*p<0.05, **p<0.01, Mann-Whitney Test). Asterisks in the '+ RNAx' columns indicate cytokines that were significantly inhibited by RNAx vs. saRNA + filler (*p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's multiple comparisons) with the magnitude of the fold inhibition indicated to the right of each histogram. Complete dataset of individual animals for saRNA-LNP-induced cytokines are shown for E) the prime or F) the boost. Created with BioRender.com



Supplementary figure 7. Impact of RNAx in cis on the antibody response from an Influenza HA saRNA-LNP vaccine. A) C57BL/6 mice (n=5 per group) were intramuscularly injected (prime and boost) with 0.05 µg or 0.5 µg of HA saRNA-LNPs with or without RNAx in cis. Serum was collected 6 h after the boost on day 28 (post prime measurement) and serum and splenocytes collected on day 42 (post boost measurement). Total anti-HA IgG in the serum (EC50) was quantified by ELISA at D) post prime and E) post boost. F) Neutralization titers were quantified by HAI assay post boost. Dotted line = lower limit of quantification, Geometric mean ± Geometric SD, Mann Whitney test (all comparisons p>0.05). Created with BioRender.com



Supplementary figure 8. Impact of RNAx on immunogenicity from Influenza HA saRNA-LNP vaccine in BALB/c mice. A) BALB/c mice (n=5 per group) were intramuscularly injected (prime and boost) with 0.05 µg or 0.5 µg of HA saRNA-LNPs. Serum was collected 6 h after the boost (post prime measurement) on day 56 and on day 70 (post boost measurement). Total anti-HA IgG in the serum (EC50) was quantified by ELISA at B) post prime and C) post boost. D) Neutralization titers were quantified by the hemagglutination inhibition (HAI) assay post boost. Dotted line = lower limit of quantification, Geometric mean ± Geometric SD, *p<0.05, **p<0.01, Kruskal-Wallis test with Dunn's multiple comparisons. Numbers indicate fold enhancement of RNAx groups relative to saRNA only. Created with BioRender.com



Supplementary figure 9. Impact of RNAx on immunogenicity following vaccination with Simplicon saRNA vector. A) C57BL/6 mice (n=5 per group) were intramuscularly injected (prime and boost) with 0.5 µg of HA saRNA-LNPs from the Simplicon vector (See Supplemental Fig. 6A). Statistical tests were conducted vs. the 32.5% filler group, denoted by 32.5^f. Serum was collected 6 h after the boost (post prime measurement) on day 28 and serum and splenocytes collected on day 42 (post boost measurement). Splenocytes were treated with 1 µg/mL Influenza HA peptide pools and analyzed for B) IFN-γ and C) IL-4 secreting cells by ELISPOT. Spot forming cells (SFC) were subtracted by untreated samples and normalized by 10^6 splenocytes. Mean ± SEM. D) Total anti-HA IgG in the serum (EC50) was quantified by ELISA at post prime and E) post boost. F) Neutralization titers were quantified by HAI assay post boost. Dotted line = lower limit of quantification, Geometric mean ± Geometric SD, *p<0.05, Kruskal-Wallis test with Dunn's multiple comparisons. Numbers indicate fold enhancement of RNAx groups relative to saRNA only.

Study	Treatment	size (nm)	PDI	EE%
HA-nLuc saRNA IVIS, PBMC	saRNA only	128	0.14	86.3
HA-nLuc saRNA IVIS, PBMC	saRNAx (36%)	130	0.21	85.7
Unmodified fLuc mRNA IVIS	fLuc	94	0.14	82.3
Unmodified fLuc mRNA IVIS	fLuc + Filler (35%)	86	0.08	85.2
Unmodified fLuc mRNA IVIS	fLuc + RNAx (9%)	86	0.09	84.5
Unmodified fLuc mRNA IVIS	fLuc + RNAx (35%)	88	0.11	83.9
HA saRNA (TC-83) immunogenicity and serum cytokines (C57BL/6 and BALB/c)	saRNA only	148	0.17	82.8
HA saRNA (TC-83) immunogenicity and serum cytokines (C57BL/6 and BALB/c)	saRNAx (1%)	148	0.14	82
HA saRNA (TC-83) immunogenicity and serum cytokines (C57BL/6 and BALB/c)	saRNAx (10%)	157	0.14	80.8
HA saRNA (Simplicon) immunogenicity and serum cytokines	saRNA only	120	0.08	77
HA saRNA (Simplicon) immunogenicity and serum cytokines	saRNA (filler, 32.5%)	120	0.1	81.4
HA saRNA (Simplicon) immunogenicity and serum cytokines	saRNAx (2%)	120	0.11	84.1
HA saRNA (Simplicon) immunogenicity and serum cytokines	saRNAx (8.1%)	125	0.1	82.4
HA saRNA (Simplicon) immunogenicity and serum cytokines	saRNAx (32.5%)	119	0.12	82.2

Supplementary table 1. Summary of RNA-LNP QC measurements. Results from DLS, including average size, polydispersity index (PDI), and encapsulation efficiency (EE) percentage.

Donor	Gender	Age	Race/ethnicity	Smoker	HBsAg	Anti-HCV	HIV-1,2
1	Female	25	Caucasian	No	Neg	Neg	Neg
2	Female	42	African American	No	Neg	Neg	Neg
3	Male	53	Caucasian	No	Neg	Neg	Neg
4	Female	34	Mixed	No	Neg	Neg	Neg

Supplementary Table 2. Demographics and health state of human PBMC donors obtained from commercial source. PBMC cells from de-identified human donors were obtained from IQ Biosciences (cat# IQB-PBMC102) (Alameda, CA) and tested for Hepatitis B virus surface antigen (HBsAg), Hepatitis C virus antibody (anti-HCV) and HIV-1,2 by IQ Biosciences. Neg=negative.