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Supplemental information

Self-amplifying mRNA bicistronic influenza vaccines raise cross-reactive immune responses in mice and prevent infection in ferrets

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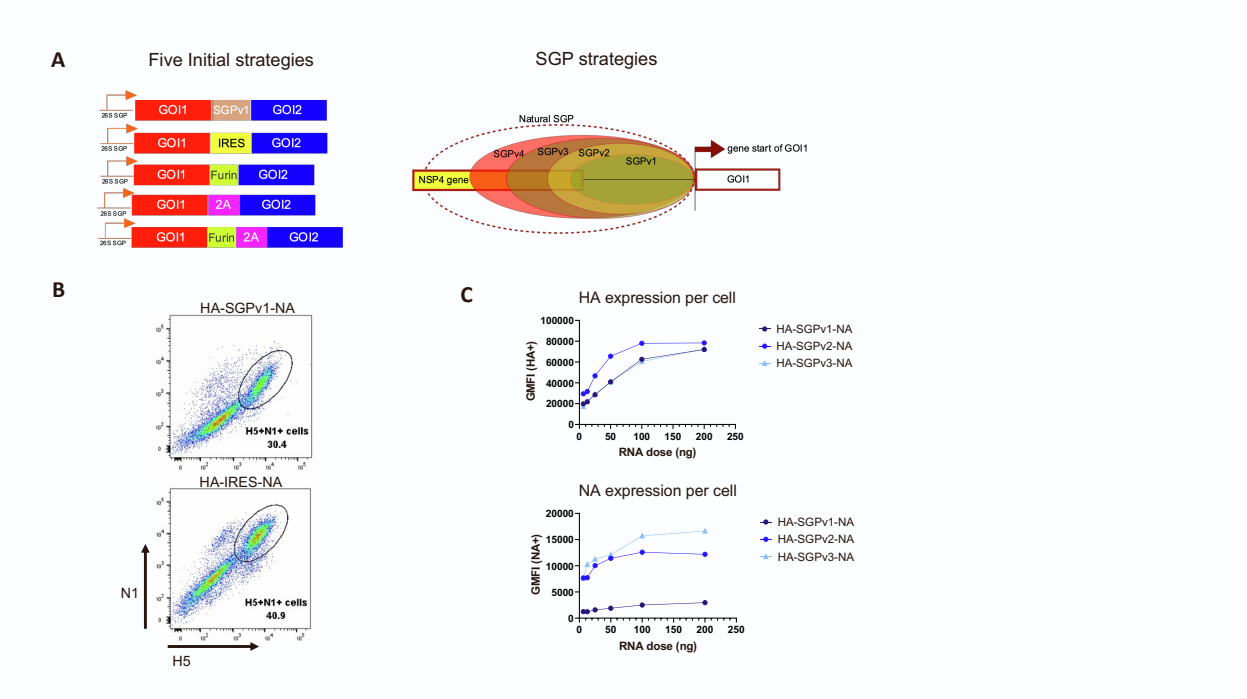
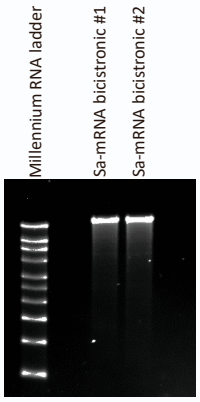


Figure S1. Initial screens and characterization *in vitro* of bicistronic strategies and SGP versions. **a** Left: schematic of initial 5 sa-mRNA bicistronic strategies. GOI: gene of interest. SGP: subgenomic promoter. Right: schematic for the relative corresponding sequence for SGPv1-v4. **b** Transfected BHK cells were analyzed for expression of HA and NA using flow cytometry; representative flow plots for cells expressing HA (X-axis) and NA (Y-axis) from different sa-mRNA bicistronic constructs are labelled above the graph. The fraction of cells that were positive for both HA and NA are circled. Data represent at least 3 independent experiments. **c** Geometric mean fluorescence intensity (gMFI) for HA+ (upper panel) and NA+ (lower panel) cells for all tested sa-mRNA concentrations. Data represent 2-3 independent experiments.

A



B

Attributes	Assay	Spec
sa-mRNA		
Capping efficacy	LC-MS	95%-100% fully capped, cap0
Double stranded RNA	ELISA	≤ 0.1%
LNP		
Particle size	DLS	90-120 nM
PDI	DLS	≤ 0.2
Zeta potential	DLS	0-50 mV

Figure S2. Quality control of sa-mRNAs and LNPs. A) Agarose gel image of sa-mRNA for RNA integrity, B) Attributes and specifications for quality control of sa-mRNAs and LNPs.

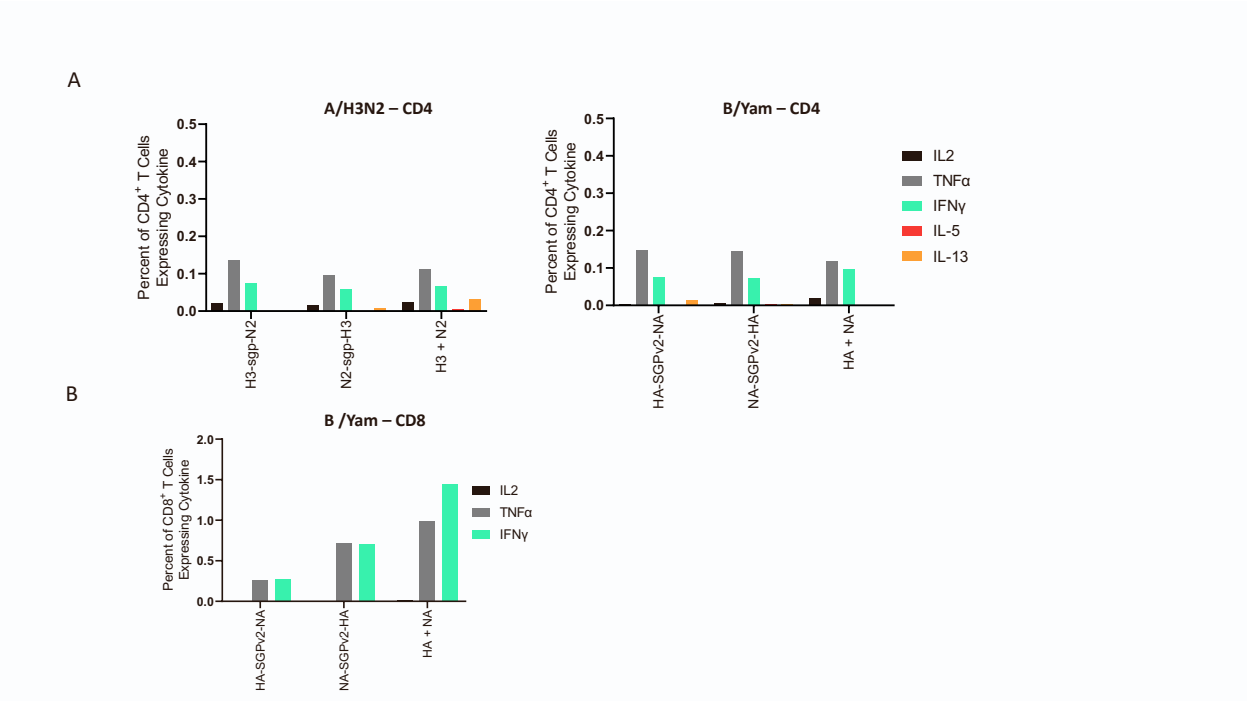


Figure S3. sa-mRNA bicistronic A/H3N2 and B/Yamagata vaccines elicit robust cellular immune responses

The frequency of intracellular cytokine expression among CD4+ (**a**) and CD8+ (**b**) in response to stimulation is displayed. Spleens from the first 5 mice/group were pooled and processed, and splenocytes were stimulated with anti-mouse CD28 in the absence or presence of cell-derived influenza monovalent bulk homologous to the vaccine, as well as an immunodominant CD8-stimulating peptide for B/Yamagata. Cells were stained with fluorescently tagged antibodies to cell surface markers CD3, CD4, CD8, and intracellular cytokines IL-2, TNFα, IFNγ, IL-5, and IL-13 were analyzed by flow cytometry. Each column represents a mean obtained from duplicate cultures of the 5 pooled spleens. Due to lack of stimulation for A/H3N2 in BALB/c mice, CD8 responses to A/H3N2 were not evaluated.

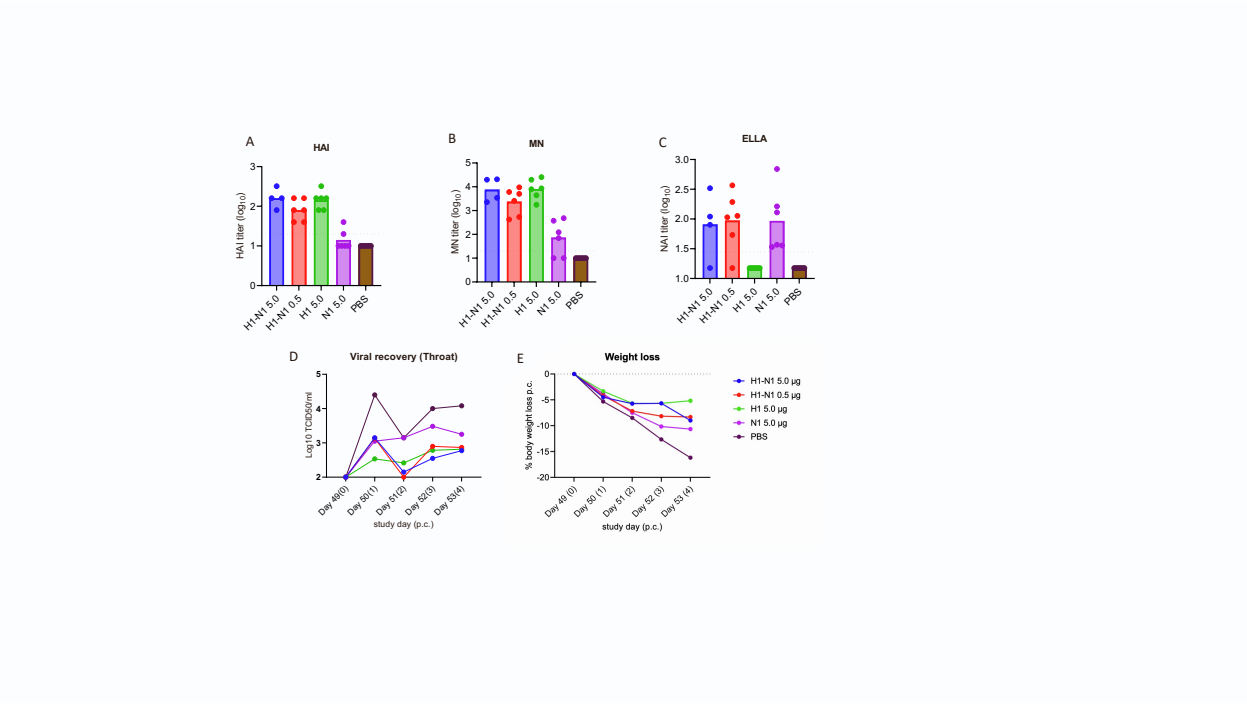


Figure S4. sa-mRNA bicistronic A/H1N1 vaccine induces potent neutralizing titers to hemagglutinin and neuraminidase and reduces viral load in throat and nose in ferrets.

Serum antibody titers measured on Day 50 before challenge by HAI assay (A), MN assay (B) and ELLA assay (C). Ferrets vaccinated with sa-mRNA vaccines containing HA showed strong anti-HA responses by HAI and MN assays, equivalent between monocistronic and bicistronic sa-mRNA vaccines. sa-mRNA vaccines containing NA showed detectable anti-NA responses by ELLA assay, comparable between monocistronic and bicistronic sa-mRNA vaccines. No antibody was detected in control ferrets. (D) Virus recovery in throat swabs during challenge. There were early and sustained high levels of virus recovered in control ferrets and a robust reduction of virus recovery in all vaccinated ferrets. (E) Body weight loss during challenge was the highest in control ferrets and reduced in all vaccinated ferrets.