

# Safety and immunogenicity of an optimized self-replicating RNA platform for low dose or single dose vaccine applications: a randomized, open label Phase I study in healthy volunteers

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Self-replicating RNA (srRNA) technology, in comparison to mRNA vaccines, has shown dose-sparing by approximately 10-fold and more durable immune responses. However, no improvements are observed in the adverse events profile. Here, we develop an srRNA vaccine platform with optimized non-coding regions and demonstrate immunogenicity and safety in preclinical and clinical development. Optimized srRNA vaccines generate protective immunity (according to the WHO defined thresholds) at doses up to 1,000,000-fold lower than mRNA in female mouse models of influenza and rabies. Clinically, safety and immunogenicity of RBI-4000, an srRNA vector encoding the rabies glycoprotein, was evaluated in a Phase I study (NCT06048770). RBI-4000 was able to elicit de novo protective immunity in the majority of healthy participants when administered at a dose of 0.1, 1, or 10 microgram (71%, 94%, 100%, respectively) in a prime-boost schedule. Similarly, we observe immunity above the WHO benchmark of protection following a single administration in most participants at both 1 and 10 microgram doses. There are no serious adverse events reported across all cohorts. These data establish the high therapeutic index of optimized srRNA vectors, demonstrating feasibility of both low dose and single dose approaches for vaccine applications.

Development of mRNA vaccines against SARS-CoV-2 revolutionized infectious disease vaccination with rapid timelines, simplified manufacturing, and reduced cost of goods as compared to previous platforms<sup>1–4</sup>. However, since the approval of these pandemic vaccines, mRNA technology has struggled to expand its utility within infectious diseases and adjacent therapeutic areas.

The utility of conventional linear mRNA technology is limited by its narrow therapeutic index (TI). In this context, the TI is defined as the ratio of the highest clinical dose reported with a favorable safety profile over the lowest clinical dose showing bioactivity (e.g.,

protective immune responses for a vaccine). For linear mRNA technology, TI is estimated to be ~3, based on this calculation from early dose escalation trials for SARS-CoV-2 vaccines<sup>1,4</sup>. A narrow TI has caused challenges for mRNA technology as exemplified by suboptimal clinical performance of multivalent vaccines<sup>5–7</sup>. Seasonal influenza vaccines highlight this challenge wherein individual mRNA constructs encoding an antigen for a single strain need to be multiplexed into a multivalent vaccine. This results in individual strain-specific mRNAs being administered at a suboptimal amount, i.e., quartered doses for each strain in the case of quadrivalent seasonal influenza vaccines, so

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the final aggregate vaccine dose can remain within the clinically tolerated range. In addition, poor durability of immune responses observed from mRNA-based vaccines is presumed to be likely due to a poor TI, as tolerable doses are insufficient to generate durable immunity for antigens with lower intrinsic immunogenicity.

Seeking to improve on the technological limitations of mRNA, alternate synthetic RNA platforms, such as self-replicating RNA (srRNA), have been explored for vaccines<sup>8–13</sup>. srRNA vaccines can be administered at an order of magnitude lower doses than mRNA and elicit robust immune responses with superior durability<sup>8,14</sup>. This is in part due to the inclusion of a viral replicase within the srRNA vector that coordinates self-limiting amplification, generating copies of RNA transcripts *in situ*<sup>15–19</sup>. An srRNA-based vaccine for SARS-CoV-2 recently approved in Japan, has demonstrated the beneficial features of this technology<sup>20</sup>. ARCT-154 is administered at a dose of 5 micrograms (mcg) which is 6–20-fold lower than approved mRNA COVID-19 vaccines. Additionally, ARCT-154 elicited sustained, durable immune responses as compared to conventional mRNA<sup>14</sup>. Although ARCT-154 was able to show clinical bioactivity at these much lower doses, its tolerability was also proportionally lower, exemplified by a maximum tolerated dose of 7.5 mcg for ARCT-21 (first iteration of ARCT-154)<sup>10</sup>. This unexpected safety profile, exhibited by local and systemic reactivity, is speculated to be driven by impurities from the large-scale manufacturing process, and/or characteristics of the drug product, such as the lipid nanoparticle (LNP) formulation<sup>21–24</sup>. Thus, first-generation srRNA platforms failed to improve the TI, but nevertheless, have successfully validated the benefits of this technology as a class.

To address this gap, we identified more potent srRNA vectors (described in refs. 25,26). In this report, we show that an optimized srRNA-based vaccine can achieve robust immunogenicity in preclinical studies using unprecedented, ultra-low picogram doses to induce both humoral and cellular immunity.

Leveraging this preclinical success, we conducted a randomized, Phase I open-label dose escalation study in adult healthy volunteers to evaluate the safety and immunogenicity of RBI-4000, a next-generation optimized srRNA vaccine, formulated in a proprietary LNP, targeting the rabies virus glycoprotein (RABV-G) as a model antigen (NCT06048770). RBI-4000 successfully induced *de novo* protective immunity in the majority of participants at all doses tested (0.1–10 mcg). Importantly, no dose-limiting toxicities or serious adverse events were reported in this study and the maximally tolerated dose was not reached. In contrast to linear mRNA and first-generation srRNA, the TI of RBI-4000 exceeds 100-fold as demonstrated by clinical bioactivity at the lowest dose tested of 0.1 mcg coupled with a lack of dose-limiting toxicities at the highest dose tested of 10 mcg. These clinical data validate the hypothesis that improved vectors and optimized long RNA manufacturing yield clinically significant improvements in TI for srRNA. These findings justify their further development in a broad array of therapeutic areas where higher doses, multiplexing, and/or chronic dosing are required.

## Results

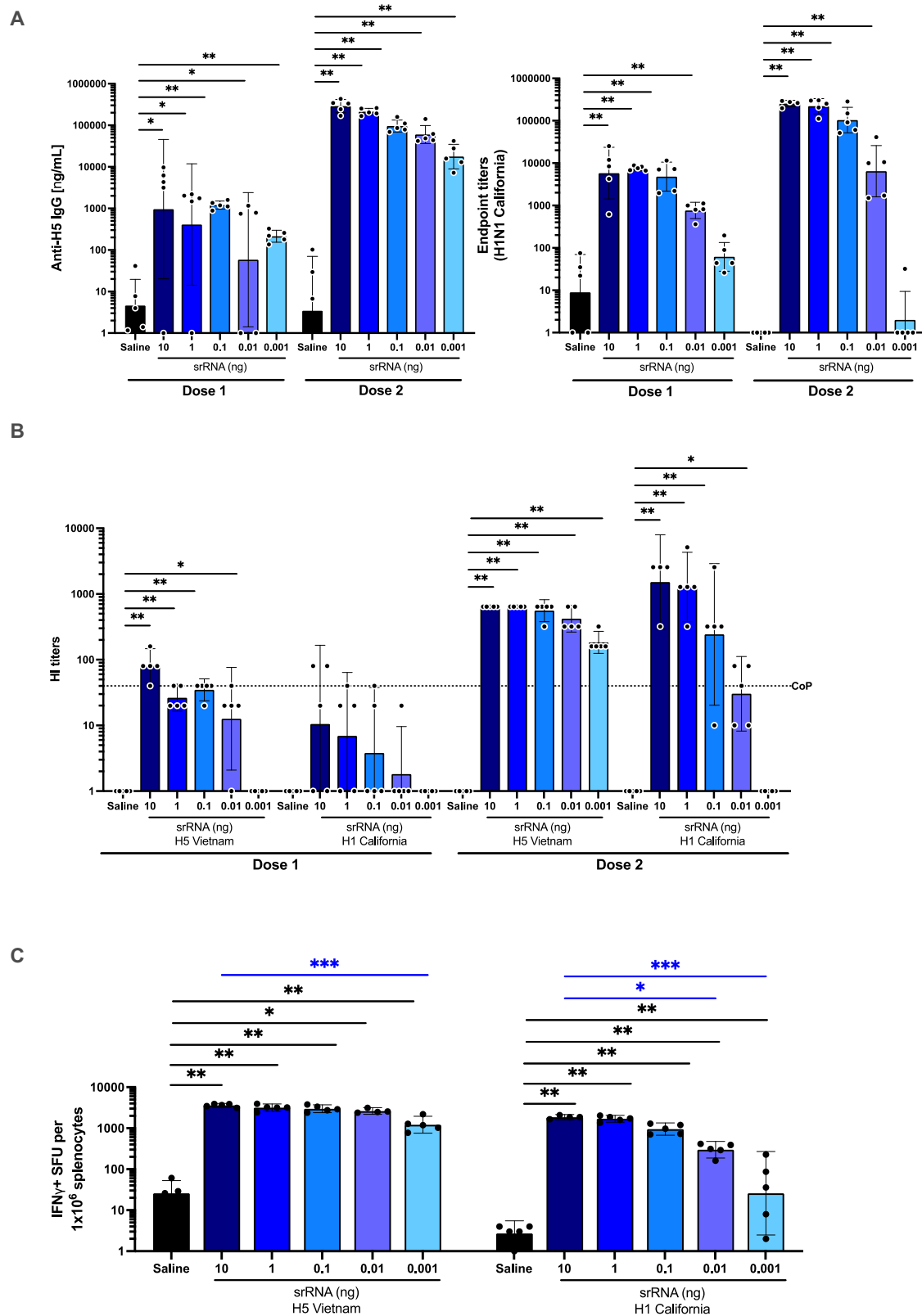
### Next-generation srRNA vectors result in protective immune responses at ultra-low doses in mice

The prototypical first-generation srRNA vaccines are mostly derived from the same alphavirus, Venezuelan Equine Encephalitis Virus (VEEV) strain TC-83, and have shown to be able to elicit equal immunogenicity to mRNA but at much lower doses<sup>27–29</sup>. This increase in the effective dose is suggested to be due to higher and more durable expression of the encoded transgene (or antigen)<sup>28–30</sup>. Although this version of the VEEV-based vector has been clinically validated and advanced in a recently approved commercial vaccine<sup>31</sup>, no intentional design improvements have been made to the technology in over three decades that confer enhanced protein expression or vaccine immunogenicity<sup>32–35</sup>. We hypothesized that bioactivity could be

optimized by more closely mimicking the natural alphavirus genome. Optimizations to the vector are summarized in Supplementary Fig 1 and ref. 25. First, we strictly designed the DNA vector to template a minimum number of ribonucleotides at the 5' and 3' ends of the srRNA that are not naturally present in alphaviruses<sup>25</sup>. Minimal cloning adapters (~30 nt) up and downstream of the gene(s) of interest on the DNA template allow for rapid, modular transgene replacement and ensure optimal expression. Additionally, while previous reports established the canon of a minimal -30 residue polyA tail being sufficient for srRNAs<sup>36,37</sup>, we increased the templated polyA tail to better match the alphavirus length (~68 residues)<sup>38</sup> and found a significant enhancement in dose-dependent bioactivity *in vitro*<sup>25</sup>. These modifications also led to a notable increase in activity *in vivo*, evidenced by enhanced protein expression as assessed in mice administered srRNA encoding a reporter transgene for firefly luciferase (Fluc) compared to the parental sequence (Supplementary Fig. 2). Longitudinal assessment of bioluminescence, as a result of active Fluc expression, over 40 days demonstrated that improvements made to the VEEV sequence resulted in a higher peak and greater durability of protein expression when compared to the original vector (labeled as prototypical VEEV TC-83). To further assess if increased protein expression from this optimized vector led to better bioactivity of vaccines, we generated constructs encoding the influenza hemagglutinin (HA) antigen from two distinct pandemic strains, H5N1 and H1N1, in this improved backbone, and evaluated these in mouse immunogenicity studies at ultra-low doses ranging from 1 pg to 10 ng (Fig. 1). A single dose of this srRNA vaccine resulted in seropositivity, as measured by the presence of HA-specific antibodies, in all dose groups for H5, and down to 10 pg for H1 strains (Fig. 1A, Dose 1). A second (boost) dose of vaccine enhanced titers of HA-specific IgG for both H5 and H1 in a dose-dependent manner (Fig. 1A, Dose 2). A WHO-defined immune correlate of protection exists for influenza and is based on the titer of HA-neutralizing antibodies as measured in a standardized hemagglutination inhibition (HI) assay<sup>39–41</sup>. An HI titer of 40 (serum diluted 1 to 40) has also been established as protective in viral challenge studies in mice<sup>42</sup>. We observed HI titers above this established threshold for protection in animals immunized with vaccine doses as low as 1 pg for the H5N1 strain after two administrations (Fig. 1B). HI titers for H1N1 were observed in animals that received 10 ng down to 10 pg of srRNA vaccine, showing the robustness of the technology for two separate influenza strains. Importantly, a single administration of vaccine led to partial neutralization for H5N1 and H1N1 at higher doses (Fig. 1B).

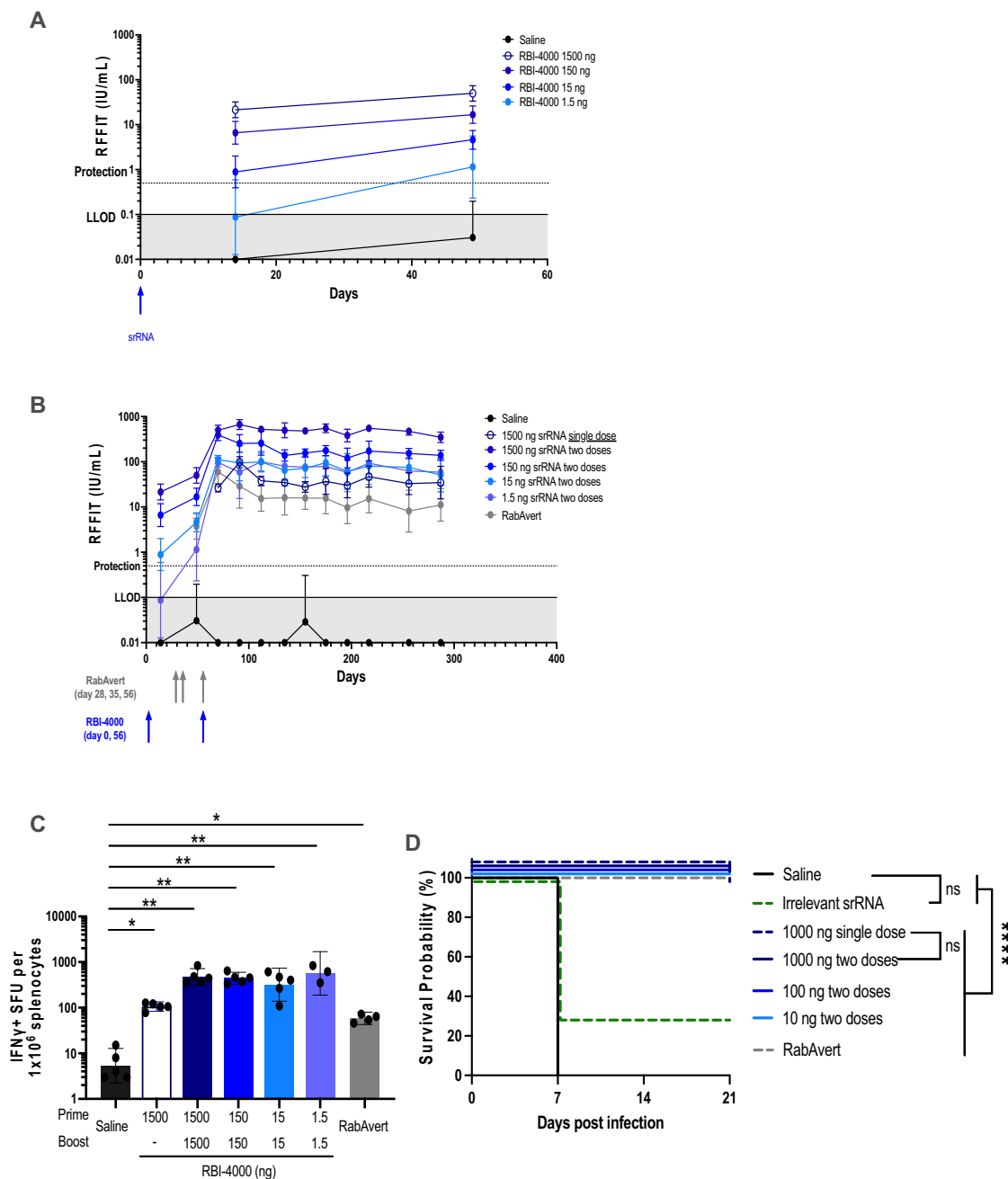
An additional strength of srRNA technology is its ability to elicit cellular immunity<sup>30,43–45</sup>. Robust HA-specific T cell responses were detected in all mice immunized with H5 at all doses of the vaccine, down to the lowest tested dose of 1 pg (Fig. 1C). HI-specific T cell responses were detectable across the tested dose range of 10 ng to 10 pg. Lack of a dose response for the higher end of the dose range suggests saturation was achieved at 1 ng for H1 and 100 pg for H5 (Fig. 1C). Additional analysis to assess the quality of the T cell responses using intracellular cytokine staining was performed. Both H5 and H1-encoding srRNA vaccines generated polyfunctional HA-specific CD8 and CD4 T cell responses, as defined by the concurrent production of several effector cytokines by the same T cell. The HA responses included CD8 T cells having a majority double- (IFN $\gamma$ , TNF) or triple-cytokine producing (IFN $\gamma$ , IL-2, TNF) effector phenotype, and also CD4 T cells with a majority triple-cytokine producing population (Supplementary Fig. 3).

To assess if the enhanced bioactivity of new srRNA vectors translated to clinical improvements, we developed RBI-4000, a next-generation srRNA vaccine encoding RABV-G. Rabies virus was selected as a model pathogen due to an immune naive participant population, as opposed to influenza, where much of the population has been previously exposed to the virus. In addition, similarly to influenza, a WHO indirect measure of protection exists for rabies virus and is



**Fig. 1 | Optimized srRNA vaccines against influenza elicit protective immune responses at ultra-low doses in mice. A** Humoral responses as measured by ELISA for anti-hemagglutinin H5 total IgG antibody titers and H1 endpoint IgG antibody titers, 14 days following one or two doses of srRNA/LNP vaccines ( $n = 5$  mice per bar). **B** Neutralizing antibodies were measured by Hemagglutination Inhibition (HI) assay against H5 and H1, using A/Vietnam/1203/04 and A/California/07/09, respectively, 14 days following one or two doses of srRNA/LNP vaccines ( $n = 5$  mice

per bar). Line refers to WHO Correlate of Protection (CoP) threshold. **C** Splenic T cell responses against H5 and H1 HA, assayed by ELISpot, 14 days following two vaccinations. Symbols represent individual animals ( $n = 5$  mice per bar). Bars represent geometric mean and error bars show 95% CI. Statistical significance was determined by using Mann–Whitney tests vs Saline (black lines) or Kruskal–Wallis test vs 10 ng dose group (blue lines), where  $*p = 0.0476$  (HI assay) and  $*p = 0.0159$  (ELISpot),  $**p = 0.0079$ ,  $***p < 0.0005$ ,  $****p < 0.0001$ .



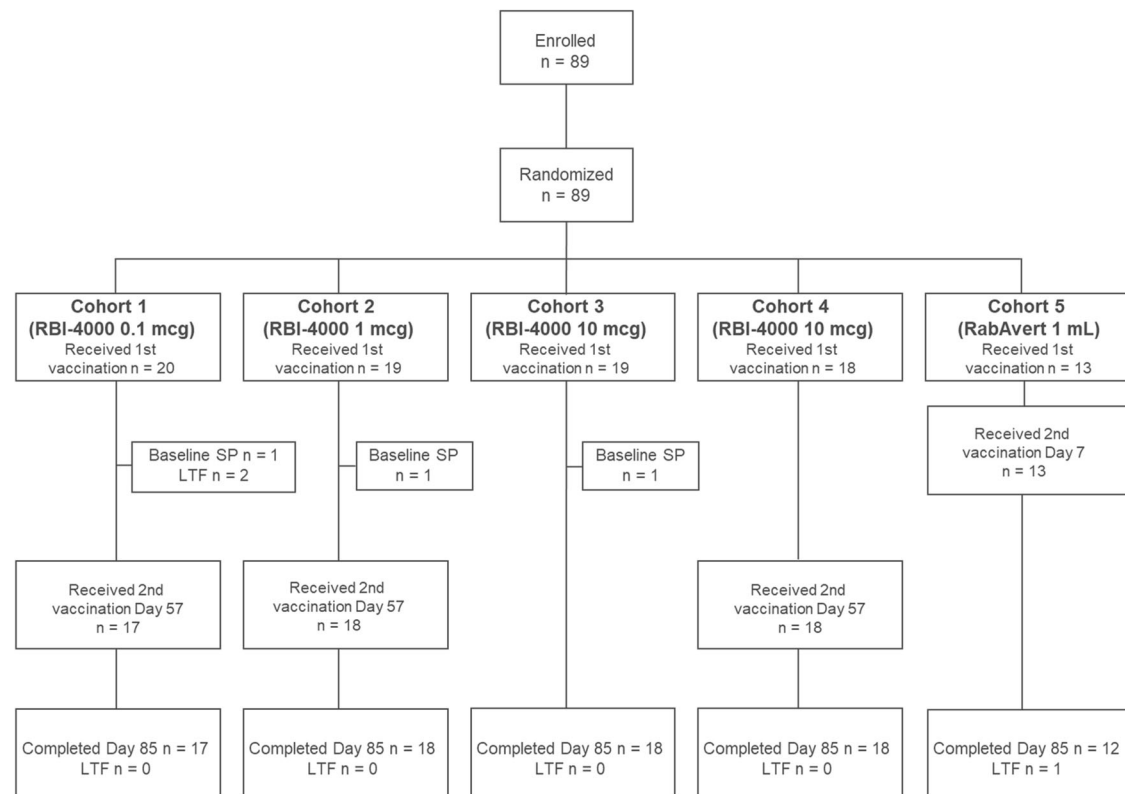
**Fig. 2 | RBI-4000 elicits protective immune responses and protects from viral challenge in mice.** Serum-neutralizing antibody titers, assessed by rapid fluorescence foci inhibition test (RFFIT), at indicated time points following one (A) or two vaccinations ( $n = 10$  mice per group per time point) (B) with RBI-4000 ( $n = 5$  mice per every time point after day 49). C Splenic T cell responses against rabies virus glycoprotein (RABV-G), assayed by ELISpot, 14 days following two vaccinations with RBI-4000. Symbols represent individual animals. D Probability of survival in a

model of rabies virus lethal challenge in previously vaccinated with one (prime) or two doses (P-B) of RBI-4000 ( $n = 10$  mice per group). Bars represent geometric mean and error bars show 95% CI. Statistical significance was determined by using a Kruskal–Wallis test for bar graphs or by two-way ANOVA with multiple comparisons for line graphs and Kaplan–Meier for survival, where  $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.0005$ ,  $****p < 0.0001$ . Exact  $p$  values are listed in the Source Data file.

defined by the presence of serum rabies virus neutralizing antibodies (RVNA) at or above 0.5 IU/mL, thus allowing clear and objective interpretation of vaccine-induced immune responses<sup>45</sup>.

Preclinical testing of RBI-4000 in mouse immunogenicity studies demonstrated that a single immunization could lead to protective RVNA titers down to a 1.5 ng dose level, the lowest dose tested (Fig. 2A). Durability of RVNA titers was established in animals that received either a single 1.5 mcg dose of RBI-4000 or two doses ranging from 1.5 ng to 1.5 mcg. Compared to an approved commercial vaccine, RabAvert, administered at 1/10th of the human dose (the

maximum dose feasible in mice), RBI-4000 generated RVNA responses that were higher in magnitude and that exhibited a slower or no decay rate over a greater than 6-month period above the 0.5 IU/mL protective threshold (Fig. 2B). These sustained neutralizing antibody responses were accompanied by strong total T cell responses to RABV-G in all animals that received RBI-4000 (Fig. 2C). Polyfunctionality in the RABV-G-specific CD8 and CD4 T cells was confirmed in animals that received RBI-4000, as demonstrated by a double or triple-cytokine-producing effector phenotype (Supplementary Fig. 4).



**Fig. 3 | Clinical trial design.** Enrollment and randomization of participants on RBI-4000-101 Phase I clinical trial.

We further confirmed that RBI-4000 could confer protective immunity in a mouse model of lethal rabies virus challenge. Mice received RBI-4000 prophylactically as a single 1 mcg administration or two administrations across a dose range of 0.01, 0.1, or 1 mcg. RabAvert provided a positive control in the study. 100% of mice that received RBI-4000 across all dose cohorts were protected from lethal rabies virus challenge, as with RabAvert (Fig. 2D). Interestingly, 30% (3/10) animals that received a srRNA construct encoding luciferase as a control (labeled as “Irrelevant”) were also protected. This non-specific effect is thought to be due to the inflammatory profile of LNP-formulated srRNA drug product that is known to trigger innate immune mediators which can lead to an anti-viral type I immune response<sup>47,48</sup>. Collectively, these data demonstrated that RBI-4000 was able to elicit robust immune responses, inclusive of polyfunctional effector T cells and RVNA titers that attained the WHO-defined metric against rabies infection.

#### Clinical evaluation of optimized srRNA platform in humans

Based on the preclinical data, we evaluated RBI-4000 in a clinical study to assess the application of the benefits of this new technology. RBI-4000-101 is a multicenter, open-label, randomized, active control Phase I clinical trial (NCT06048770) to assess the safety and immunogenicity of RBI-4000. The study enrolled 89 healthy volunteers, aged 18–45 years with no prior exposure or vaccination to rabies (Fig. 3, Demographics in Table 1). The study included four cohorts of ascending doses of RBI-4000 and an active comparator of the commercially available rabies vaccine, RabAvert. Participants in Cohorts 1 (0.1 mcg), 2 (1 mcg), and 4 (10 mcg) received a prime-boost dosing schedule of RBI-4000, 8 weeks apart, while Cohort 3 participants received a single dose of 10 mcg. The 8-week interval for RBI-4000 dosing was selected as it is optimal for generation of immune responses, specifically for srRNA-based vaccines<sup>9</sup>. RabAvert was administered as per the CDC guidelines, i.e., two doses 1 week apart, on Days 1 and 8<sup>49</sup>.

Immunogenicity and safety data through 85 days post initial vaccination is included in this report. Long-term durability of RBI-4000 immunogenicity is continuing to be assessed.

#### Clinical safety showed minimal reactogenicity at all doses tested

Safety analysis includes all participants who received any dose of RBI-4000 or RabAvert. Vaccination was generally well tolerated with no anaphylactic reactions. There were no serious adverse events, no dose-limiting adverse events, and a maximally tolerated dose of RBI-4000 was not reached. Adverse events were limited to grades 1 and 2, with the exception of a single self-reported grade 3 fever event in a Cohort 2 participant (1 mcg dose, first administration only), which was not medically confirmed. Local reactogenic adverse events were more common than systemic, with pain or tenderness at the injection site being the most common symptoms. The most common systemic adverse event was headache. Myalgia and fatigue were also reported in over half of the participants at the 10 mcg dose. Overall, the boost dose (second administration) appeared better tolerated than the prime with fewer participants reporting adverse events, though more of the reported events were grade 2 after the boost versus grade 1 after the initial administration. Rates of adverse events are summarized in Fig. 4. There were no unsolicited related adverse events.

#### De novo protective immune responses were achieved at all doses clinically tested in humans

Serial analysis of sera from immunized participants showed detectable RVNA titers in a dose-dependent manner following a single administration of RBI-4000 (Fig. 5A, gray bars). Furthermore, a single administration of RBI-4000 led to protective (RVNA  $\geq$  0.5 IU/mL) titers in the majority (67%) of participants that received a 1 mcg dose (Fig. 5A, Cohort 2, blue bars), while 89% and 94% of participants achieved RVNA titers at or above the protective threshold with a single dose of 10 mcg in Cohort 3 and 4, respectively.



**Table 1 | Demographic characteristics of the participants of RBI-4000-101 clinical trial**

|                        | Cohort 1<br>RBI-4000<br>0.1 mcg | Cohort 2<br>RBI-4000<br>1 mcg | Cohort 3<br>RBI-4000<br>10 mcg<br>(prime only) | Cohort 4<br>RBI-4000<br>10 mcg | Cohort 5<br>RabAvert<br>1 mL | Total   |
|------------------------|---------------------------------|-------------------------------|--|--------------------------------|------------------------------|---------|
| <b>Total—n</b>         | 20                              | 19                            | 19   | 18                             | 13                           | 89      |
| <b>Age (years)</b>     |                                 |                               |  |                                |                              |         |
| Median                 | 35.5                            | 34.0                          | 36.0   | 34.0                           | 39.0                         | 35      |
| Range                  | 23–45                           | 25–45                         | 22–45  | 20–43                          | 27–44                        | 20–45   |
| <b>Sex—n (%)</b>       |                                 |                               |  |                                |                              |         |
| Male                   | 10 (50)                         | 10 (53)                       | 3 (16)   | 8 (44)                         | 7 (54)                       | 38 (43) |
| Female                 | 10 (50)                         | 9 (47)                        | 16 (84)  | 10 (56)                        | 6 (46)                       | 51 (57) |
| <b>Race—n (%)</b>      |                                 |                               |  |                                |                              |         |
| Black                  | 5 (25)                          | 4 (21)                        | 2 (11)   | 4 (22)                         | 4 (31)                       | 19 (21) |
| White                  | 15 (75)                         | 15 (79)                       | 17 (89)  | 12 (67)                        | 8 (62)                       | 67 (75) |
| Other                  | 0 (0)                           | 0 (0)                         | 0 (0)  | 2 (11)                         | 1 (8)                        | 3 (3)   |
| <b>Ethnicity—n (%)</b> |                                 |                               |  |                                |                              |         |
| Hispanic/Latino        | 10 (50)                         | 9 (47)                        | 10 (53)  | 10 (55)                        | 6 (46)                       | 45 (51) |
| Other                  | 10 (50)                         | 10 (53)                       | 9 (47)   | 8 (45)                         | 7 (54)                       | 44 (49) |

As expected, a booster dose led to an increase in RVNA titers in Cohorts 1, 2, and 4. Two administrations of RBI-4000 at the lowest dose level tested of 0.1 mcg (Cohort 1), resulted in detectable RVNA in 95% of participants and over 70% of participants achieved the WHO-defined measure of indirect protection (RVNA  $0.5 \geq$  IU/mL). A dose-dependent increase in RVNA titers was observed with 100% of participants in the 1 mcg cohort (Cohort 2) with detectable RVNA titers after boost, with 94% achieving the WHO-established metric. Boosting at the highest dose level tested of 10 mcg (Cohort 4) resulted in 100% of participants achieving RVNA titers above 0.5 IU/mL, indicative of adequate responses to the rabies vaccine<sup>50</sup>.

Longitudinal analysis of RVNA titers post administration of RBI-4000 showed a clear dose response with peak RVNA titers at Day 29 following the first, i.e., prime, administration (Fig. 5B, C). A dose-dependent enhancement of RVNA titers was observed in Cohorts 1, 2, and 4 that received a second or booster vaccination of RBI-4000, peaking 15 days following administration of RBI-4000. RVNA titers from participants who received RabAvert, an inactivated whole rabies virus vaccine, had differing kinetics from RBI-4000 (all dose levels), peaking at Day 15 following two administrations, proceeded with a rapid decay over the 85-day follow-up period (Fig. 5B). Assessing durability of the humoral immune responses is ongoing.

Analysis of the RVNA responses at their peak (Day 29 for RBI-4000) showed a clear dose response (Fig. 5C), suggesting that although protective immune responses were achieved in the majority of participants in Cohort 2, 3, and 4, immune responses were not saturated. This, combined with the favorable safety profile, suggests that further dose escalation is possible and may yield even better immunity. After boosting, peak titers (Day 71 for RBI-4000) were higher across all levels tested and equivalent titers were observed for the 1 and 10 mcg dose cohorts (Fig. 5D, Cohorts 2 and 4). Importantly, peak titers of the RVNA response post-boost were not statistically different for Cohort 4 (Day 71) and RabAvert (Day 15). Also, the fold change of the boost was highest in those cohorts where the baseline RVNA level was lowest at Day 57, suggesting that the interval between prime and boost can be optimized to coincide with full contraction of the humoral immune response to achieve the highest boost of RVNA titers. This is in agreement with recent studies in both RNA and non-RNA-based vaccine platforms<sup>51–54</sup>.

Three participants initially enrolled in the trial were subsequently identified as seropositive at baseline for RVNA. These participants were

distributed one across each dose level and received a single administration of RBI-4000, prior to being replaced to complete the dose cohorts. They remained in the study for safety and RVNA evaluation, although excluded from the data presented in the main cohorts. Regardless of dose level of RBI-4000 received, each showed boosting of RVNA titers between 6 and 11-fold above their baseline levels, with little to no decay over time with RBI-4000 (Supplementary Fig. 5).

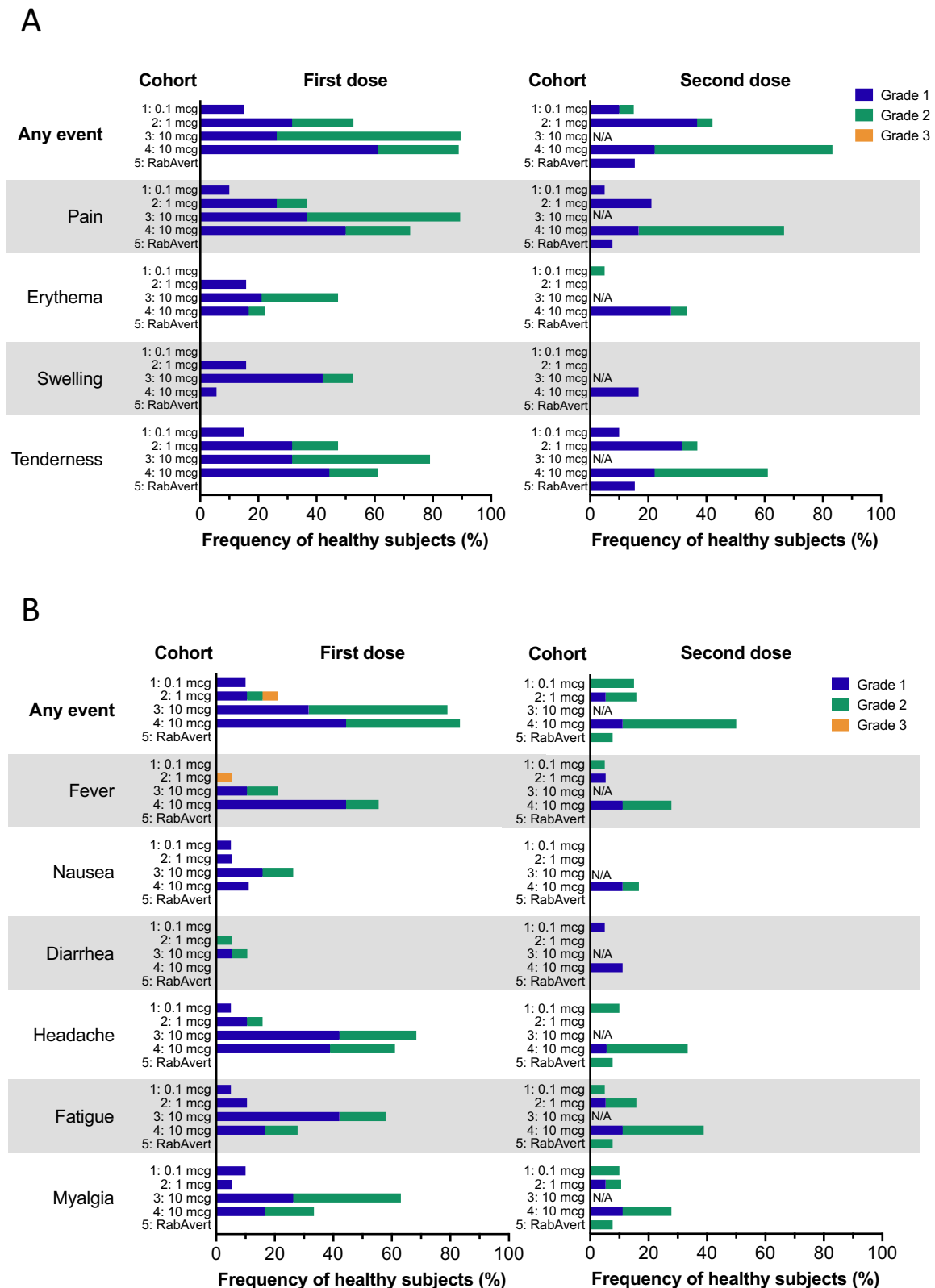
Rabies-specific T cell responses were also assessed following each administration of RBI-4000 in a limited subset of participants based on sample availability at the time of this report. RBI-4000 was able to elicit antigen-specific T cells in a dose-dependent manner (Supplementary Fig. 6).

## Discussion

This is the first report on the clinical use of next-generation, optimized srRNA technology. We conducted a Phase I clinical trial to assess safety and immunogenicity of a srRNA rabies vaccine in healthy volunteers.

In contrast to prior srRNA vaccines for SARS-CoV-2<sup>8–11</sup>, this study is the first demonstration of priming de novo immune responses by a srRNA vaccine, i.e., in participants with no confirmed prior history of antigen exposure or vaccination; an important step in the validation of a vaccine platform. De novo priming of immune responses as measured by RVNA titers above the WHO measure of indirect protection was detected in the majority of participants at all dose levels, including the lowest dose level tested of 0.1 mcg. The vaccine demonstrated a dose-dependent priming effect with no saturation, suggesting that higher doses of RBI-4000 may be well tolerated and result in even more striking immune responses. As clinical head-to-head technology assessments are challenging from several angles, we relied on cross-trial comparisons to assess gains in bioactivity with the next-generation RNA vaccine vector used for RBI-4000. The immunogenicity achieved with 0.1 mcg doses is unprecedented and is the lowest reported dose for any RNA platform that can achieve protective immunity in humans: 0.1 mcg represents a 100–1000-fold reduced dose when compared to approved mRNA vaccines for SARS-CoV-2<sup>1,2</sup>. Furthermore, this demonstration of clinical bioactivity at sub-microgram doses is differentiated from unoptimized first-generation srRNA platforms<sup>9</sup>.

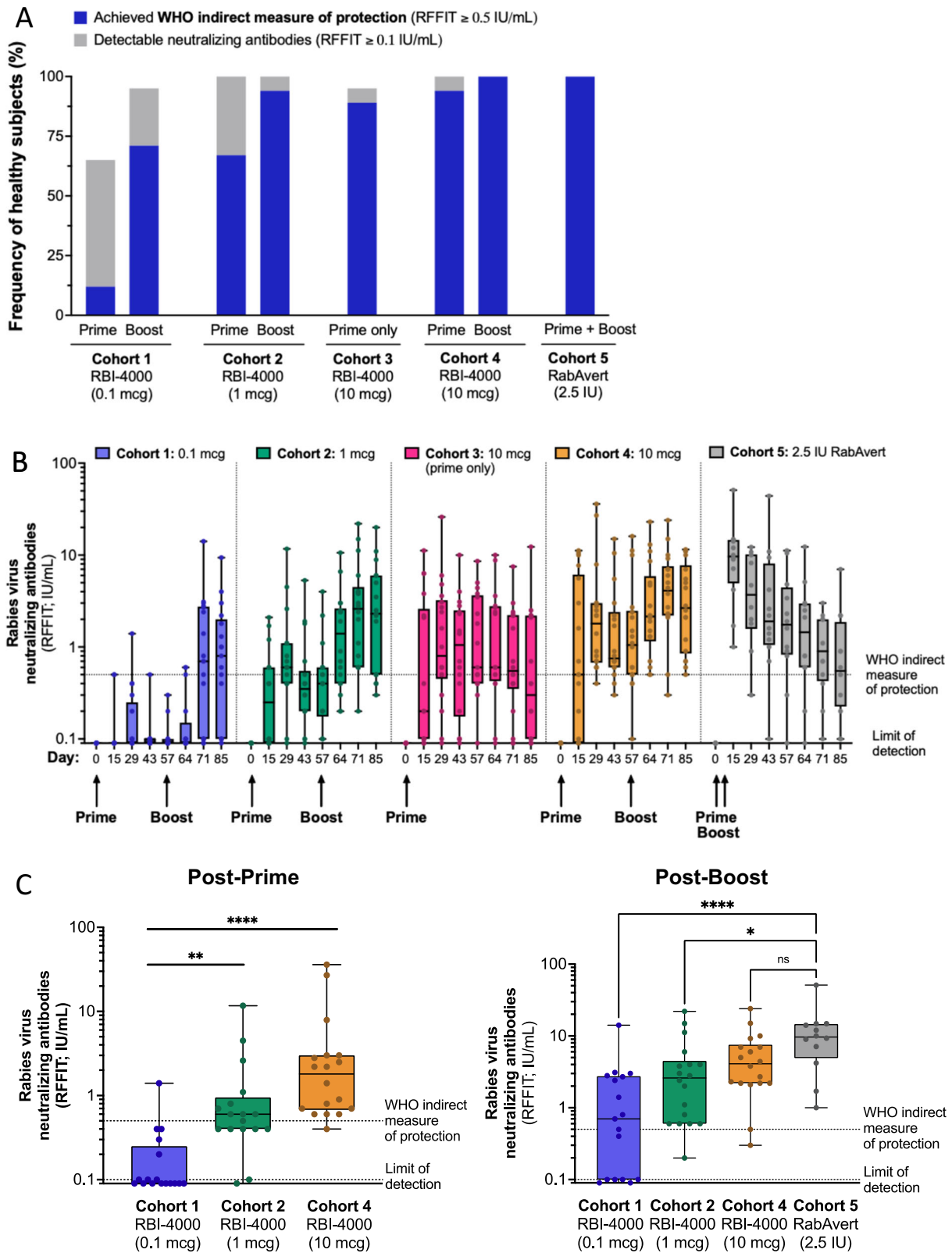
Data shown here and from others<sup>8,10,11</sup> show that srRNA technology is not inherently more reactogenic than mRNA-based vaccines incorporating modified nucleotide bases to avoid innate immune



**Fig. 4 | Clinical safety of RBI-4000. A** Local solicited and **B** Systemic solicited adverse reactions following 1 and 2 doses of RBI-4000 and RabAvert. Cohort 3 only received a single dose of RBI-4000. Maximum severity of each symptom is plotted. Reactogenicity was described as grade 1 (blue), grade 2 (green), or grade 3 (yellow).

sensors. RBI-4000 was well tolerated at all dose levels with no serious adverse events reported and a maximum tolerated dose was not defined in this study. In some instances, dose-limiting toxicities have been reported by other groups with srRNA vaccines administered at comparable doses<sup>9,10</sup>. Although unconfirmed, differences in

tolerability and reactogenicity are believed to be attributable to different drug product compositions and/or manufacturing processes that could introduce impurities, such as dsRNA, that trigger innate inflammatory pathways<sup>21,22,24</sup>. Optimization of these factors allowed dose escalation of RBI-4000 up to 10 mcg without any dose-limiting



toxicities, suggesting that higher doses are likely to be clinically feasible.

In addition to RBI-4000, an srRNA vaccine for rabies, we have demonstrated that bioactivity of improved srRNA vectors is also evident for alternative antigens, such as influenza hemagglutinin. In preclinical models, influenza vaccines administered at picogram doses led to functional antibody responses that achieved titers established as

protective in mice<sup>42</sup>, representing a 1000-fold improvement over previously reported first-generation srRNA vaccines for the same antigen<sup>55</sup>. This increase in bioactivity, over first-generation srRNA, is a result of higher levels of protein expression from vector engineering and optimization (Supplementary Fig. 2).

Thus, we have shown that vaccines utilizing optimized srRNA technology show a substantial improvement in bioactivity for multiple



**Fig. 5 | RBI-4000 elicits RVNA response in all dose cohorts in humans. A** Serum of trial participants was assayed for RVNA titers by RFFIT, after a single (Prime) or two (Boost) administrations of RBI-4000. Percentage of participants within each cohort with an RVNA titer above the LOD of the assay at any time during the study is plotted in gray bars. Percentage of participants within each cohort with a RVNA titer above the WHO-defined indirect immune measure of protection threshold (RVNA  $\geq 0.5$  IU/mL) at any time during the study is plotted in blue bars ( $n = 17$  Cohort 1,  $n = 18$  Cohort 2–4 and  $n = 12$  Cohort 5). **B** Longitudinal analysis of serum RVNA titers as measured by RFFIT in each cohort is plotted. Dashed line represents the WHO-defined indirect protection threshold (RVNA  $\geq 0.5$  IU/mL). Symbols

represent individual titers. Box represents 25–75th percentile, line represents median and whiskers represent min and max values ( $n = 17$  Cohort 1,  $n = 18$  Cohort 2–4 and  $n = 12$  Cohort 5). **C** RVNA titers following immunization with RBI-4000 at Day 29 (Prime only) and Day 71 (Prime-Boost) are shown for each dose level. Peak RVNA titers for RabAvert are shown for Day 15. Dashed line represents the WHO-defined indirect protection threshold (RVNA  $\geq 0.5$  IU/mL). Symbols represent individual titers ( $n = 17$  Cohort 1,  $n = 18$  Cohort 2–4 and  $n = 12$  Cohort 5). Box represents 25–75th percentile, line represents median and whiskers represent the min and max values. Statistical significance was determined by using a Kruskal–Wallis analysis, where  $*p < 0.0402$ ,  $**p < 0.0041$ ,  $***p < 0.0001$ .

antigens. Importantly, in a first-in-human study, RBI-4000 achieved clinical bioactivity, while maintaining a favorable safety profile, across the tested dose range of 0.1–10 mcg. These findings broaden the TI of RNA technology which has wide-ranging implications. A superior bioactivity at low doses can favorably impact deployability and accessibility for widespread use, such as in pandemic settings<sup>56</sup>. Additionally, a broadened therapeutic window suggests expanded use of this platform outside of simple vaccines. For example, for multi-genic or multivalent vaccines, this technology can be multiplexed with no compromise on its effective dose based on safety. Furthermore, for alternative therapeutic uses outside infectious disease vaccines, such as cancer vaccines and protein replacement therapies, where high, repeat dosing is expected to achieve the required levels of antigen or protein expression<sup>57–63</sup>, a broad therapeutic window is critical to avoid toxicity. We have generated preclinical and clinical evidence that improvements to srRNA vectors can greatly expand their potential applications. Coupled with a favorable safety profile, these next-generation vectors have a broader utility for future vaccine and drug development.

## Methods

### Preparation of srRNA

srRNA was transcribed *in vitro* using linearized plasmids encoding the transgene(s) under the control of a VEEV-based srRNA vector as template<sup>25</sup>. The coding sequences for Flury-LEP-C RABV-G (Genbank: ACL98057.1), A/California/MA-07/2009 H1 HA (Genbank: AT121640.1), and A/Vietnam/1203/2004 H5 HA (Genbank: AAW80717.1) were codon optimized for human expression (IDT). No engineering to stabilize RABV-G in the pre-fusion form was performed. For preclinical material, *in vitro* transcription was performed using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB, Cat#E2050S) and purified by LiCl precipitation. The RNA product was capped using Vaccinia Capping System (NEB, Cat# M2080S) and mRNA Cap 2'-O-Methyltransferase (NEB, Cat# M0366S) and purified by LiCl precipitation. For the clinical study, the GMP srRNA drug substance was produced by Curia (Hopkinton, MA, USA).

### LNP formulation

For the preclinical influenza vaccine formulations, a lipid mix was prepared at a concentration of 12.5 mM in ethanol, including an ionizable lipid, ALC-0315 ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate); Broad Pharma), helper lipids, cholesterol, (distearoyl-sn-glycero-3-phosphocholine (DSPC), and ALC-0159 (2-[(polyethylene glycol)-2000]-N,N ditetradecylacetamide; Broad Pharma) at molar ratios of 46.3:42.7:9.4:1.6 using standard methodology<sup>64</sup>. Lipids in ethanol and srRNA in aqueous buffer were mixed to form LNPs on a NanoAssemblr® Ignite™ (Precision NanoSystems Inc), concentrated and sterile-filtered. Formulations were stored at  $-80^{\circ}\text{C}$  until use.

For the preclinical rabies vaccine formulations, preclinical srRNA was formulated in LNPs as above, but with a proprietary LNP which contained a proprietary ionizable lipid<sup>65,66</sup>. For the clinical study, the GMP srRNA drug product was produced at Precision NanoSystems Inc.

(Vancouver, Canada). The RBI-4000 drug product is supplied as a frozen suspension ( $-80^{\circ}\text{C}$ ) at 0.1 mg/mL. Prior to patient dosing, it is freshly thawed and diluted with sterile 0.9% sodium chloride USP for injection to the required concentration so that administration of 0.5 mL via intramuscular injection achieves the required dose.

### Mice

Female BALB/c mice were procured from Charles River Laboratories (CRL). Mice were age-matched and within 6–12 weeks old at the time of study initiation. On the day of dosing, LNP-formulated srRNAs were administered intramuscularly in one or both quadriceps muscles. Animals were monitored for body weight and other general observations throughout each study. RabAvert was administered at 1/10th of the human dose intramuscularly. All procedures were conducted in compliance with all the laws, regulations, and guidelines of the National Institutes of Health (NIH) and with the approval of CRL Animal Care and Use Committee. CRL (formerly Explora Biolabs) is an AAALAC-accredited facility.

For rabies virus challenge studies, animal experiments were conducted under protocol 201565, approved by the Wistar Institute's Institutional Animal Care and Use Committee (IACUC). Female ICR mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the Wistar Institute's AAALAC-accredited animal Facility.

For luciferase reporter studies female BALB/c mice ( $n = 3$ , Jackson Laboratory) were administered 5 mcg of LNP-formulated srRNA encoding Fluc or PBS per leg via intramuscular injection in a volume of 50  $\mu\text{L}$ . For imaging at the indicated time points, mice were administered an intraperitoneal injection of 100  $\mu\text{L}$  of 30 mg/mL filter-sterilized D-Luciferin (PerkinElmer, Cat#122799) dissolved in PBS. After 7 min, the mice were placed under anesthesia using 2.5% isoflurane + 2%  $\text{O}_2$  for 3 min. Thereafter, eye lubricant was applied to the mice before being placed into the IVIS instrument for a 3-min exposure. Background signal was based on total flux from PBS injection. All studies described were reviewed and approved by the Institutional Animal Care Committee at the University of British Columbia.

### Peptides and proteins

An overlapping peptide library for RABV-G (Flury-LEP-C) protein was custom made, consisting of 15-mer peptides with a 10-amino-acid overlap. Individual peptides were then pooled into a single cocktail for the peptide library stimulations. The commercially available PepMix™ for Influenza A (H5 and H1) was purchased from JPT Innovative Peptide Solutions (Cat# PM-INFA-HACal for H1 and PM-INFA-HAIndo for H5). Immunodominant H5 CD4 (KSSFFRNVVWLIKKN) and CD8 (IYST-VASSL) peptides were synthesized by BioSynth.

### ELISpot

For preclinical studies, single-cell suspensions of homogenized spleens were stimulated for 16–18 h with indicated peptides. T cell responses were assessed using Mouse IFN $\gamma$  ELISpot PLUS kit (HRP) kit as per the manufacturer's instructions (Mabtech Cat# 3321-4HST-10). Developed ELISpot plates were sent to Cellular Technology Limited for

spot counting and quality control. Samples with high experimental background signal (exceeding 100 SFU per  $1 \times 10^6$  splenocytes in mock-treated wells) were omitted from the analysis.

For clinical studies, IFN $\gamma$  ELISpot was performed by stimulating PBMCs, collected at the indicated time points, with an overlapping peptide library spanning the length of RABV-G protein (CellCarta, Montreal, Canada). DMSO was used as a negative control for mock stimulation and these values were subtracted from RABV-G stimulated counts. Samples with viability below 70% prior to stimulation (after thawing and rest) were excluded from the analysis. All evaluable samples based on this criterion were included in the analysis. Testing was conducted in accordance with the applicable regulatory agencies as stated in the clinical protocol RBI-4000-101. Applicable portions of the Good Clinical Practices (GCP) and Good Clinical Laboratory Practices guidelines were followed.

### Intracellular cytokine staining

Splenocytes were stimulated with indicated peptides, in the presence of anti-CD28 (Life Technologies, Cat# 16-0281-86) and anti-CD49d (Life Technologies, Cat# 16-0492-85) or with PMA/Ionomycin (eBioscience, Cat# 00-4970-03) as a positive control. Intracellular cytokine staining was performed as per antibody manufacturer instructions. Data were acquired on an Aurora analyzer (Cytek) and analyzed using FlowJo (BD Biosciences).

### ELISAs

The endpoint titer total IgG ELISA against HI A/California/07/2009 (Sino Biological I1085-V08B) was performed by BioQual, Inc. For total IgG ELISA against H5, recombinant HA from A/Vietnam/1203/2004 (Immune Technology Corp, Cat# IT-003-0051p) was used as a coating antigen. Concentrations were interpolated from a standard curve using known concentrations of anti-H5 IgG, clone 2B7 (Abcam, Cat# Ab135382).

### Neutralization assays

Sera were used to perform rapid fluorescent foci inhibition tests (RFFIT) to quantify RVNA titers using a validated assay<sup>46</sup> by Rabies Laboratory, Kansas State University. The lower limit of detection for this assay is <0.1 IU/ml and any value below this limit is plotted as 0.09. A standard HI assay was performed for influenza studies using A/California/07/09 for HI and A/Vietnam/1203/04 for H5 (BioQual Inc), the lower limit of detection of this assay is 10, any value below this is plotted as 0.1.

### Rabies viral challenge

Six- to eight-week-old female ICR mice were administered RBI-4000 as a single dose or prime-boost regimen at the indicated dose levels at Day 0 and 56 or RabAvert at 1/10th of the human dose as per the recommended schedule (i.e. two doses, 7 day interval), both intramuscularly. Animals were serially sampled for serum RVNA levels. At Day 90, mice were challenged intramuscularly with  $7 \times LD_{50}$  of rabies virus (CVS-11 strain). Mice were monitored for body weight and general clinical observations and were euthanized at first sign of CNS infection. The animals that did not show signs of rabies infection, were kept on study for a total of 21 days after rabies infection.

### Clinical trial

RBI-4000-101 is a multicenter, open-label, randomized, active control Phase I clinical trial (NCT06048770) to assess the safety and immunogenicity of RBI-4000. The study enrolled 89 healthy volunteers, aged 18–45 years with no prior exposure or vaccination to rabies. Participants also had to be seronegative to hepatitis B, hepatitis C, and HIV and have no history of immune dysregulation, myocarditis, pericarditis, or malignancy within the past 5 years. All participants had to

verify their willingness not to initiate a pregnancy for at least 60 days following the completion of vaccination. Enrollment was completed at two sites in the United States.

Upon arrival at the clinical facility, subjects were randomly assigned subject numbers that corresponded to a previously generated randomization scheme. The randomization schedule was generated by Syneos Health using a validated proprietary computer software program (Statistical Analysis System [SAS®]) Version 9.4 and was reviewed by a biostatistician.

Demographics for the study are shown in Table 1. Volunteer gender and racial composition reflected the US population and were balanced between groups except for Cohort 3 which had a predominance of women. Enrollment was open to individuals of any gender. Enrolled subjects' sex was recorded by the study investigator. No sex or gender-based analyses are planned on these Phase I data and the study design did not account for sex or gender as no sex/gender differences in toxicity or safety were expected. Approximately half of the enrolled participants self-identified as of Latinx or Hispanic ethnicity, consistent with the population network of one of the two enrolling sites. Following each vaccination participants were evaluated for acute reactogenicity by medically qualified personnel and then sent home with diaries for self-reporting of reactogenicity for 7 days. Safety analysis included all dosed participants, solicited and unsolicited adverse events through March 21st 2024. Participants were enrolled between September 1st 2023 and December 21st 2023. The study was terminated at the Sponsor's request based on completion of study data analysis on July 31, 2024. The study was conducted in accordance with the protocol, applicable laws, and regulatory requirements, as well as International Council for Harmonization GCP guidelines, and the consensus ethical principles derived from international guidelines, including the Declaration of Helsinki and Council for International Organizations of Medical Sciences International Ethical Guidelines. The protocol was approved by the central institutional review board (Advarra, Inc., Columbia, MD) prior to study initiation, and written informed consent was obtained from all participants before enrollment.

Immunogenicity was assessed at predefined time points prior and post-immunization to measure serum RVNA using a RFFIT validated assay (Rabies Laboratory, Kansas State University) or rabies-specific T cells in total PBMCs using an IFN $\gamma$  ELISpot qualified assay (CellCarta, Montreal, Canada).

### Data collection and statistical analysis

Statistical analysis was performed in GraphPad Prism, version 10.1.1, and is reported for each figure in the corresponding figure legend. Clinical reactogenicity data were retrieved and processed by Stat One LLC using SAS for Windows release 9.4 (SAS Institute Inc. Cary, NC, USA) in accordance with FDA guidelines.

Clinical data collection was performed using RAVE EDC version 2022.3.1 (Medidata Solutions, New York City, NY, USA).

Any significance determined by these tests was denoted in the appropriate data graphs.

### Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

### Data availability

The Clinical Study Protocol is provided with the Supplementary Information. Preclinical source data have been provided with this paper as supplemental information. Additional clinical data will be available upon request once the study is formally completed, and a Clinical Study Report is prepared. Source data are provided with this paper.

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## Competing interests

The authors declare the following competing interests: C.J.M., S.J.M.-S., D.S.S., G.P., A.C., E.D.B., M.D.O., C.C.D., H.J.L., T.T.G., J.L.P., J.G., T.L.B., N.S.W., A.J.G., Z.G., and P.A. are employees of Replicate Bioscience Inc. D.B.W. has received grant funding, participates in industry collaborations, has received speaking honoraria, and has received fees for consulting, including serving on scientific review committees. Remunerations received by D.B.W. include direct payments and equity/options. D.B.W. also discloses the following associations with commercial partners: Geneos (consultant/advisory board), AstraZeneca (advisory board, speaker), Inovio (board of directors, consultant), Sanofi (advisory board), BBI (advisory board), Pfizer (Advisory Board), and Advaccine (consultant). A.K.B. has received grant funding, participates in industry collaborations, has received speaking honoraria, and has received fees for consulting, including serving on scientific advisory boards. Remunerations received by A.K.B. include direct payments and equity/options. A.K.B. also discloses the following associations with commercial partners: Genvax Technologies (consultant/advisory board), Pasture Biosciences (advisory board), Moderna (consultant), Epitopea (consultant), and the Cystic Fibrosis Foundation (consultant). The remaining authors declare no competing interests. Funders did not contribute to the design, execution, analysis, or reporting of data included in this manuscript.

## Ethics approval

All collaborators of this study have fulfilled the criteria for authorship required by Nature Portfolio journals and have been included as authors. The work described in this manuscript aligns with the inclusion and ethical guidelines embraced by Nature Portfolio editorial policies.

## Additional information

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41467-025-55843-9>.

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