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Impact of pre-existing anti-replicase immunity on the efficacy of self-amplifying mRNA vaccines

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Xiaole Cui[1], Laura Amelinck[2,3], Oriane Gillon[1], João Paulo Portela Catani[2,3], Ya Gao[4], Qing Sun[1], Elisabeth De Sutter[1], Pieter Vervaeke[1], Janne Snoeck[1], Stefan Lienenklaus[5], Xavier Saelens[2,3], Zifu Zhong[6,7] & Niek N. Sanders[1,7]

Self-amplifying mRNA vaccines use a viral replicase for self-amplification. It is currently unclear whether anti-replicase immunity occurs after saRNA vaccination, and whether such pre-existing anti-replicase immunity impacts the efficacy of subsequent saRNA vaccines. Using female mice, this study demonstrates that an initial saRNA vaccination schedule elicits replicase-specific immunity, which reduces the in vivo expression and impairs the Th1 cell responses of subsequent saRNA vaccines. Passive transfer experiments demonstrate that the suppression of T cell responses is driven by a synergistic effect of both anti-replicase antibodies and anti-replicase T cells. Interestingly, pre-existing anti-replicase immunity does not affect the ability of an influenza saRNA vaccine to provide full protection against an H5N1 challenge in female mice. Taken together, these results provide crucial insights on the effects of anti-replicase immunity induced by a first saRNA vaccination schedule on the expression, immunogenicity and protection efficacy of a subsequent saRNA vaccine.

Self-amplifying mRNAs (saRNAs) have emerged as a promising platform for vaccine development. Two saRNA vaccines were recently approved in India (Gemcovac®, 2022), Japan (Kostaive®, 2023) and EU (Kostaive®, 2025), marking a significant milestone in saRNA vaccine technology[1,2]. Besides, saRNAs also hold promise for applications in cancer treatment and protein replacement therapies. SaRNA is unique as it temporarily self-replicates within cells by the aid of an encoded viral replicase, which is primarily derived from alphaviruses or flaviviruses such as Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus, and Sindbis virus. These viruses contain a positive-sense single-stranded RNA with a 5′ cap, 3′ poly-A tail and two open reading frames (ORFs). The first ORF encodes 4 non-structural proteins (nsP1-4), which together form the replicase complex[3]. Each nsP plays an essential role, for instance, nsP1 acts as a capping enzyme[4], nsP2 functions as a helicase[5], nsP3 interacts with host factors critical for replication[6], and nsP4 serves as an RNA-dependent RNA polymerase with adenylyltransferase activity[7,8]. In saRNA constructs, the second ORF, which typically encodes viral structural proteins, is replaced by a gene of interest (GOI). Upon cellular entry, saRNA first directs the translation of nsP1-4 that form the replicase complex, which in turn drives the synthesis of negative-stranded RNA and high amounts of subgenomic RNA containing the GOI, leading to robust gene expression[9].

For viral-based vaccine platforms, such as adeno-associated virus (AAV) and adenovirus (Ad) -based vaccines, it has been reported that after their first administration, T cell responses[10] and neutralizing

[1]Laboratory of Gene Therapy, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium. [2]Center for Medical Biotechnology, VIB, Ghent, Belgium. [3]Department of Biochemistry and Biotechnology, Ghent University, Ghent, Belgium. [4]Department of Translational Physiology, Infectiology and Public Health, Ghent University, Merelbeke, Belgium. [5]Institute for Laboratory Animal Science, Hannover Medical School, Hannover, Germany. [6]Department of Pharmaceutics, Ghent University, Ghent, Belgium. [7]Cancer Research Institute (CRIG), Ghent University, Ghent, Belgium. e-mail: zifu.zhong@ugent.be; niek.sanders@ugent.be

antibodies[11] are elicited against viral proteins[12]. Viral-specific T cells can limit the efficacy of subsequent vaccinations using the same viral-based platform by accelerating the clearance of transduced cells[13–15]. Similarly, pre-existing humoral immunity against the viral vector's surface proteins can hinder cellular binding and reduce intracellular delivery[16–18]. Additionally, prior exposure to AAV or Ad due to natural infections is common[12,19], further complicating the effectiveness of these vaccine platforms.

Unlike AAV and Ad vectors, saRNA vaccines are delivered via lipid nanoparticles (LNPs)[3] or other delivery vehicles such as polymers, cell-penetrating peptides[20] and cationic nanoemulsions[3], allowing for repeated administration without compromising delivery efficiency. However, it is unknown if adaptive immune responses are induced against the viral replicase encoded by saRNA vaccines. Moreover, the impact of such anti-replicase immunity induced by an initial saRNA administration on the expression and efficacy of subsequent administered saRNA vaccines is also unknown. Even the immune response to alphavirus replicases after a viral infection remains largely unexplored. Some studies suggest that antibodies targeting the replicase could potentially serve as therapeutic agents for controlling alphavirus infections. For example, anti-replicase antibodies could be elicited using fusion proteins composed of beta-galactosidase and partial amino acid sequences of nsP1-4[21], or through the conjugation of nsP1-4 peptides with carrier proteins[22]. Whether saRNA vaccines can induce immunity against the encoded replicase remains unknown and requires further investigation.

In sum, it is currently not known whether saRNA vaccines elicit anti-replicase immunity and whether such anti-replicase immunity could affect subsequent saRNA vaccine expression and efficacy. Therefore, in this work we investigated the influence of pre-existing anti-replicase immunity on the expression, and especially the immune responses and protection efficacy of saRNA vaccines. Our results demonstrated that pre-existing anti-replicase immunity, induced by pre-treating mice with saRNA^{Fluc}, impaired the Th1 cell response of a subsequent saRNA^{HA} vaccine. This decrease was not mitigated by increasing the interval from two weeks to eight weeks between the saRNA^{Fluc} pretreatment and the saRNA^{HA} vaccination schedules. Using passive transfer experiments, we demonstrated that this inhibition was mediated via a coordinated effect of anti-replicase antibodies and T cells. Moreover, when pre-existing anti-replicase immunity was induced by a high dose of saRNA or when a low immunogenic antigen was used in the second schedule, a reduction in the T cell response, along with a modest yet significant decrease in the antibody titers of the second saRNA vaccination schedule, was observed. Although pre-existing anti-replicase immunity impaired immune responses of subsequent saRNA vaccines, it did not compromise the protective efficacy of a saRNA influenza vaccine in a mouse challenge experiment.

Results

Self-amplifying mRNAs elicit adaptive immune responses against the replicase that affect the expression of subsequent saRNAs

To investigate whether antibody and T cell responses are induced against the VEEV replicase of saRNA, we injected mice with LNP-formulated saRNA encoding firefly luciferase (saRNA^{Fluc}) on day 0, followed by a second injection 3 weeks later (Fig. 1a). Three weeks after the first saRNA^{Fluc}-LNP injection, IgG antibody titers against the replicase were found in the sera of all mice. After a second saRNA^{Fluc}-LNP injection, the anti-replicase IgG titers further increased and were significantly higher than in the PBS control group (Fig. 1b). Moreover, significant levels of replicase-specific IFN-γ$^+$CD4$^+$ and IFN-γ$^+$CD8$^+$ T cells were found in the saRNA^{Fluc}-LNP injected mice one week after the boost, while no such cells were found in the PBS control group (Fig. 1c).

To investigate the influence of pre-existing anti-replicase immunity on the in vivo translation of saRNA, we injected mice once or twice with 1 μg LNP formulated saRNA encoding H5 HA protein (saRNA^{HA}, Supplementary Fig. 1) to induce replicase-specific immune responses. Mice in the reference group received PBS injections instead (Fig. 1d). Three weeks after the single saRNA^{HA}-LNP injection or 2 weeks after the second saRNA^{HA}-LNP injection, all mice were injected with 1 μg saRNA^{Fluc}-LNP, and the in vivo firefly luciferase expression was monitored for 24 days (Fig. 1d, e). Mice that were pre-injected with saRNA^{HA}-LNPs showed, compared to the PBS pretreated group, lower luciferase expression during the first 2 days post-injection. Unexpectedly, from day 14 onward, saRNA^{HA}-LNP pre-injected mice maintained higher luciferase expression levels than the PBS pretreated mice (Fig. 1e). A lower expression of saRNA^{Fluc}-LNPs was also observed when mice were pretreated with 4 μg saRNA^{HA}-LNPs (Supplementary Fig. 2a–c).

Sera collected 24 days after saRNA^{Fluc}-LNP injection were analyzed for humoral responses against firefly luciferase (Fluc), which is known to be a weak antigen[23]. Anti-Fluc IgG titers were reduced in the saRNA^{HA}-LNPs pretreated group compared to PBS control mice. Moreover, the IgG1/IgG2a ratio was elevated in the saRNA^{HA}-LNP pretreated groups, suggesting suppression of Th1 immune response (Fig. 1f, g and Supplementary Fig. 2d, e).

Pre-existing anti-replicase immunity from a first saRNA vaccination schedule lowers antigen-specific T cell responses of a subsequent saRNA vaccination schedule

To further investigate the impact of pre-existing anti-replicase immunity on subsequent saRNA vaccination, we first administered two doses of 1 μg saRNA^{Fluc}-LNPs or PBS to mice, with a 3-week interval between the injections. Subsequently, 2, 4 and 8 weeks after the last saRNA^{Fluc}-LNP injection, vaccination with saRNA^{HA}-LNPs using a prime-boost schedule with 3 weeks in between was initiated (Fig. 2a). The anti-replicase antibody levels were measured just before the first saRNA^{HA}-LNP injection. The anti-replicase IgG titers peaked 2 weeks after the second injection of saRNA^{Fluc}-LNP and declined slightly after 4 weeks with no further decline after 8 weeks (Fig. 2b). Subsequently, vaccination of saRNA^{Fluc}-LNP pre-exposed mice with saRNA^{HA}-LNPs resulted, independent of the gap period between the last saRNA^{Fluc}-LNP injection and first saRNA^{HA}-LNP injection, in anti-HA IgG titers that were not significantly different from the anti-HA IgG titers in the PBS-HA group, which were not pre-exposed to saRNA^{Fluc}-LNPs (Fig. 2c). In contrast, mice pre-exposed to saRNA^{Fluc}-LNPs displayed significantly reduced frequencies of HA-specific IFN-γ$^+$CD4$^+$ and IFN-γ$^+$CD8$^+$ T cells compared to PBS-pretreated controls, with a calculated effect size of d = 2.6 (CD4$^+$, 2 weeks gap), 3.0 (CD8$^+$, 2 weeks gap), 3.2 (CD4$^+$, 4 weeks gap), 3.0 (CD8$^+$, 4 weeks gap), 4.3 (CD4$^+$, 8 weeks gap), 6.0 (CD8$^+$, 8 weeks gap) (Fig. 2e). Together with the elevated IgG1/IgG2a ratio with a calculated effect size of d = 1.1 (Fig. 2d), these findings indicate that pre-exposure to the saRNA platform attenuates Th1 T cell responses of a subsequent saRNA^{HA}-LNP vaccination schedule. Of note, this suppression of Th1 cell responses was independent of the time gap between the last saRNA^{Fluc}-LNP injection and the first saRNA^{HA}-LNP vaccine injection.

Pre-existing anti-replicase T cells induced by mRNA^{Rep} are not sufficient to reduce T cell responses of a saRNA^{HA} vaccine

To determine how pre-existing anti-replicase immunity affects the T cell response of subsequent saRNA vaccines, we constructed a non-replicating modified mRNA encoding the VEEV replicase (mRNA^{Rep}, expression data shown in Supplementary Fig. 3a), a non-replicating modified mRNA encoding firefly luciferase (mRNA^{Fluc}, expression data shown in Supplementary Fig. 3b), and a self-replicating RNA only encoding VEEV replicase (saRNA^{Rep}), as shown in Supplementary Fig. 1. Four groups of mice were injected twice with either 1 μg saRNA^{Fluc}, 1 μg mRNA^{Fluc}, 1 μg saRNA^{Rep}, or 1 μg mRNA^{Rep} followed by two 1 μg saRNA^{HA} vaccine injections. All mRNA or saRNAs were formulated with LNPs and administered intramuscularly. A PBS-injected group served as a

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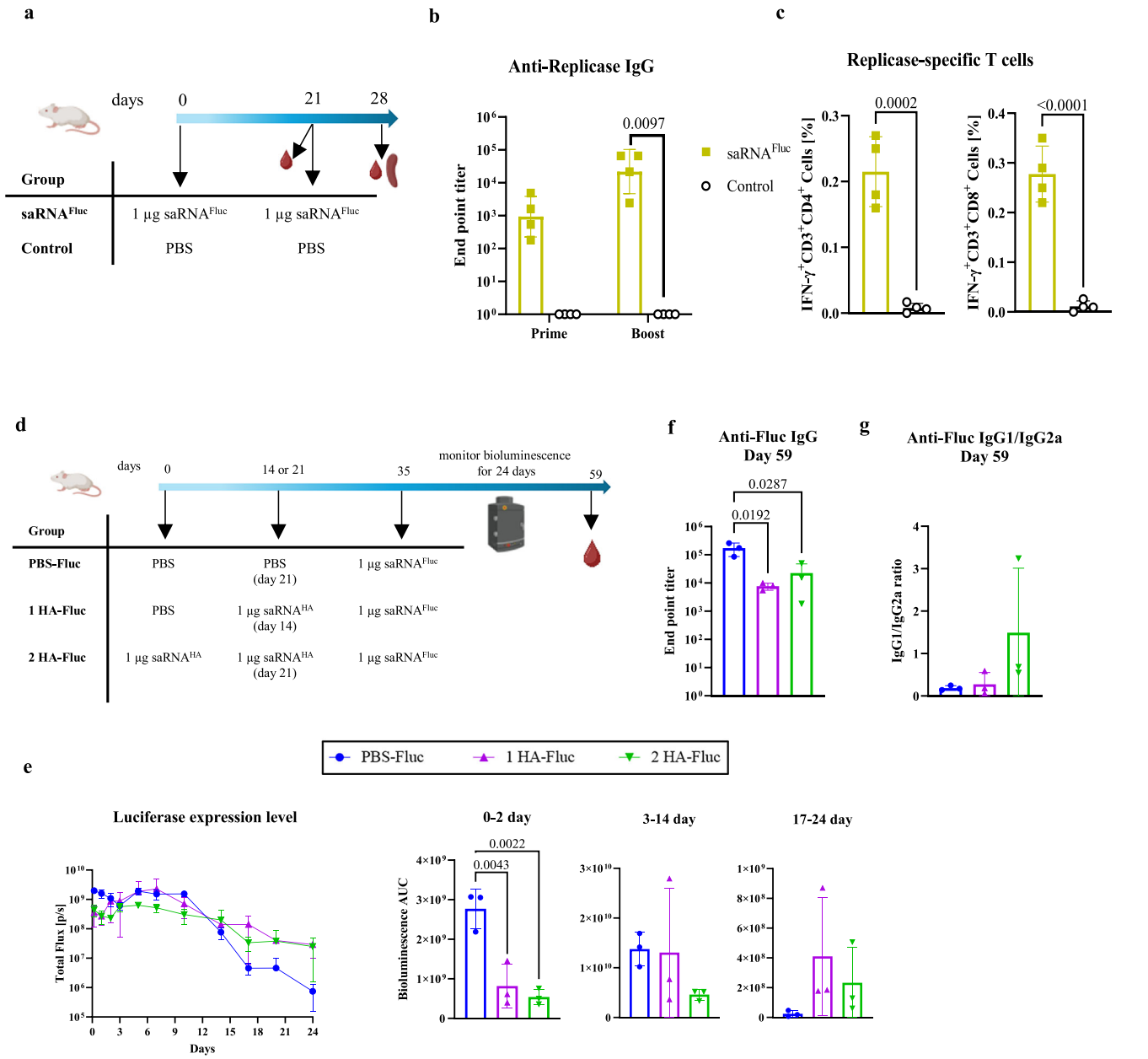

Fig. 1 | Replicase-specific antibody and T cell responses following saRNA^{Fluc}-LNP injections and impact of pre-existing anti-replicase immunity on subsequent saRNA^{Fluc} expression. a Schedule showing the injection, blood and spleen sampling times. BALB/cJRj mice received two intramuscular injections 3 weeks apart of either 1 µg saRNA^{Fluc}-LNPs (yellow filled squares) or PBS (black open circles). Blood samples were collected 3 weeks after the 1st injection. One week after the 2nd injection, the mice were euthanized and blood samples and spleens were collected. **b** Anti-replicase IgG antibody titers in serum 3 weeks after the 1st saRNA^{Fluc}-LNP injection (prime) or 1 week after the 2nd saRNA^{Fluc}-LNP injection (boost). **c** Percentage of replicase-specific IFN-γ$^+$CD4$^+$ (left) and IFN-γ$^+$CD8$^+$ (right) T cells in splenocytes isolated one week after the 2nd saRNA^{Fluc}-LNP injection. **d** Schedule showing the injection time points and the in vivo bioluminescence imaging period. BALB/cJRj mice received two intramuscular injections 3 weeks apart of either 1 µg saRNA^{HA}-LNPs (2 HA-Fluc group, green solid downward triangles) or PBS (PBS-Fluc group, blue filled circles). Two weeks later, mice were injected with 1 µg saRNA^{Fluc}-LNPs. Another group of mice received a single injection of 1 µg saRNA^{HA}-LNPs followed by 1 µg saRNA^{Fluc}-LNPs 3 weeks later (1 HA-Fluc group, purple solid upward triangles). **e** Firefly luciferase expression kinetics following saRNA^{Fluc}-LNP injection, as well as the areas under the curve (AUC) between days 0 and 2, 3 and 14, and 17 and 24. **f** Serum anti-firefly luciferase IgG titers and **g** IgG1/IgG2a ratios on day 59. Data are presented as geometric mean with geometric SD (**b**, **f**) or mean ± standard deviation (**c**, **e**, **g**) and biological replicates n = 4 (**b**, **c**) or n = 3 (**e**, **f**, **g**). Statistical analysis was performed using two-way ANOVA with Bonferroni's multiple comparisons (**b**), t-test (**c**) or one-way ANOVA with Tukey's multiple comparison test (**e**, **f**). Exact P values are indicated above the comparison bars in the figure. All tests were two-sided. The schematic elements in (**a**), (**d**) were created with BioRender.com. Source data are provided as a Source Data file.

control (Fig. 3a). Prior to the saRNA^{HA}-LNP vaccination, the immune responses against the replicase were measured. Interestingly, mice that received saRNA^{Fluc} or saRNA^{Rep} generated replicase-specific antibodies and T cell responses, whereas mice that received the mRNA^{Rep} elicited only low levels of T cell responses against the replicase (Fig. 3b–c). This difference, which is most likely due to a higher expression and innate immunity stimulation of the saRNA platform (Supplementary Fig. 3b–c), allowed us to investigate if pre-existing replicase-specific T cells induced by mRNA^{Rep} are sufficient to lower the T cell responses elicited by a subsequent saRNA vaccine. To that end, replicase pre-exposed mice were vaccinated with saRNA^{HA}-LNPs (Fig. 3a). One week after the saRNA^{HA}-LNPs boost, the anti-HA antibody

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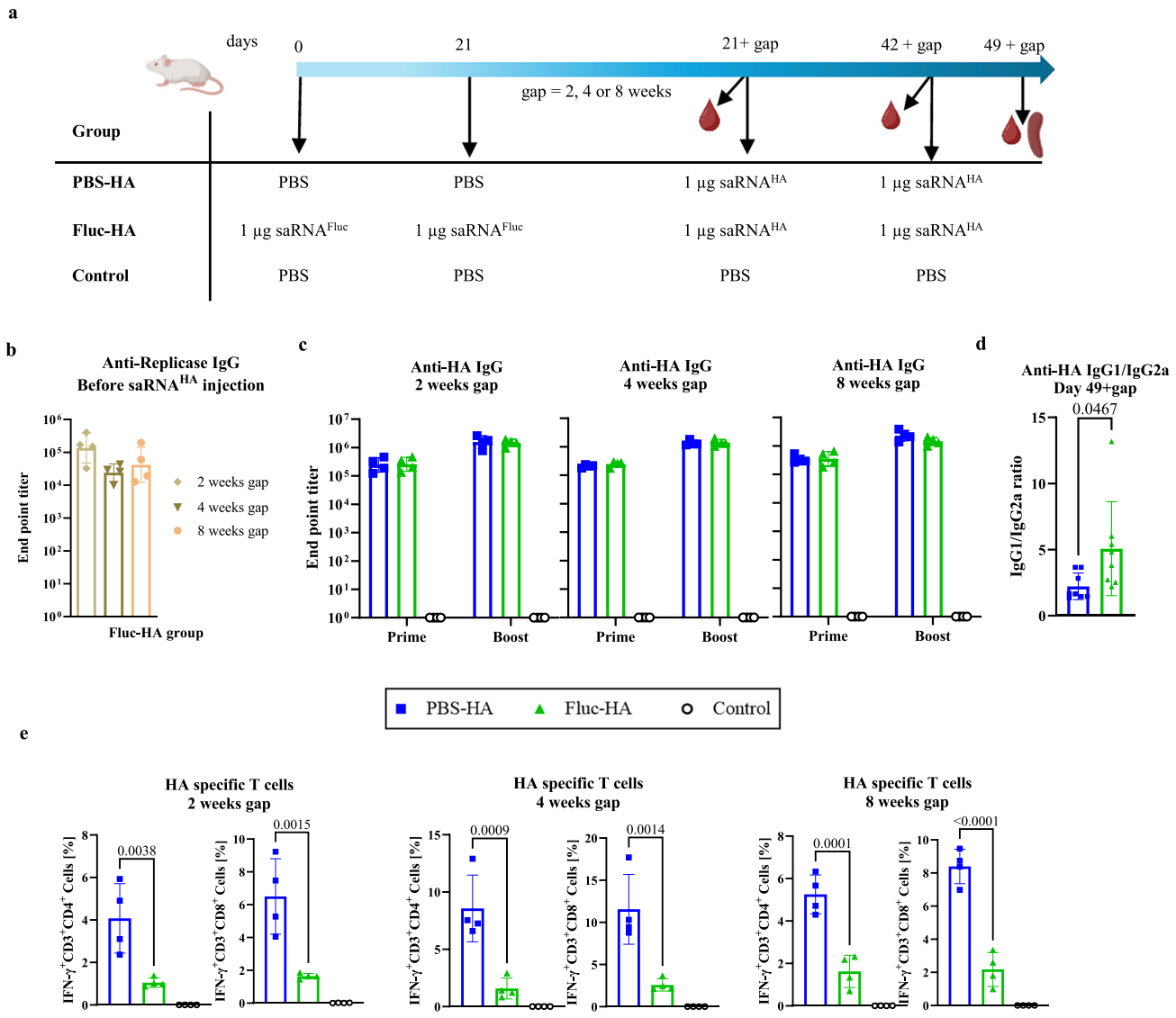

Fig. 2 | Pre-existing anti-replicase immunity induced by a first saRNA vaccination schedule impairs Th1 cell responses of a subsequent saRNA vaccination schedule. a Replicase pre-exposure and saRNA^{HA} vaccination schedule showing injection, blood and spleen sampling times. Anti-replicase immunity was induced in BALB/cJRj mice by two intramuscular injections of 1 µg saRNA^{Fluc}-LNPs (Fluc-HA group, green filled upward triangles) given 3 weeks apart. The reference group, without pre-existing replicase immunity, received two injections of PBS (PBS-HA group, blue filled squares) 3 weeks apart. Two, four and 8 weeks after the last saRNA^{Fluc}-LNP injection, blood samples were collected to measure anti-replicase antibodies, and the mice were subsequently intramuscularly immunized with a first shot of 1 µg saRNA^{HA}-LNP vaccine. Three weeks later, blood samples were collected, and mice were intramuscularly boosted with 1 µg saRNA^{HA}-LNPs. One week after the boost, blood and spleen samples were collected to measure the anti-HA immune responses. Control group only received PBS (black open circles). **b** Anti-replicase IgG antibody titers (n = 4) in serum 2 weeks, 4 weeks or 8 weeks after the last saRNA^{Fluc}-LNP injection in the Fluc-HA group. **c** Serum anti-HA IgG antibody titer (n = 4) 3 weeks after the saRNA^{HA}-LNP prime or 1 week after the saRNA^{HA}-LNP boost. The time interval between last saRNA^{Fluc}-LNP injection and the first saRNA^{HA}-LNP vaccination is 2 weeks (left), 4 weeks (middle) or 8 weeks (right). **d** Serum anti-HA IgG1/IgG2a ratio 1 week after the saRNA^{HA} boost, samples (n = 8) were from 2 weeks and 8 weeks gap groups. **e** Percentage of HA specific IFN-γ$^+$CD4$^+$ and IFN-γ$^+$CD8$^+$ T cells (n = 4) in splenocytes isolated 1 week after the saRNA^{HA}-LNP boost in mice that were vaccinated 2 weeks (left) 4 weeks (middle) or 8 weeks (right) after the last saRNA^{Fluc}-LNP injection. Data are presented as geometric mean with geometric SD (**b**, **c**) or mean ± standard deviation (**d**, **e**). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. All samples are biological replicates. Exact P values are indicated above the comparison bars in the figure. All tests were two-sided. The schematic elements in (**a**) were created with BioRender.com. Source data are provided as a Source Data file.

titers were measured, with the mRNA^{Rep} pretreated group showing anti-HA titers that were slightly higher than those in the other groups (Fig. 3d). A similar result was observed in IFN-β$^{+/Δβ\text{-}luc}$ reporter mice (Supplementary Fig. 4f). The IgG1/IgG2a ratio was again increased in the saRNA pretreated groups (Fig. 3e) in line with previous results (Figs. 1g and 2d). One week after the saRNA^{HA}-LNPs boost, the HA-specific cellular immune responses were measured. The mRNA^{Rep} pretreated group, which had only low levels of pre-existing anti-replicase T cells and no anti-replicase antibodies, showed no reduction in anti-HA IFN-γ$^+$ T cell responses (Fig. 3f) compared to mice without pre-existing anti-replicase immunity (mRNA^{Fluc} group). In contrast, the saRNA^{Fluc} and saRNA^{Rep} groups, which had both high pre-existing T cell and antibody responses against the replicase, elicited lower anti-HA IFN-γ$^+$ T cell responses compared to mice without pre-existing anti-replicase immunity (mRNA^{Fluc} group) (Fig. 3f). This suggests that low levels of pre-existing anti-replicase T cells immunity without anti-replicase antibodies induced by mRNA^{Rep} do not impair T cell response of subsequent saRNA vaccines.

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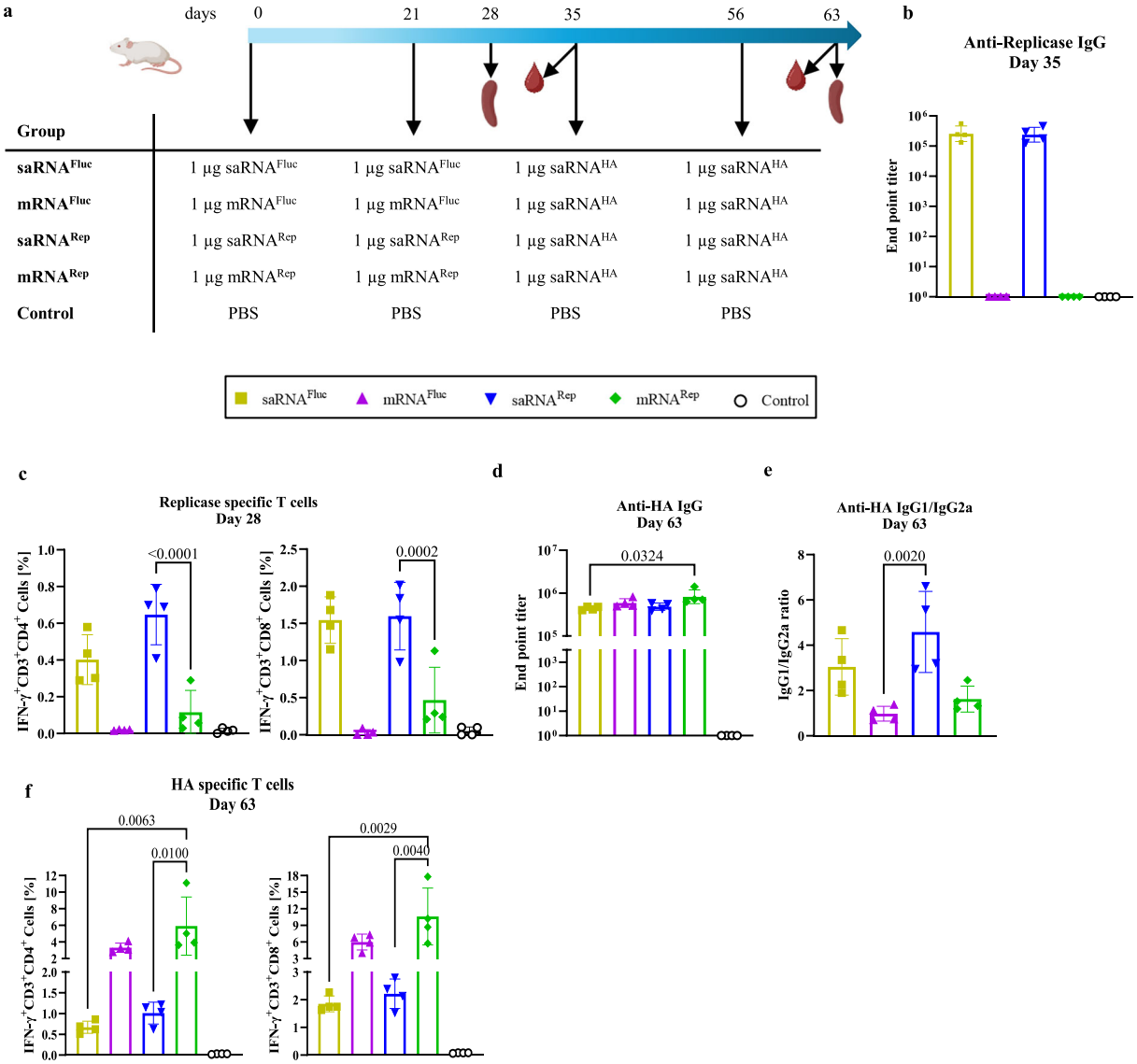

Fig. 3 | Low levels of only pre-existing anti-replicase T cells do not impair T cell responses of subsequent saRNA vaccines. a Replicase pre-exposure and saRNA^{HA} vaccination schedule showing injection, blood and spleen sampling times. BALB/cJRj mice were pretreated with two intramuscular injections of either 1 µg saRNA^{Fluc}-LNP (yellow filled squares), 1 µg mRNA^{Fluc}-LNP (purple solid upward triangles), 1 µg saRNA^{Rep}-LNP (blue solid downward triangles), 1 µg mRNA^{Rep}-LNP (green solid diamonds), or PBS (black open circles). The interval between the two injections was 3 weeks. One week after the last pretreatment, spleens from saRNA^{Fluc}, mRNA^{Fluc}, saRNA^{Rep}, mRNA^{Rep} and control groups were collected to measure anti-replicase T cell immunity. Two weeks after the last pretreatment, blood samples (n = 4) were collected to measure anti-replicase antibodies. Subsequently, the pretreated mice were vaccinated with the saRNA^{HA}-LNP vaccine using a prime-boost schedule with a 3-week interval. Blood samples (n = 4) were collected 3 weeks after the prime. Spleens (n = 4) were harvested 7 days after the saRNA^{HA}-LNP boost. **b** Anti-replicase antibody titers (n = 4) in serum samples collected just before the saRNA^{HA}-LNP prime. **c** Percentage of replicase-specific IFN-γ$^+$CD4$^+$ (left) and IFN-γ$^+$CD8$^+$ (right) T cells in splenocytes isolated 1 week after the last pretreatment of mice with RNAs (n = 4). **d** Anti-HA antibody titers and **e** anti-HA IgG1/IgG2a ratio in serum samples collected 1 week after the saRNA^{HA}-LNP boost (n = 4). **f** Percentage of HA-specific IFN-γ$^+$CD4$^+$ (left) and IFN-γ$^+$CD8$^+$ (right) T cells in splenocytes isolated 1 week after the saRNA^{HA}-LNP boost (n = 4). Data are presented as mean ± standard deviation (**c**, **e**, **f**) or geometric mean with geometric SD (**b**, **d**). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. All samples are biological replicates. Exact P values are indicated above the comparison bars in the figure. All tests were two-sided. The schematic elements in panel **a** were created with BioRender.com. Source data are provided as a Source Data file.

Type I interferons like IFN-β play an important role in inducing T cell responses[24,25] after mRNA vaccination. To determine whether pre-existing anti-replicase immunity affects the innate immune response to subsequent saRNA vaccination, the experiment described in Fig. 3a was repeated in IFN-β$^{+/\Delta\beta\text{-luc}}$ reporter mice using saRNA^{mCherry} instead of saRNA^{Fluc} to pre-expose the mice to the replicase (Supplementary Fig. 4a). Replicase-specific IFN-γ$^+$ T cells were significantly higher in the mRNA^{Rep} group compared to the mRNA^{mCherry} pretreated group after saRNA^{HA} injection (Supplementary Fig. 4b–c), confirming the pre-existing replicase-specific IFN-γ$^+$ T cells in mRNA^{Rep} group after pre-treatment. Consistent with the data in Fig. 3, lower percentages of HA-specific IFN-γ$^+$CD4$^+$ and IFN-γ$^+$CD8$^+$ T cells were present in mice that were pre-exposed to the saRNA, while the highest HA-specific IFN-γ$^+$ T cells after saRNA^{HA} boost were in mRNA^{Rep} pre-treated group (Supplementary Fig. 4d–e). Interestingly, saRNA^{mCherry} group displayed, after the first saRNA^{HA}-LNP injection, the lowest IFN-β expression at the injection site (Supplementary Fig. 4g). Hence, the drop in T cell responses after saRNA vaccination in mice that were pre-exposed to the saRNA replicase may not only be related to lower antigen expression (Fig. 1e), but also to lower inflammatory cytokine levels after saRNA vaccination in mice that were pre-exposed to the saRNA platform.

The highest reduction in T cell response of saRNA vaccines occurs when both anti-replicase antibodies and T cells are present

To further validate that adaptive immunity against the replicase is responsible for limiting T cell responses induced by subsequent saRNA vaccination, a passive transfer strategy was employed (Fig. 4a). To this end, sixteen mice were twice injected with 4 μg saRNA^{Fluc}-LNPs, and serum and splenocytes were harvested 2 weeks after the second saRNA^{Fluc}-LNP injection. Splenocytes were stimulated with nsP peptides overnight (Supplementary Fig. 5a) and T cells were subsequently separated (Supplementary Fig. 5b). Subsequently, the anti-replicase serum (350 μL), T cells (1.4×10^6 cells) or both were intravenously transferred to naïve mice (n = 8). These groups were designated as the Serum-trans, T cells-trans and Serum & T cell-trans groups, respectively (Fig. 4a). A reference group that received PBS intravenously was also included (Non-trans group). One day after the passive transfer, the mice received their first saRNA^{HA}-LNP vaccine. The successful transfer of anti-replicase antibodies was confirmed as anti-replicase antibodies were detected in sera collected from passive transferred groups before saRNA^{HA} vaccination (Fig. 4b). T cell transfer and homing after passive transfer was confirmed using CellTrace™ labeled T cells (Supplementary Fig. 5c). One week after the saRNA^{HA}-LNP booster vaccination, high anti-HA titers were established with no significant differences between the groups (Fig. 4c). This is in line with the data in Fig. 2c, where pre-existing immunity against the replicase did also not affect humoral responses of subsequent saRNA^{HA} vaccination. However, in this passive transfer experiment we did not find an increased anti-HA IgG1/IgG2a ratio as found in Fig. 2d (Fig. 4d). Interestingly, mice that received serum and T cells from mice with anti-replicase immunity before saRNA^{HA} vaccination had significantly lower HA-specific CD4$^+$ and CD8$^+$ T cell responses than the Non-trans group. Mice that received only serum or T cells from mice with anti-replicase immunity had slightly non-significantly reduced CD8 + T cell responses (Fig. 4e). This suggests that both pre-existing anti-replicase antibodies and T cells induced by saRNA play a role in inhibiting the T cell responses of subsequent saRNA vaccines. To rule out any influence from the passive transfer of external serum and splenocytes, we also included mice that received serum and splenocytes from PBS-injected mice just before the prime and boost vaccination with saRNA^{HA}-LNPs (Supplementary Fig. 6a). The anti-HA antibody responses (Supplementary Fig. 6b), as well as the percentages of HA-specific IFN-γ$^+$CD4$^+$ T cells and IFN-γ$^+$CD8$^+$ T cells (Supplementary Fig. 6c) in these passive transfer control groups, showed no significant differences compared to the Non-trans group (Supplementary Fig. 6).

Pre-treating mice with a high saRNA^{Fluc} dose impairs both cellular and humoral responses of a subsequent saRNA^{HA} vaccine

It is possible that a higher saRNA dose during the first exposure schedule results in a stronger impairment of the immune response elicited by a subsequent saRNA^{HA} vaccine. Therefore, in the next experiment, mice were injected twice with 4 μg instead of 1 μg saRNA^{Fluc}-LNPs prior to saRNA^{HA} vaccination (Fig. 5a). Interestingly, the higher saRNA^{Fluc}–LNP pretreatment not only reduced the T cell response but also the humoral responses of a subsequent saRNA^{HA}-LNP vaccine (Fig. 5b). Additionally, after the saRNA^{HA}-LNP prime, HI titers were undetectable in the saRNA^{Fluc}-LNP pretreated mice (Fig. 5d), while 3 out of 4 mice in the PBS-HA group exhibited HI titers above 16. Interestingly, after the saRNA^{HA}-LNP boost, the anti-HA IgG antibody levels and HI titers in the saRNA^{Fluc}-LNP pre-treated group strongly increased and approached levels that were similar to the PBS-HA reference group (Fig. 5b–d). However, despite the almost equal HA antibody levels in both groups after the saRNA^{HA}-LNP boost, the HA-specific IFN-γ$^+$CD4$^+$ T and IFN-γ$^+$CD8$^+$ T cell responses in the saRNA^{Fluc}-LNP pre-treated group remained significantly lower than in the PBS-HA group, with a calculated effect size of d = 4.7 (CD4$^+$), 3.5 (CD8$^+$) (Fig. 5e). Consistent with our previous observations (Figs. 1g, 2d and 3e), again, the mice with pre-existing anti-replicase immunity, i.e. the saRNA^{Fluc}-LNP pretreated group, showed an increased IgG1/IgG2a ratio (Fig. 5c).

The inhibitory effect of pre-existing replicase immunity on subsequent saRNA vaccination also appears to depend on the immunogenicity of the antigen encoded by the subsequently administered saRNA vaccine. Indeed, when we used saRNA^{HA}-LNPs to induce pre-existing replicase immunity and saRNA^{Fluc}-LNPs as a vaccine, we observed that pre-exposure to two injections of 1 μg saRNA^{HA}-LNPs was sufficient to reduce anti-luciferase antibody titers after the prime (Fig. 1f). Given that luciferase is a weak immunogen[23], this indicates that the effect of pre-existing anti-replicase immunity on subsequent saRNA vaccination is higher when the saRNA encodes a weak immunogen.

Pre-existing anti-replicase immunity does not impair the protective efficacy of a saRNA vaccine against H5N1 influenza virus

Next, a challenge experiment was performed to assess the effect of pre-existing anti-replicase immunity, due to a previous saRNA exposure, on the protective efficacy of a saRNA vaccine against H5N1 (Fig. 6a). To this end, a new saRNA$^{HA\text{-}RG}$ was generated that encoded the HA of the challenge virus clade (expression data shown in Supplementary Fig. 7). Mice were pretreated with two injections, given 3 weeks apart, of 1 μg (Group F) or 4 μg (Groups B and D) of saRNA^{Fluc}-LNPs. Groups A, C, and E were pretreated with two injections of PBS also administered 3 weeks apart. Two weeks after pretreatment, groups B, C, and F received two injections of 1 μg saRNA$^{HA\text{-}RG}$-LNP 3 weeks apart. Groups D and E received a single 1 μg saRNA$^{HA\text{-}RG}$-LNP injection 5 weeks after the last pretreatment. Two weeks after the final saRNA$^{HA\text{-}RG}$-LNP or PBS injection, the HI titers were measured and the mice were challenged with 5 LD_{50} of H5N1 virus (NIBRG14, clade 1). Robust HI titers were detected in all groups that received two doses of saRNA$^{HA\text{-}RG}$-LNPs (Groups B, C, and F), with no significant difference observed between PBS-pretreated and saRNA^{Fluc}-LNP-pretreated mice (Fig. 6b). In contrast, HI titers were undetectable in groups that received only one dose of saRNA$^{HA\text{-}RG}$-LNP, regardless of pretreatment (groups D and E). All vaccinated mice, including those pre-exposed to two doses of either 1 or 4 μg of saRNA^{Fluc}-LNPs and those receiving only one dose of the saRNA$^{HA\text{-}RG}$-LNP vaccine, were fully protected against a lethal H5N1 challenge. The latter is notable because the single-dose vaccinated mice did not have detectable HI titers at the time of the challenge. Perhaps low, undetectable levels of HI titers are present 2 weeks after priming and are further boosted by the challenge, as shown in Fig. 5d, high levels of HI titers occurring after the boost. Mice that were not vaccinated (group A) rapidly lost weight and succumbed to the challenge by 7 days post-challenge (Fig. 6c, d). These results demonstrate that pre-existing anti-replicase immunity does not compromise the protective efficacy of a saRNA$^{HA\text{-}RG}$-LNP vaccine, even when only one shot is given.

Discussion

Self-amplifying RNA vaccines encode both the desired antigen(s) and a viral replicase that transiently amplifies the saRNA and, in particular, the subgenomic mRNA encoding the antigen(s). The replicase consists of four non-structural proteins, most commonly derived from VEEV or other alphaviruses. Because of this self-amplification, currently licensed saRNA vaccines (Gemcovac® and Kostaive®) are used at a 6-fold lower dose than non-amplifying modified mRNA vaccines[26,27]. Although saRNA vaccines have been approved for human use, the impact of pre-existing immunity to the replicase on vaccine efficacy remains unclear. In this study, we demonstrated that both antibody and cellular immune responses against the VEEV replicase were induced following saRNA administration in mice. In addition, mice with pre-existing immunity to the replicase showed altered saRNA^{Fluc}

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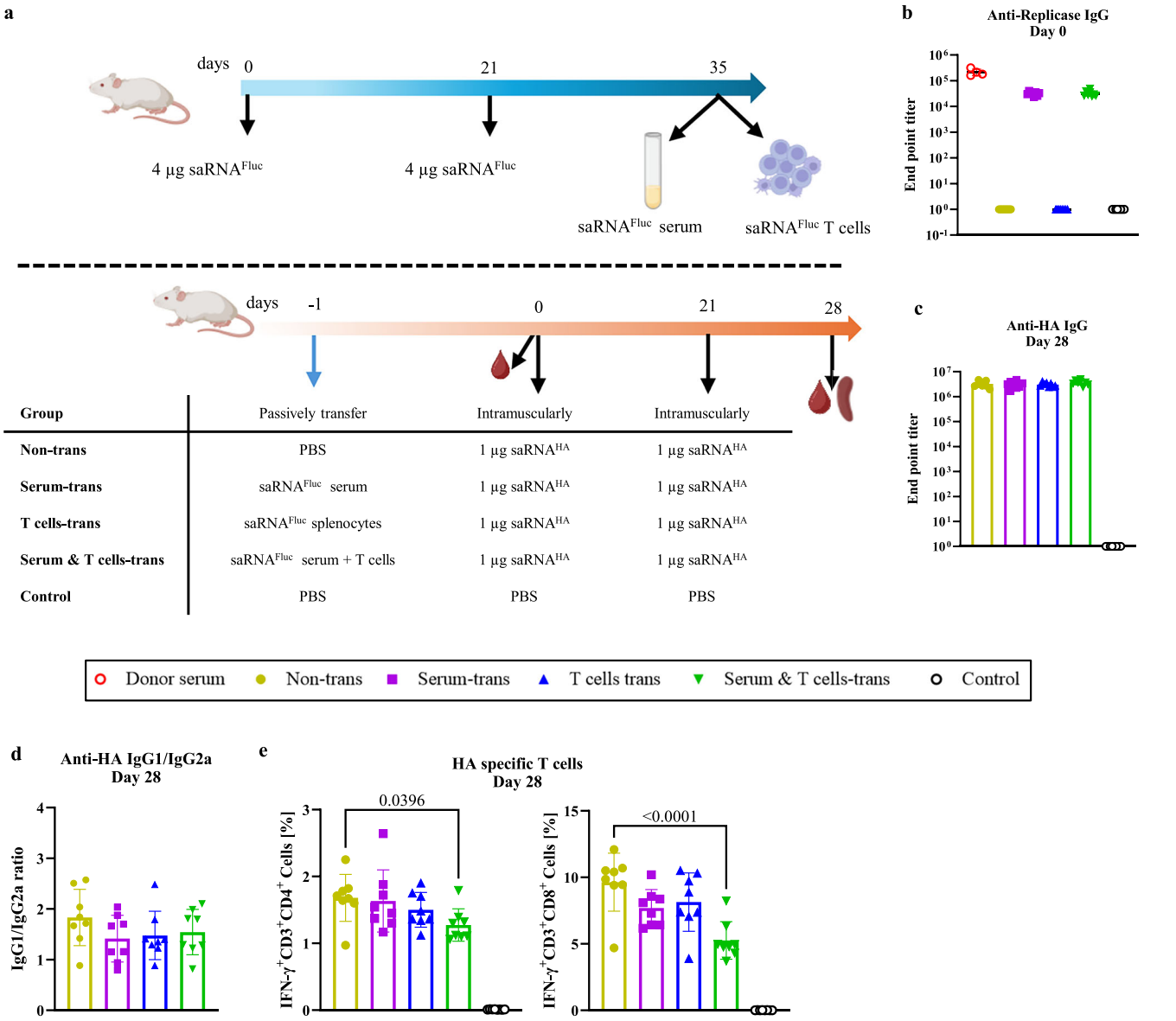

Group	Passively transfer	Intramuscularly	Intramuscularly
Non-trans	PBS	1 μg saRNA^{HA}	1 μg saRNA^{HA}
Serum-trans	saRNA^{Fluc} serum	1 μg saRNA^{HA}	1 μg saRNA^{HA}
T cells-trans	saRNA^{Fluc} splenocytes	1 μg saRNA^{HA}	1 μg saRNA^{HA}
Serum & T cells-trans	saRNA^{Fluc} serum + T cells	1 μg saRNA^{HA}	1 μg saRNA^{HA}
Control	PBS	PBS	PBS

Fig. 4 | Adaptive immune responses after saRNA^{HA} vaccination in mice that received anti-replicase serum, T cells, or both via passive transfer. a Schematic representation of the experimental setup and timeline of the passive transfer experiments. Anti-replicase immunity was first induced in sixteen BALB/cJRj mice by giving them two intramuscular injections of 4 μg saRNA^{Fluc}-LNPs 3 weeks apart (blue timeline). Two weeks after the last saRNA^{Fluc}-LNP injection, serum and T cells from spleens were collected and intravenously transferred into naïve BALB/cJRj mice (n = 8). The serum transfer (Serum-trans, purple filled squares) group received 350 μL serum, the T cells transfer (T cells-trans, blue solid upward triangles) group received 1.4×10^6 T cells in 350 μL PBS, the serum and T cells transfer (Serum & T cells-trans, green solid downward triangles) group received 1.4×10^6 T cells in 350 μL serum, and the reference (Non-trans, yellow filled circles) group received 350 μL PBS (orange timeline and table). **b** The presence of anti-replicase antibodies in the blood of mice that received serum, T cells or both from anti-replicase positive mice was measured by ELISA one day after the passive serum transfer (n = 8). The anti-replicase antibody level in the serum used for transfer is shown by the open red circles (donor serum, red open circles). One day after passive transfer, mice were vaccinated with 1 μg saRNA^{HA}-LNPs. Three weeks later, the mice received a saRNA^{HA}-LNP boost injection. Blood and spleen samples were harvested 1 week after the boost. A control group (black open circles) that received only PBS at each timepoint was also included. **c** Anti-HA IgG antibody levels (n = 8) and **d** anti-HA IgG1/IgG2a ratio (n = 8) in serum samples collected 1 weeks after the saRNA^{HA}-LNP boost. **e** The percentage of HA specific IFN-γ$^+$ CD4$^+$ (left) and IFN-γ$^+$ CD8$^+$ (right) T cells (n = 8) in splenocytes collected 1 week after the saRNA^{HA}-LNP boost. Data are presented as geometric mean with geometric SD (**b**, **c**) or mean ± standard deviation (**d**, **e**). Anti-replicase antibodies in donor serum were measured repeatedly using the same sample. Statistical analysis was performed using one-way ANOVA (**e**) with Tukey's multiple comparisons test. All samples, except for donor mice serum, are biological replicates. Exact P values are indicated above the comparison bars in the figure. All tests were two-sided. The schematic elements in (**a**) were created with BioRender.com. Source data are provided as a Source Data file.

expression kinetics, with a lower expression in the first few days, but surprisingly also a longer expression than observed in mice without pre-existing anti-replicase immunity (Fig. 1e). The altered expression kinetics might be due to a rapid clearance of highly transfected cells shortly after saRNA^{Fluc}-LNP injection by replicase-specific immune responses, which will be discussed in more detail below. The longer expression might be due to lower anti-luciferase immunity in mice with pre-existing anti-replicase immunity (Fig. 1f).

Sequential administration of saRNA vaccines targeting different pathogens is likely in the future. However, our data demonstrated that injection of saRNA results in anti-replicase immunity that decreases the expression of subsequent saRNAs. To investigate if

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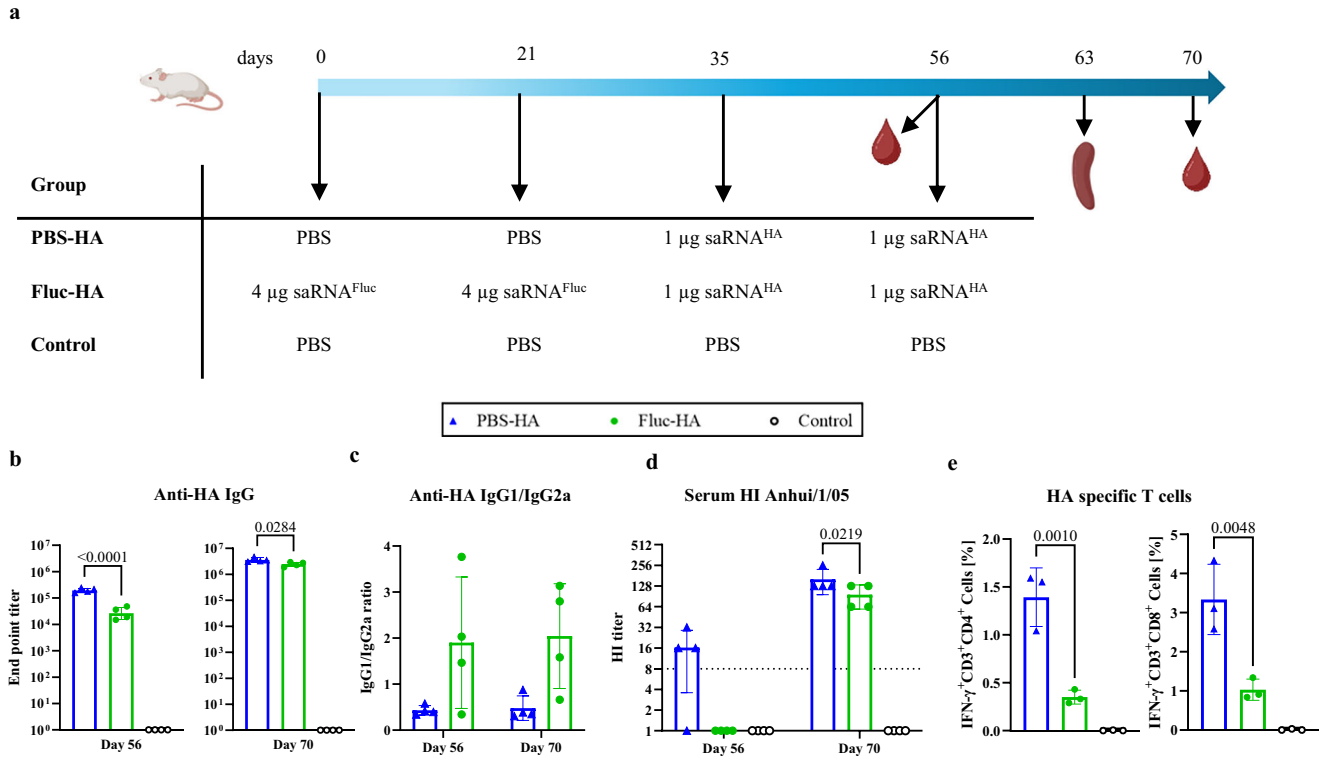

Fig. 5 | Pre-treating mice with a 4-fold higher saRNA^{Fluc}-LNP dose than the subsequent saRNA^{HA}-LNP vaccine dose affects both anti-HA T cell and antibody responses. a Replicase pre-exposure and saRNA^{HA} vaccination schedule showing injection, blood and spleen sampling times. Anti-replicase immunity was induced in BALB/cJRj mice by two intramuscular injections of 4 µg saRNA^{Fluc}-LNPs (Fluc-HA group, green solid circles) given 3 weeks apart. The reference group, having no pre-existing replicase immunity, received two injections of PBS (PBS-HA group, blue solid upward triangles) 3 weeks apart. Two weeks after the last saRNA^{Fluc}-LNP injection the mice were vaccinated with 1 µg saRNA^{HA}-LNPs using a prime-boost schedule with a 3-week interval. Control group only received PBS (black open circles). Blood samples (n = 4) were collected 3 weeks after the saRNA^{HA}-LNP prime and 2 weeks after the saRNA^{HA}-LNP boost. Spleens (n = 3) were collected 7 days after the saRNA^{HA}-LNP boost. **b** Anti-HA IgG antibody titers (n = 4), **c** anti-HA IgG1/IgG2a ratio (n = 4) and **d** hemagglutination inhibition (HI) titers in serum samples (n = 4) collected 3 weeks after the prime and 2 weeks after the saRNA^{HA}-LNP boost. The HI detection limit was 8 and is indicated as a dashed line, and titers below this threshold are considered as undetectable titers and shown as 1. **e** The percentage of HA specific IFN-γ$^+$ CD4$^+$ (left) and IFN-γ$^+$ CD8$^+$ T cells (right) in splenocytes isolated 1 week after the boost (n = 3). Data are presented as geometric mean with geometric SD (**b**, **d**) or mean ± standard deviation (**c**, **e**). Statistical analysis was performed using two-way ANOVA (**c**, **d**) or one-way ANOVA (**b**, **e**) with Tukey's multiple comparisons test. All samples are biological replicates. Exact P values are indicated above the comparison bars in the figure. All tests were two-sided. The schematic elements in (**a**) were created with BioRender.com. Source data are provided as a Source Data file.

this phenomenon affects the effectiveness of sequentially administered saRNA vaccines, two sequential saRNA prime-boost schedules were performed using interim periods of 2, 4 and 8 weeks between the two schedules. For the first two shots saRNA^{Fluc}-LNPs were used, while for the subsequent two shots of a HA encoding saRNA^{HA}-LNP vaccine[28] was used (Fig. 2a). Equal saRNA doses were used for both schedules. This experiment demonstrated that HA-specific IFN-γ$^+$ T cell responses were significantly impaired by the anti-replicase immunity induced by the first two saRNA^{Fluc} shots, while anti-HA IgG titers were not affected. Moreover, increasing the time between the two vaccination schedules to 8 weeks did not restore the effectiveness of the last saRNA^{HA}-LNP vaccination schedule. This is consistent with the observation that 8 weeks after the last shot of the first vaccination schedule, the anti-replicase IgG antibodies had not declined (Fig. 2b). Pre-existing anti-replicase immunity induced by pretreating IFN-β$^{+/Δβ\text{-luc}}$ mice with saRNA^{mCherry}-LNPs resulted in a similar impairment of the T cell responses elicited by subsequent saRNA^{HA} vaccination (Supplementary Fig. 4).

Interestingly, pre-exposure of mice with non-replicating mRNA^{Rep}-LNPs resulted only in low levels of anti-replicase T cells, which did not affect the capacity of a subsequent saRNA^{HA} vaccine to elicit IFN-γ$^+$ T cells. This underscores fundamental differences in the immune responses elicited by mRNA and saRNA vaccines. The higher efficacy of saRNA vaccines is likely due to the higher and longer antigen expression together with a stronger innate immune response that may cause transfected cells to undergo apoptosis[29].

By passive transfer of anti-replicase antibodies, T cells or both from mice with anti-replicase immunity to naïve mice just before their saRNA^{HA}-LNP shots, we showed that T cell responses to saRNA vaccines are significantly impaired when pre-existing anti-replicase antibodies and T cells are present (Fig. 4e). Nevertheless, small non-significant reductions in T cell responses were also observed after saRNA^{HA} vaccination of mice that received only anti-replicase serum or T cells. The inhibitory effect of anti-replicase T cells on a subsequent saRNA^{HA} vaccination can be explained by the killing of saRNA^{HA} infected cells by replicase-specific CD8$^+$ T cells. To unravel how anti-replicase antibodies impair the immunity of subsequent saRNA vaccines, we performed several studies. First, we ruled out the possibility that anti-replicase antibodies killed living, saRNA transfected cells by antibody-dependent effector functions as we demonstrated that passively transferred anti-replicase antibodies did not alter the in vivo expression of saRNA^{Fluc}-LNPs (Supplementary Fig. 8) nor did they bind to the surface of living saRNA-transfected cells (Supplementary Fig. 9). In contrast, serum of saRNA pretreated mice that contained anti-replicase antibodies bound to the intracellular replicase after permeabilization of saRNA transfected cells (Supplementary Fig. 9). This indicates that the replicase proteins are located inside the cells rather than exposed on the plasma membrane. We hypothesize that anti-

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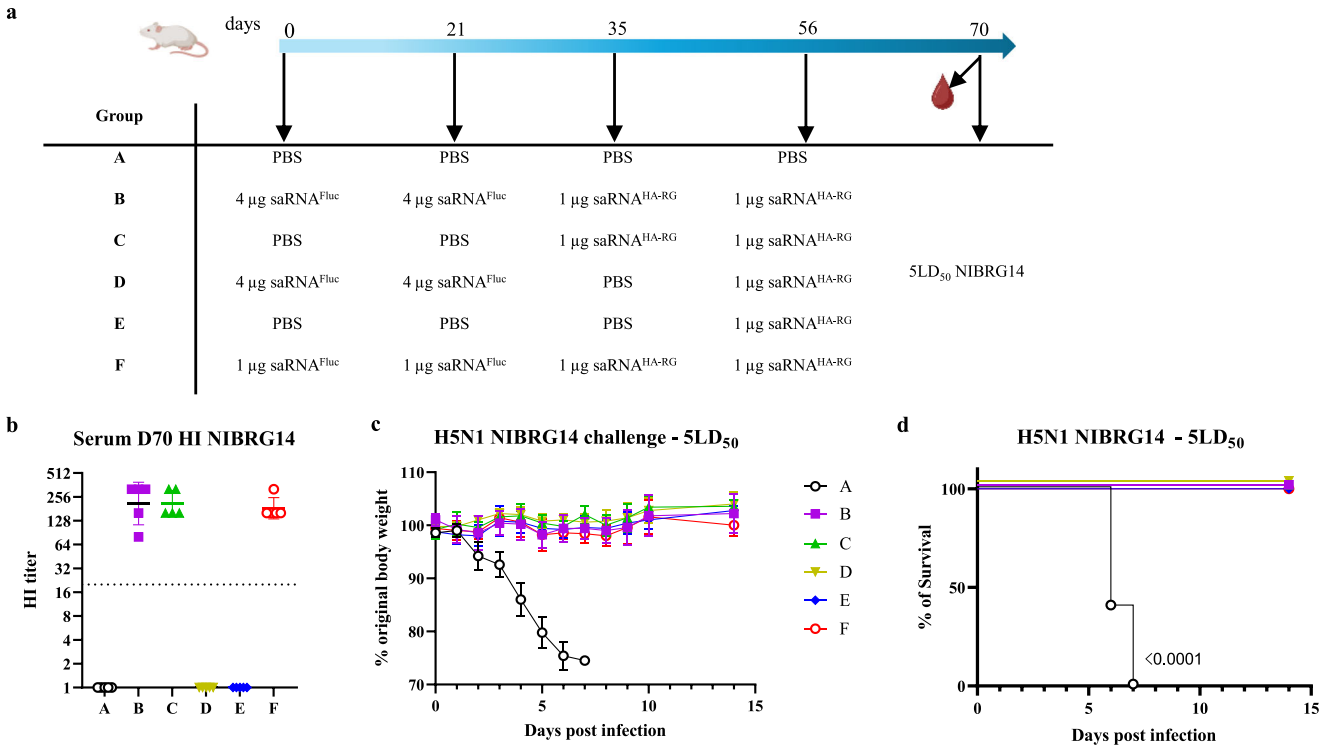

Fig. 6 | Pre-existing anti-replicase immunity does not affect the protection efficacy of a saRNA^{HA} vaccine against a lethal H5N1 challenge. a Schematic representation of the experimental setup. Anti-replicase immunity was induced in BALB/cJRj mice (n = 5) by two intramuscular injections of PBS (group A, black open circles; C, green solid upward triangles; and E, blue filled diamonds), 4 µg saRNA^{Fluc}-LNPs (group B, purple filled squares; and D, yellow solid downward triangles) or 1 µg saRNA^{Fluc}-LNPs (group F, red open circles) given 3 weeks apart. Two weeks after the last saRNA^{Fluc}-LNP injection, the mice in groups B, C and F were vaccinated twice with 1 µg of saRNA$^{HA\text{-}RG}$-LNPs, with a three-week interval between doses. The mice in groups D and E were vaccinated with a single dose of 1 µg saRNA$^{HA\text{-}RG}$-LNPs. Mice in group A were not vaccinated. Two weeks after the final saRNA$^{HA\text{-}RG}$-LNP injection, blood was collected to determine HI titers and all mice were challenged with 5 LD_{50} NIBRG14. **b** HI titers (n = 5) in serum samples collected 2 weeks after the last saRNA$^{HA\text{-}RG}$-LNP injection. The HI detection limit was 20 and is indicated as a dashed line, and titers below this threshold are shown as 1. **c** Mice body weight change after virus challenge (n = 5). **d** The survival rate after challenge (n = 5). Data are presented as geometric mean with geometric SD (**b**) or mean ± standard deviation (**c**). Statistical analysis was performed using Gehan-Breslow-Wilcoxon test (**d**). All samples are biological replicates. Exact P values are indicated above the comparison bars in the figure. All tests were two-sided. The schematic elements in (**a**) were created with BioRender.com. Source data are provided as a Source Data file.

replicase antibodies are a result of the release of the non-structural proteins from saRNA-transfected cells that underwent apoptosis or necrosis due to saRNA-triggered inflammation, as recently reported[29–31]. Moreover, intracellular antigen can also relocate to the surface of apoptotic cells[32]. Hence, when anti-replicase antibodies are present before saRNA^{HA} vaccination, they can bind to apoptotic saRNA^{HA}-transfected cells and trigger a rapid clearance of these cells by antibody-dependent cellular phagocytosis (ADCP). This process could indirectly influence the immune response by limiting the availability of antigen for dendritic cells[33], thereby reducing cross-presentation and MHC-peptide display to T cells. Moreover, high levels of immune complexes can drive macrophage polarization toward an M2-like phenotype[34], which produces immunosuppressive cytokines such as IL-10, further dampening Th1 and $CD8^+$ T cell activation.

We also found in this work that pre-exposure to a first saRNA vaccination schedule impacted both cellular and humoral responses of a subsequent saRNA vaccination schedule when a 4-fold higher saRNA dose was used in the first vaccination schedule (Fig. 5b–d). Notably, the anti-HA antibody and HI titers after the prime were drastically reduced. However, after the saRNA^{HA}-LNP boost, anti-HA antibody levels sharply increased, though they remained slightly lower than in mice without pre-existing replicase immunity (Fig. 5b–d). Moreover, also the immunogenicity of the antigen matter. Indeed, humoral immune responses against luciferase, which is a weak antigen, were much lower when the mice were immunized with the saRNA encoding luciferase after an influenza saRNA vaccination schedule.

In a final experiment the effect of pre-existing anti-replicase immunity on the protective efficacy of an saRNA^{HA} vaccine was investigated in a challenge experiment. To increase the likelihood of observing an effect of pre-existing replicase immunity, we also included a group that was pretreated with saRNA^{Fluc} at a dose four times higher than the saRNA^{HA} doses. Additionally, the effect of pre-existing anti-replicase immunity on the protection efficacy of a single dose of the saRNA^{HA} vaccine was tested, as our data showed that the effect of pre-existing anti-replicase immunity was most pronounced after the prime (Fig. 5b, d). However, surprisingly, none of the pre-existing anti-replicase immunity conditions did decrease the protection efficacy of the saRNA^{HA} vaccine, which was 100% protective in all conditions. Even mice that received only one dose of the saRNA^{HA} vaccine were fully protected against a lethal H5N1 challenge, despite having no detectable HI titers at the time of the challenge. This underscores the strong protective potential of the saRNA vaccine platform that elicits both humoral and cellular responses.

Our observation that a first saRNA vaccination schedule significantly reduces the Th1 responses of a subsequent saRNA vaccine could have significant implications for the efficacy of saRNA vaccines that target cancer. Indeed, the efficacy of cancer vaccines largely depends on their capacity to elicit Th1 cells and especially $CD8^+$ T cells, as these cells can directly kill cancer cells. Moreover, the emerging neoantigen based RNA cancer vaccines that showed promising results in clinical trials require many (8 or more) repeated injections[35,36]. Future studies are urgently needed to investigate to what extend

anti-replicase immunity will impair the efficacy of saRNA cancer vaccines. To overcome anti-replicase immunity induced by an initial saRNA vaccine, subsequent saRNA vaccines could employ saRNA platforms that utilize replicases derived from other alphaviruses, like e.g. Semliki Forest virus, Sindbis virus, Eastern equine encephalitis virus, Tonate virus, or Chikungunya virus[37] or even from unrelated viral families such as flaviviruses or picornaviruses. It is important to stress that in contrast to viral vector vaccines like adenovirus-based vaccines, where repeated injection shortly after the prime demonstrated no significant boost effect due to the induction of adenovirus-neutralizing antibodies after the first application[38,39], the saRNA vaccine platform still elicits robust antibody responses even when given shortly (i.e. 2 weeks) after a previous saRNA vaccination schedule.

In conclusion, our findings demonstrate that pre-existing immunity against the VEEV replicase significantly impairs the Th1 cell response to subsequent saRNA vaccines. Increasing the time window between two saRNA vaccination schedules from 2 to 8 weeks does not restore the T cell responses of the second saRNA vaccine to normal levels. This is in line with the observation that anti-replicase antibodies levels persist at a stable level for at least 8 weeks after the last saRNA-LNP injection of the first schedule. Furthermore, when the first vaccination schedule was administered at a higher saRNA dose, the pre-existing anti-replicase immunity could also affect the antibody response to subsequent saRNA vaccines, albeit that this effect was largely mitigated following the boost vaccination, suggesting that repeated dosing may overcome some of the inhibitory effects of pre-existing replicase immunity on the humoral response of subsequent saRNA vaccines. These data together with our challenge results, indicate that saRNA vaccines can be used for sequential immunizations against different pathogens, especially for infections where viral clearance is mainly antibody-dependent. However, for applications where Th1 cell mediated immunity is critical, such as cancer vaccines, it is important to consider that an initial saRNA vaccination schedule will negatively impact Th1 cell responses of a subsequent saRNA vaccination schedule.

Methods

Mice

Female BALB/cJRj mice (6–7 weeks old) were purchased from Janvier (France). Heterozygous BALB/c IFN-β reporter female mice (IFN-$\beta^{+/\Delta\beta\text{-luc}}$) (6–7 weeks old) were bred in-house, with the original breeding pairs obtained from the Institute for Laboratory Animal Science, Hannover Medical School (Germany)[40]. All the in vivo experiments were strictly reviewed and approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC2023/026) and the VIB-UGent Institutional Ethical Committee on Experimental Animals (EC2025-035). All intramuscular injections were performed in the quadriceps muscle using 50 µl. Blood samples (maximal 50 µl) were collected from the tail vein under anaesthesia or via cardiac puncture for collecting blood for passive transfer. The mice were euthanized before cardiac puncture or spleen collection. Mice were housed in ventilated cages with high-efficiency particulate air filters and in temperature-controlled (22–24°) and humidity-controlled (40–60%), air-conditioned BSL1 or BSL2 facilities. Mice had access to food and water ad libitum. All animal experiments were conducted according to the Belgian legislation (Belgian Law 14/08/1986 and Belgium Royal Decree 06/04/2010) and European legislation on the protection of animals used for scientific purposes (EU directives 2010/63/EU and 86/609/EEC).

Synthesis and purification of saRNAs and mRNAs

An overview of the saRNAs and modified mRNAs that were designed and used in this work is shown in Supplementary Fig. 1. The 5′ UTR, the 3′ UTR, the non-structural proteins (nsP) 1-4, and the subgenomic promoter in all saRNAs were based on the Venezuelan Equine Encephalitis Virus (VEEV) TC-83 strain (GenBank: L01443.1). In the $saRNA^{Fluc}$, $saRNA^{HA}$, $saRNA^{HA\text{-}RG}$ and $saRNA^{mCherry}$, the subgenomic RNA encoded for respectively firefly luciferase (GenBank: ABA41662.1), hemagglutinin (HA) of A/Anhui/1/2005(H5N1)[28] (GenBank: HM172104), hemagglutinin (HA) of A/Vietnam/1194/2004 (H5N1) (GeneBank: AY651333.1), and mCherry (GenBank: OQ335096.1). The subgenomic gene in the $saRNA^{Rep}$ construct was deleted, resulting in a replicon that retains only the nsP genes, 5′ UTR, subgenomic promoter, and 3′ UTR of VEEV TC-83 strain (GenBank: L01443.1). All saRNAs were designed, produced and validated within our team. Two non-replicating mRNAs encoding either firefly luciferase (GenBank: ABA41662.1) or the VEEV replicase (GenBank: L01443.1) were also designed and produced in our lab. For $mRNA^{Fluc}$ and $mRNA^{Rep}$, we used the 5′ UTR of human α−1-globin and the 3′ UTR of the Pfizer-BioNTech BNT162b2 COVID-19 vaccine (GenBank: OR134577.1). The $mRNA^{mCherry}$ was purchased from Trilink Biotechnologies (San Diego, USA, Cat # L-7203) and contains a CleanCap and the 5-methoxyuridine modification.

The saRNAs were synthesized through in vitro transcription (IVT) as previous described[28]. Briefly, the IVT template was synthesized by PCR using primers (Forward: CAGGGTAATTAATACGACTCACTATAATG Reverse: TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAAATATTAAAAACAAAATCCGATTC), and confirmed by examining the template DNA through agarose gel electrophoresis. The template was purified using silica spin columns (PCR & DNA cleanup kit, T1030S, New England Biolabs). The purified templates were then employed in an IVT reaction (AM1334, Thermo fisher), with co-transcriptional capping using CleanCap technology (CleanCap AU N711410, Trilink BioTechnology, USA), following the manufacturer's instructions. The IVT template for mRNA were also synthesized by PCR using primers (Forward: TAGGTAATACGACTCACTATA Reverse: GCCTTTTGCTCAATTACCCTG), and confirmed by examining the template DNA through agarose gel electrophoresis. The template was purified in the same manner as the saRNA template and employed in an IVT reaction with co-transcriptional capping (CleanCap AG N711310, Trilink) and 50% Pseudouridine (N1019-1, Trilink). The resulting saRNA and mRNA were purified using an RNA cleanup kit (T2050L, New England Biolabs) and subjected to cellulose-based purification to minimize the IFN response induced by dsRNA, as described elsewhere[41]. In brief, cellulose was prewashed with chromatography buffer (10 mM HEPES [pH 7.2], 0.1 mM EDTA, 125 mM NaCl, and 16% ethanol), and the saRNA or mRNA was added to the prewashed cellulose and incubated for 30 min with shaking. The ssRNA was collected by centrifugation with removal of dsRNA that bind to the cellulose. The mRNAs and saRNAs were precipitated using isopropanol, dissolved in RNase-free water, and stored at −80 °C for further use. HA expression of the $saRNA^{HA}$ vaccine was confirmed as previously described[28] by denaturing gel electrophoresis and Western blotting. HA expression of the $saRNA^{HA\text{-}RG}$ was confirmed in Supplementary Fig. 7.

Lipid nanoparticle formulation and characterization

RNAs were formulated in lipid nanoparticles (LNPs) by rapid mixing three volumes of an aqueous solution containing the saRNA or mRNA (in sodium acetate, pH 4.5) with one volume of an ethanolic solution (100% ethanol) containing the ALC-0315 lipid (HY-138170, MedChemExpress, USA), DMG-PEG2K (880151 P, Avanti Polar Lipids, USA), cholesterol (700100 P, Avanti Polar Lipids) and DOPE (850725 P, Avanti Polar Lipids) at a molar ratio of 50: 1.5: 38.5: 10. After formulation, the saRNA-LNPs or mRNA-LNPs were dialyzed using a dialysis cassette (66003, Thermo Fisher) against DPBS (14190144, Thermo Fisher) to remove the ethanol. Size and zeta potential was determined by dynamic light scattering in 20 mM HEPES buffer. The average sizes were between 100 nm to 170 nm and zeta potentials of the saRNA-LNPs were between −5mV to 2 mV and encapsulation efficacy was always >90%.

Animal experiments

Anti-replicase immunity and saRNA expression. To evaluate whether the saRNA vaccine platform results in anti-replicase immunity, mice received two intramuscular injections of 1 μg saRNA^{Fluc}-LNPs 3 weeks apart. Blood samples were collected from the tail vein 3 weeks after the first injection and one week after the second injection to determine anti-replicase antibodies by ELISA. Spleens were harvested 1 week after the second injection to quantify the anti-replicase CD4$^+$ IFN-γ$^+$ and CD8$^+$ IFN-γ$^+$ T cells in splenocytes using intracellular staining and flow cytometry.

Effect of pre-existing replicase immunity generated by a first saRNA exposure on the immunogenicity of a subsequent influenza saRNA vaccine. To investigate whether a first saRNA vaccination schedule could affect the immune responses of a subsequent saRNA vaccination schedule, mice received first two intramuscular injections of 1 μg saRNA^{Fluc}-LNPs or PBS with 3 weeks apart. Subsequently, after 2, 4 or 8 weeks mice were vaccinated with 1 μg saRNA^{HA}-LNPs using a prime-boost schedule with a 3-week interval. Blood samples and spleens were isolated at several time points to study the effect of pre-existing replicase immunity on the anti-HA immune responses. The experimental setup and timelines are shown in Fig. 2a.

Inducing pre-existing anti-replicase immunity with other saRNAs and mRNAs. To elucidate how the source and type of pre-existing anti-replicase immunity affects subsequent saRNA vaccination, BALB/cJRj mice were pretreated with two intramuscular injections, given 3 weeks apart, of either 1 μg saRNA^{Fluc}-LNPs, saRNA^{Rep}-LNPs, mRNA^{Fluc}-LNPs or mRNA^{Rep}-LNPs (Supplementary Fig. 1). A similar experiment was performed in BALB/c IFN-β reporter mice, which were pretreated with 1 μg saRNA^{mCherry}-LNPs, mRNA^{mCherry}-LNPs or mRNA^{Rep}-LNPs. The IFN-β reporter mice allowed us to monitor whether the pretreatments affected the innate immunity of the subsequent saRNA^{HA}-LNP vaccines. Two weeks after the pretreatment the mice were vaccinated with 1 μg saRNA^{HA}-LNPs using a prime-boost schedule with a 3-week interval. Blood samples and spleens were isolated at several time points to study the effect of pre-existing anti-replicase immunity on the anti-HA immune responses. The experimental setup and timeline are shown in Fig. 3a and Supplementary Fig. 4a.

Passive transfer of anti-replicase serum and splenocytes. To elucidate how pre-existing anti-replicase antibodies or T cells affect saRNA vaccine efficacy, passive transfer experiments were performed with either 350 μl PBS, 350 μl anti-replicase serum, 1.4×10^6 T cells or 350 μl anti-replicase serum plus 1.4×10^6 T cells from mice having anti-replicase immunity. Spleens were homogenized using a syringe plunger, and the red blood cells were lysed with ACK lysing buffer (A1049201, Thermo Fisher). Splenocytes were diluted to 2×10^6/ml in RPM-1640 medium with 10% FBS and stimulated with the nsP peptide mix overnight (final concentration 1 μg/ml of each peptide). The next day, T cells were separated by EasySep™ Mouse T Cell Isolation Kit (19851, Stemcell technologies). The passive transfer of serum and T cells was performed via intravenous injection through the tail vein. Successful passive transfer of T cells was confirmed by labeling the T cells with CellTrace™ Far Red Cell Proliferation Kit (C34572, Thermo Fisher Scientific). Subsequently, 1.4×10^6 labeled T cells in 350 μL PBS were injected intravenously into naive mice. Three days post-transfer, recipient mice were euthanized, and the spleen and lymph nodes were isolated to generate single-cell suspensions. These were stained with eBioscience™ Fixable Viability Dye eFluor™ 506 (65-0866-14, Invitrogen) for 30 min at room temperature. Following a washing step, Fc receptors were blocked with Rat anti-mouse CD16/CD32 antibody (553142, BD Biosciences) for 15 min. Cells were then stained with Alexa Fluor® 488-conjugated anti-mouse CD3 antibody (clone 17A2, lot B354286, 100210, BioLegend) and analyzed by flow cytometry. To confirm successful serum transfer, blood samples were taken one day after the serum transfer and the levels of anti-replicase antibodies was measured by ELISA. One day after the transfers, mice were vaccinated with two injections of 1 μg saRNA^{HA}-LNPs given 3 weeks apart. Before the first vaccination, blood samples were collected to measure the passively transferred anti-replicase antibodies. Blood and spleen samples were harvested 1 week after the last saRNA^{HA}-LNP vaccination to readout antibody titers and cellular immune response. A control group that received only PBS at each time point was also included. The experimental setup and timelines are shown in Fig. 4a.

In vivo bioluminescence imaging

Luciferase expression kinetics was measured on several time points after intramuscular injection of saRNA^{Fluc}-LNPs. To that end, mice received a subcutaneous injection of 200 μl D-luciferin (15 mg/ml) and 10 min later in vivo bioluminescence was measured using a Lumina III In Vivo Imaging System (IVIS, Revvity PerkinElmer, USA).

Antibody titers

Serum was obtained from blood samples after clotting and centrifugation. HA-specific, replicase-specific or luciferase-specific antibody levels were determined in the sera using an ELISA assay. In brief, Nunc MaxiSorp plates (44-2404-21, Thermo fisher) were coated overnight at 4 °C with one of the following: 25 ng/well A/Anhui/1/2005 Hemagglutinin HA Protein (11048-V08B, Sino Biological, China) for anti-HA antibodies, 100 ng/well Recombinant Venezuelan equine encephalitis virus Polyprotein P1234 (CSB-EP329710VAZ, Cusabio, USA) for anti-replicase antibodies, or 1 μg/well luciferase (E1702, Promega, USA) for anti-luciferase antibodies. The plates were then washed three times with washing buffer (PBS with 0.05% tween-20) and blocked with assay buffer (DS98200, Thermo fisher) for 1 h at room temperature. After washing, serially diluted mouse sera were added to the plates and incubated at room temperature for 2 h. Subsequently, the plates were washed three time, and were incubated with a HRP-conjugated anti mouse IgG (H+L) (1:10,000 dilution, 31430, Invitrogen) for 1 h at room temperature. The plates were then washed 4–5 times. Next, 100 μL of TMB solution (10445723, Invitrogen) was added to each well. After 15 min incubation, 100 μL of stop solution was added. Absorbance at 450 nm was measured using a Cytation 5 (Agilent Technologies, Santa Clara, USA). Antibody levels were calculated as endpoint titers, defined as the highest dilution of serum that produced an absorbance value above the cut-off. The cut-off was determined as the mean of the control wells plus three times the standard deviation.

Cellular immune responses

Spleens were harvested from the mice 1 week after the first or second saRNA-LNP injection. Subsequently, spleens were homogenized using a syringe plunger, and the red blood cells were lysed with ACK lysing buffer (A1049201, Thermo Fisher). A total of 10^6 splenocytes were stimulated for 16 h with PepMix™ Influenza A (HA/Indonesia (H5N1) (PM-INFA-HAIndo, JPT Peptide Technologies GmbH, Germany) or a customized replicase-targeting peptides mix (Table S1) synthesized by JPT Peptide Technologies GmbH, or with a cell stimulation cocktail as a positive control (00-4970, Invitrogen). Cytokine secretion was inhibited by adding a mixture of Monensin Solution (00-4505-51, Thermo Fisher) and eBioscience™ Brefeldin A Solution (00-4506-51, Thermo Fisher). Next, the splenocytes were incubated with eBioscience Fixable Viability dye eFluor 506 (lot 2696732, 65-0866-14, Invitrogen) for 30 min. Then, after washing,

the Fc receptors were blocked with Rat anti-mouse CD16/CD32 (553142, BD Biosciences, USA) for 15 min. The splenocytes were then incubated with Alexa Fluor® 488 anti-mouse CD3 antibody (clone 17A2, lot B354286, 100210, Biolegend, USA), PerCP/Cyanine5.5 anti-mouse CD4 Antibody (clone RM4-4, lot B374471, 116011, Biolegend), Alexa Fluor® 700 anti-mouse CD8a Antibody (clone 53-6.7, lot B386942, 100729, Biolegend) for 30 min. After fixation and permeabilization using eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (88-8824-00, Invitrogen), the splenocytes were stained with PE anti-mouse IFN-γ Antibody (lone W18272D, lot B358874, 163503, Biolegend). The fluorophores were then measured using a CytoFLEX flow cytometer (Beckman Coulter, USA). Flow cytometry data were analysed using FlowJo v10. The gating strategy is shown in Supplementary Fig. 10.

Hemagglutination inhibition titers

To determine the levels of protective anti-HA antibodies in the collected serum samples, an HI assay was performed as previously described[42]. HA titration of the H5N1 virus particle (07/290, NIBSC, UK) was first performed using a 0.75% turkey blood cell suspension to quantify the virus particles needed for the hemagglutination inhibitor assay. Subsequently, 10 μL of mouse serum was mixed with 30 μL cholera filtrate (c8772, sigma) and incubated overnight at 37 °C. The mixture was then incubated at 56 °C for 30 min to inactivate cholera filtrate. Each well of a 96 V-bottom plate was filled with 25 μL PBS. The first wells of each row received 25 μL of serum mixture, followed by 2-fold serial dilutions across the plate, with 25 μL discarded from the final wells. The lowest dilution factor of the serum was 8. Next, 25 μL of the inactivated strain matched H5N1 virus, containing 4 HA units, was added to each well. The plate was gently tapped and incubated for 30 min at room temperature. Finally, 50 μL of 0.75% turkey blood cell suspension was added to each well and incubate for 30 min. The plate was then tilted vertically for 25 s before reading out. After obtaining the results, HI titers were subsequently analyzed using GraphPad Prism (version 10.1.10), and the "geometric mean with geometric SD" was selected for the analysis.

Challenge experiment

The H5N1 influenza A virus NIBRG-14 was obtained from the UK National Institute for Biological Standards and Control. NIBRG-14 is a reassortant generated by reverse genetics (RG) from A/Vietnam/1194/2004 (H5N1) virus, in which the polybasic HA cleavage site has been excised, and A/PR/8/34 (H1N1) virus. In contrast to A/Anhui/1/2005(H5N1), NIBRG-14 causes lethal infections in mice[43]. Specific-pathogen-free female BALB/c mice (6 weeks old) were purchased from Janvier (France) and pre-exposed to saRNA^{Fluc}-LNPs to induce anti-replicase immunity. Subsequently, these mice were vaccinated with saRNA$^{HA\text{-}RG}$-LNPs as summarized in Fig. 6a. A non-pretreated and non-vaccinated control group received only PBS. Two weeks after the final saRNA$^{HA\text{-}RG}$ vaccination or PBS injection, mice were challenged intranasally with 5 LD_{50} of NIBRG-14 diluted in 50 μl PBS. This volume was equally divided over both nostrils. HI titers were determined using blood collected from a tail vein 2 weeks after the final saRNA$^{HA\text{-}RG}$ vaccine (just before challenge). The humane endpoint for euthanasia was defined as ≥25% body weight loss relative to the day of challenge. To determine the HI titers in these mice a slightly different HI assay was used. Briefly, 10 μL serum was mixed with 40 μL of receptor-destroying enzyme (RDE) and incubated overnight at 4 °C. The next day, 50 μL of 1.5% sodium citrate was added to the mixture. Then, the mixture was heat-inactivated at 56 °C for 30 min to inactivate residual RDE activity. For the HI assay, 25 μL of the treated serum was mixed with 25 μL PBS in the first well, resulting in a starting serum dilution of 1:20. The rest of the procedure was similar to the HI assay described above.

Data collection and analysis

CytExpert 2.5 (Beckman Coulter) was used for collecting the flow cytometry data. Image Lab 6.1 (Bio-Rad) was applied for collecting Western blot data. Gen5 3.12 (Agilent) was used to read out the ELISA results. The living image 4.7.2 (64-bit) (Revvity) software was applied for reading out and analysing in vivo bioluminescence data. Flowjo 10.8.1 was used to analyse the flow cytometry data.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 10.1.10). Effect size Cohen's *d* was computed as the difference in group means divided by the pooled SD. The antibody endpoint titers were represented as geometric means and other data were represented as arithmetic means. Error bars represent the standard deviation (SD). Detailed information on the statistical analyses can be found in the figure legends. All samples were obtained from distinct sources unless otherwise stated.

Reporting summary

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

Data availability

All results are shown in the main manuscript or in Supplementary Data 1, while all raw data are available in the Source Data file. All RNA sequences used in this study can be found in the supplementary information file. Source data are provided with this paper.

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Author contributions

X.C., Z.Z., and N.N.S. designed the experiments. X.C. performed the experiments and wrote the first drafts of the manuscript. Z.Z. and N.N.S. corrected the manuscript. O.G., Y.G., S.L., and Q.S. helped with the in vivo experiments. J.P.P.C., L.A., and X.S. performed the challenge experiment. E.D.S., P.V., and J.S. contributed to the methodology. N.N.S. conceived the project and supervised together with Z.Z. all aspects of the research.

Competing interests

N.N.S. is the inventor of 2 granted patents on mRNA carriers (WO2021122871A1 and WO2022207938A1), 1 patent on a saRNA SARS-CoV-2 vaccine (WO2021255270A1) and 2 pending patents, one on saRNAs and one on (sa)-mRNA vaccination strategies. The remaining authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Zifu Zhong or Niek N. Sanders.

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