Pre-exposure to mRNA-LNPs reprograms adaptive and innate immune responses in an inheritable fashion

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ABSTRACT

Billions of SARS-CoV-2 mRNA-LNP vaccine doses have already been administered to humans. However, we lack a comprehensive understanding of the immune effects of this platform. Here we bring experimental evidence that pre-exposure to mRNA-LNPs or its LNP component has effects on both innate and adaptive immune responses. Pre-exposure to mRNA-LNPs led to long-term, platform-specific inhibition of the adaptive immune responses. As such, mice responded with lower antibody responses when exposed the second time to the mRNA-LNP platform, but with undisturbed adaptive immune responses to protein-based Alum or AddaVax-adjuvanted vaccines. On the other hand, we report that after pre-exposure to mRNA-LNPs, resistance of mice to heterologous infections with influenza virus and *Candida albicans* is likely enhanced. Interestingly, mice pre-exposed to mRNA-LNPs can pass down the acquired immune traits to their offspring, providing better protection. In summary, the mRNA-LNP vaccine platform induces long-term immunological changes that can affect both humoral responses and heterologous protection against infections. More studies are needed to understand in-depth the mechanisms responsible for these effects.
INTRODUCTION

The mRNA-LNP vaccine platform gained much attention with the ongoing SARS-CoV-2 pandemic. Initially, this vaccine platform was thought to be non-inflammatory since the mRNA has been modified and purified to limit innate immune activation (Karikó et al., 2005, 2008, 2011). At the same time, the lipid nanoparticle (LNP) component was considered an inert carrier and protector of the mRNA. However, recently has been shown that the synthetic ionizable lipid component of the LNPs is highly inflammatory (Ndeupen et al., 2021), and this is a critical component to support induction of adaptive immune responses. These LNPs mixed with proteins induce comparable responses to mRNA-LNPs (Alameh et al., 2021).

The acute side effects reported with the mRNA-LNP vaccine platform are diverse and likely associated with its highly inflammatory nature and partially mediated by innate immune responses (Igyártó et al., 2021; Ndeupen et al., 2021). In addition to the induction of specific T- and B-cell activation, certain vaccines or infections can affect long-term innate immune responses by either increasing or decreasing the activation of innate immune cells (Netea et al., 2020). Furthermore, the innate immune reprogramming induced by certain vaccines can interfere with the immune responses induced by other vaccines (Netea et al., 2020). The possible short and long-term immunological changes of the mRNA-LNP vaccine outside the induction of antigen-specific anti-SARS-CoV-2 responses are unknown. A recent human study awaiting peer-review reported innate and adaptive immune reprogramming with this platform (Föhse et al., 2021), while single-cell RNA-seq studies on human white blood cells derived from vaccinated people also revealed significant changes in innate immune
cells (Arunachalam et al., 2021). Whether the reported changes are long-lasting and can influence immune fitness or interfere with the responses induced by other vaccines remains to be determined.

Here, using an mRNA-LNP animal vaccination model, we show that pre-exposure to mRNA-LNP inhibits antibody responses. The inhibition was specific to the mRNA-LNP platform and did not interfere with protein vaccines efficacy. At the same time however, this vaccine platform enhances innate immune fitness, and this can even be passed down to the offspring.

RESULTS

**Pre-exposure to LNPs or mRNA-LNPs inhibit adaptive immune responses**

The LNPs used in preclinical animal studies are highly inflammatory (Ndeupen et al., 2021). The critical inflammatory component of the LNPs is the synthetic ionizable lipid, which for the Pfizer SARS-CoV-2 vaccine has been estimated to have a 20–30-day in vivo half-life (Comirnaty, 2021). The LNPs used for preclinical studies and the Pfizer vaccine are similar and produced by Acuitas Therapeutics (Ndeupen et al., 2021, 2022). The immune system under chronic stimulation often responds with exhaustion and non-responsiveness (Wherry and Kurachi, 2015). Since the mRNA-LNP platform is highly inflammatory and has a long in vivo half-life, we sought to test whether pre-exposure to this platform affects subsequent adaptive immune responses. We used an intradermal immunization model developed in our laboratory (Ndeupen et al., 2022) to test this. Adult WT mice were exposed to PBS, 2.5 μg of mRNA-LNPs coding for eGFP, or 2.5 μg empty LNPs intradermally, as shown in **Figure 1A**. Two weeks later, the mice were injected in the same area with 2.5 μg of mRNA-LNP coding for PR8 influenza HA. Two
weeks post-inoculation, the anti-HA responses were determined in the serum using ELISA and the GC B cell responses in the skin draining lymph nodes monitored by flow cytometry (Supplemental Figure 1), as we previously described (Ndeupen et al., 2022). We found that pre-exposure to mRNA coding for an irrelevant protein (eGFP) or empty LNPs significantly decreased the anti-HA responses, both on antibody and GC B cell levels (Figure 1B and C). We found no difference between mRNA-LNP and empty LNP groups (Figure 1B and C). Thus, these data suggest that pre-exposure to this platform can inhibit subsequent adaptive immune responses and that the LNPs play a critical role in this.

The inhibition of adaptive immune responses by mRNA-LNPs is systemic, but more pronounced at the site of injection

Humans often receive follow-up and booster shots in the same arm with similar locations (deltoid muscle). In mice, the adaptive immune responses with the mRNA-LNP platform are primarily localized to the lymph node draining the injection site (Ndeupen et al., 2022). Thus, we next sought to determine whether the inhibition we observed is localized or systemic. To define this, we performed similar experiments as detailed above, but one group of mice received the second shot in an area distinct from the first inoculation site (Figure 2A). We found that the mice injected in a different location still showed a significant decrease in anti-HA responses compared to PBS pretreatment (Figure 2B and C). However, the inhibition was significantly less pronounced than in mice injected into the same area (Figure 2B and C). Thus, pre-exposure to LNPs shows injection-site dominance with systemic traits.
Duration of the inhibitory effects of mRNA-LNPs on the adaptive immune responses

From a human health perspective, defining the length of the inhibitory effects caused by exposure to LNPs is critical to minimize its impact and devise preventive measures. To determine how long the inhibition lasted, WT mice were exposed to either PBS or mRNA-LNPs, and at 2, 4, or 8 weeks later injected into the same area with mRNA-LNPs coding for influenza HA as presented above (Figure 3A). Two weeks post-inoculation, we found that even mice injected four weeks post pre-exposure showed a significant decrease of anti-HA responses (Figure 3B and C). The downward trend was still present at week eight, but with the limited dataset, it did not reach significance for anti-HA responses (Figure 3B and C). Thus, inhibition of the adaptive immune responses by pre-exposure to mRNA-LNPs is long-lasting but it is likely to wane with time.

The inhibition of adaptive immune responses by mRNA-LNPs is platform-specific

We observed that the LNPs significantly inhibited the immune responses triggered by the mRNA-LNP platform. However, it is crucial to determine whether this inhibition affects adaptive immune responses triggered by other vaccines and alters immune protection. To determine the spectrum of the inhibition the PBS and eGFP-coding mRNA-LNP pre-exposed mice were injected either with mRNA-LNPs coding for influenza PR8 HA or PR8 HA protein mixed with Alum or AddaVax (Figure 4A). We found that pre-exposure to mRNA-LNP did not inhibit the adaptive anti-HA responses induced by Alum and AddaVax (Figure 4B and C). Thus, these preliminary data suggest that the inhibition by LNPs is likely specific to the mRNA-LNP platform and does not interfere with protein-based adjuvanted vaccines.
Since the protein-based vaccine’s efficacy was not affected by pre-exposure to mRNA-LNP, we hypothesized that a possible mechanism of inhibition might lie with mRNA degradation, translation, etc., limiting the production of the antigen coded by the mRNA. The decreased amount of antigen could lead to lower overall adaptive immune responses. To test whether pre-exposure to mRNA-LNP leads to decreased antigen production, animals exposed to PBS, mRNA-LNP coding for luciferase (Luc) or PR8 HA were injected two weeks later with Luc mRNA-LNPs and imaged using IVIS daily for 7 days (Supplemental Figure 2A). We found that pre-exposure to Luc or PR8 HA mRNA-LNP significantly decreased the signals in comparison to PBS or no-exposure controls (Supplemental Figure 2B-D). Thus, these data suggest that pre-exposure to mRNA-LNPs might affect antigen levels by regulating mRNA half-life or translation.

Pre-exposure to mRNA-LNPs enhances resistance to heterologous infections

Innate immune cells are sensitive to inflammatory signals and respond with epigenetic modifications that promote or suppress the subsequent innate immune responses (Netea et al., 2020). Subsequently, if such effects are induced by certain vaccines, they can impact susceptibility to heterologous infections. To assess whether pre-exposure to mRNA-LNPs affects susceptibility to heterologous infections, we exposed mice to PBS or mRNA-LNP coding for eGFP. Two weeks later, we inoculated the mice with either a sublethal dose of influenza intranasally or Candida albicans intravenously. Disease progression was monitored by taking the weight of the mice daily (Figure 5A). We found that mice pre-exposed to mRNA-LNP showed significantly higher resistance to Candida infection and lost less weight than the PBS-exposed mice (Figure 5B). We also found significantly less Candida fungal burden in the mRNA-LNP-pre-exposed
mouse kidneys (Figure 5C), which are the target organ in this mouse model of disseminated candidiasis. No differences in fungal loads were observed in the liver (Figure 5D). Similar enhanced resistance in an influenza infection model was observed after administration of mRNA-LNP (Figure 5E). Thus, these preliminary data suggest that pre-exposure to mRNA-LNPs might increase innate immune activation and immune fitness.

**Immune changes induced by pre-exposure to mRNA-LNP can be inherited**

Transmission of immune traits to the next generation in vertebrates has been recently reported (Bomans et al., 2018; Lim et al., 2021; Rokade et al., 2021). In humans, lower overall mortality has been reported in infants whose fathers had been vaccinated with BCG (Berendsen et al., 2021), and maternal SARS-CoV-2 infection has been associated with increased cytokine functionality and nonspecific immune imprinting in neonates (Gee et al., 2021). Finally, trained immunity has been shown to be transmitted in newborn infants of hepatitis B virus-infected mothers (Hong et al., 2015). Since the mRNA-LNP platform is highly inflammatory, and we observed that pre-exposure to it enhances resistance to heterologous infections, we hypothesized that some of these traits might be inherited by the offspring. To test our hypothesis, we immunized adult WT B6 male and female mice intradermally with 10 μg of mRNA-LNP coding for influenza PR8 HA, as we previously described (Ndeupen et al., 2022). Two weeks post-immunization, the mice were screened for successful immunization by anti-HA ELISA (Supplemental Figure 3A-B) and then mated (Figure 6A) as follows: immunized males with unimmunized (DI; dad immunized) or immunized females (DMI; dad-mom immunized), and immunized females with unimmunized males (MI; mom immunized).
Non-immunized males mated to non-immunized females served as controls (DMN; dad-mom non-immunized). Offspring from 1\textsuperscript{st}, 2\textsuperscript{nd}, and 4\textsuperscript{th} litters at eight-ten weeks of age were intranasally inoculated with a sublethal dose of PR8 influenza and weight monitored for 14 days (Figure 6A). We found that mice from the 1\textsuperscript{st} litter from the DI group showed significantly better resistance to influenza infection and lost less weight than the litters derived from naïve parents (Figure 6B). Mice from MI or DMI groups showed complete protection from weight drop (Figure 6B), which was likely in large mediated by passive immunity provided by the maternal anti-HA antibodies (Supplemental Figure 3C). The second litters derived from the DI group were no longer different from those of non-immunized parents (Figure 6B). MI litters showed a significant drop in protection, but still above the litters from DMN parents (Figure 6B). Interestingly, litters from DMI still showed complete protection from weight loss (Figure 6B). With the 4\textsuperscript{th} litters, the DI and DMN mice remained similar, while the MI and DMI mice were comparable but still significantly protected compared to DMN litters (Figure 6B). Thus, these data all together support that the immune changes induced by the mRNA-LNP vaccine in parents can be passed down to the offspring, and both male and female mice play an important role in this transmission.

DISCUSSION

The new anti-COVID-19 mRNA vaccines’ immunological effects beyond inducing certain protection against SARS-CoV-2 infection are poorly understood. Based on our earlier studies demonstrating the pro-inflammatory properties of the LNP platform used in these vaccines, we report that pre-exposure to the mRNA-LNP platform has long-
term impacts on both innate and adaptive immune responses, with some of these traits being even inherited by the offspring.

The first aim of our study was to assess whether a previous exposure to mRNA-LNPs influences the response to secondary vaccination. Interestingly, we found that indeed the antibody response was inhibited after an earlier administration of mRNA-LNPs. This inhibition of adaptive immune responses was relatively long-lasting, with effects seen for at least 4 weeks, while starting to wane after 8 weeks. Humans receive a 2-dose standard regimen of mRNA-LNP vaccines at 3 to 4 weeks intervals (Walsh et al., 2020) and booster shots at different time points. Our data are strongly supported by recent studies showing that a delay of the second dose of an mRNA vaccine from 3 weeks to 3 months significantly improves the antibody response (Hall et al., 2022; Ooi and B, 2022; Parry et al., 2022). Indeed, inflammation has been related to a poor responsiveness to vaccination in earlier studies (Trzonkowski et al., 2003), and it is rational to hypothesize that the acute inflammatory side effects of the LNP platform negatively impedes induction of antibody responses during the second dose administration. Increasing the interval between vaccination doses, thus giving time to the immune system to return to homeostasis, is likely to improve the effects of the second dose of the vaccine. Thus, our findings have important implications for improving the schedules of administration for the current mRNA vaccines. However, more studies are needed in the future to assess these effects in more detail.

Whether multiple pre-exposures lead to an even more drastic inhibition of the adaptive immune responses and how much overlap there is between mouse and human data remains to be determined. The inhibition of the adaptive immune responses was more
pronounced if the second shot was delivered into the site of pre-exposure. This is in concordance with our recent data showing that the adaptive immune responses with the mRNA-LNP platform are mounted in the lymph node(s), draining the injection site (Ndeupen et al., 2022), while largely sparing the other secondary lymphoid organs. These data suggest that the inhibition of adaptive immune responses by the mRNA-LNP could be partially mitigated if the follow-up shots are delivered far away from the first injection sites. Interestingly, our data support that the inhibition of the adaptive immune responses might be limited to the mRNA-LNP platform. This is a highly relevant finding from a human health perspective. The lack of interference with protein/subunit vaccines is reassuring that their efficacy will not be hindered by the pre-exposure to the mRNA-LNP platform. Indeed, it has been also shown that combination of mRNA vaccines with adenovirus-based anti-SARS-CoV-2 vaccines may actually even improve the serological responses and protection (Atmar et al., 2022; Liu et al., 2021; Spencer et al., 2021). However, whether the effectiveness of live attenuated viral vaccines, such as influenza is affected, remains to be determined.

Since the protein-based vaccine's efficacy was not affected by pre-exposure to mRNA-LNP, a possible mechanism of inhibition might lie with mRNA degradation, translation, etc., limiting the production of the antigen coded by the mRNA. The decreased amount of antigen could lead to lower overall adaptive immune responses. Our recent preliminary data support this hypothesis (Supplemental Figure 2). If further studies confirm that pre-exposure to mRNA-LNP indeed inhibit subsequent adaptive immune responses through regulation of mRNA degradation and translation, that could indicate that the nucleoside modification step of the mRNA might be less important. The
nucleoside modification and removal of double stranded RNAs were aimed to lower innate immune recognition, activation, and IFNα secretion to decrease side effects and prolong the mRNAs’ half-life (Karikó et al., 2005, 2008, 2011). However, our preliminary data indicate that the LNP component likely through its highly inflammatory nature might at least partially counteract the benefit of nucleoside-modification and RNA purification.

The effects of the mRNA-LNP platform reported here on the innate immune responses is in line with recently published human data generated three weeks after the first shot, in which PBMCs isolated from individuals vaccinated with one dose of the anti-COVID-19 Pfizer-BioNTech mRNA vaccine responded with significantly higher production of TNFα and IL-1β after stimulation with Candida albicans compared with the response before vaccination (Föhse et al., 2021). However, stimulation of cells with other stimuli such as influenza and different TLR ligands showed no significant difference before and after 1st vaccination, and these effects seem to be lost after subsequent doses (Föhse et al., 2021). If the potential innate immune fitness benefits from the first exposure to mRNA-LNPs reported here for mice and supported by human in vitro restimulation data might be dampened by the subsequent shots, then we would expect that people exposed to multiple mRNA-LNP shots ultimately become more vulnerable to infections. However, a recent RNA-seq analyses of blood immune cells after the booster shot revealed enhanced innate immune signatures (Arunachalam et al., 2021) pointing towards a possible overall better innate immune fitness. Further research will be needed to establish the long-term effect of multiple mRNA-LNP shots on immune fitness of humans and mice.
In humans, many vaccines, including the mRNA-LNP vaccines are administered intramuscularly for ease of use (Ols et al., 2020). The preclinical animal intradermal and intramuscular vaccine studies revealed induction of similar adaptive immune responses, with slightly better responses with the intradermal delivery (Laczkó et al., 2020; Pardi et al., 2018a). The inflammatory reactions in nature and magnitude induced by the mRNA-LNP platform are independent of the delivery route (Ndeupen et al., 2021). They are characterized by robust and transient neutrophil influx and activation of multiple different inflammatory pathways and the production of inflammatory cytokines such as IL-1β and IL-6. Thus, it is unlikely that the immune reprogramming observed here would be limited to intradermal exposure, especially since data from mRNA-LNP-vaccinated individuals collaborate our findings (Föhse et al., 2021). The vaccine doses used in mouse and human studies differ significantly, and human vaccines doses adjusted to weight are much lower than in mice (Nair and Jacob, 2016). In our studies, we used doses at the lower end of the amounts used for mouse vaccine studies (Laczkó et al., 2020). While we did not perform a wide dose range for our studies, we observed that pre-exposure to 2.5 μg of empty LNPs and 2.5 μg mRNA-LNPs (2.5 μg refers to the amount of mRNA, which is complexed with LNPs at ~1:20 weight ratio, i.e., ~50 μg of LNP) resulted in similar inhibition of adaptive immune responses. Thus, while dose might play a role in the magnitude and length of inhibition, our data combined with human data support that the LNPs have similar effects in a wide dose range.

A very interesting observation was that the improved heterologous protection induced by mRNA-LNPs on influenza infection was successfully passed down to the offspring. A number of recent studies reported evidence for transmission of either trained immunity
or tolerance across generations in mice (Bomans et al., 2018; Katzmarski et al., 2021), although not all of them (Kaufmann et al., 2022). Our independent study initiated and performed before these data become public seem to support the existence of transgenerational inheritance of immune traits. The highly inflammatory properties of the mRNA-LNP platform might have induced the inherited changes, and it would be very important to determine whether any such immune inheritance may be observed in humans vaccinated with mRNA vaccines. Cross-generational protection of infants of parents vaccinated with BCG has recently suggested in epidemiological studies (Berendsen et al., 2021). Whether these changes in innate immune genes could also directly or indirectly affect adaptive immune responses remains to be determined. Our experimental platform was able to detect the innate contribution of males. The passively transferred maternal antibodies largely masked the innate female contribution. Nevertheless, we observed that the 2\textsuperscript{nd} litters from the DMI group outperformed the MI ones. These data suggest that innate immune traits inherited from the father could provide essential advantages in protection even in the presence of maternal-derived antibodies. However, since the 2\textsuperscript{nd} litters from the DI group were no better than the litters from DMN, but the DMI litters outperformed the MI litters, these data suggest that an immune female counterpart might further boost the benefits provided by the immune traits inherited from males. Nevertheless, the overall protection levels fell across the board with later litters (data not shown), suggesting that such heterologous effects do not persist for the entire life of an animal.

In conclusion, we describe important immunological properties of the mRNA-LNP platform used in mRNA vaccines against COVID-19. These findings have important
biological and clinical implications. First, our data show that the administration of mRNA-LNPs inhibits humoral responses to a second dose of the vaccine for at least several weeks: this finding is highly relevant from a human health perspective as it suggests that a second dose of a mRNA vaccine may be more effective if given at a later timepoint and different location than currently used. This conclusion is strongly supported by recent human studies suggesting that indeed the delay of the second dose of the Pfizer/BioNTech vaccine leads to a better humoral and cellular response (Hall et al., 2022; Parry et al., 2022). Second, our study suggest increased heterologous protection against fungal and viral infections by the mRNA-LNPs platform, that may thus expand its beneficial effects. Third, our study also shows the capacity of these vaccines to transmit protection trans-generationally, thus supporting the concept of Lamarckian inheritance in mammals (Skinner and Nilsson, 2021). However, our study only partially opens the door towards understanding the various immunological effects of mRNA-LNP platform. Considering the broad exposure of a large proportion of human populations to vaccines based on this novel technology, more studies are warranted to fully understand its broad immunological and physiological effects.

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AUTHOR CONTRIBUTION

Z.Q. performed all the experiments and analyzed the data. B.Z.I. conceptualized the study, interpreted the data, and wrote the manuscript.

DECLARATION OF INTERESTS

Authors declare no conflict of any sort.

FIGURE LEGENDS

Figure 1. Pre-exposure to mRNA-LNPs or LNPs significantly inhibits subsequent adaptive immune responses induced by the mRNA-LNP vaccine. A). Experimental model. Animals were shaved and intradermally inoculated in the left upper spot with either PBS, mRNA-LNP coding for eGFP (eGFP) or empty LNP (eLNP). Two weeks later the same areas were injected with PR8 HA mRNA-LNP (HA). Serum and skin draining lymph nodes were harvested 2 weeks later and the anti-HA serum antibody levels determined using ELISA and the germinal center (GC) B cell responses using flow cytometer (please see Materials and Methods for details on data normalization). B). Serum anti-HA antibody levels detected by ELISA. OD450 readings (top) at different serum dilutions. Summary graph of the relative area under the curve (AUC) for each sample (middle). C). GC B cell responses (CD38− GL7+) from the same mice. Each dot represents a separate mouse. Data from at least two separate experiments pooled and
are shown as mean ±SD. One-way ANOVA was used to establish significance. ns = not significant. **p<0.005, ***p<0.0005, ****p<0.0001.

**Figure 2. Pre-exposure to mRNA-LNPs has systemic effects. A).** Experimental model. As in Figure 1, but one of the groups was injected at a spot distinct from the primary exposure. B). ELISA OD450 readings and summary AUC graph on serum anti-HA antibody levels. C). Relative GC B cell responses from the same mice. Each dot represents a separate mouse. Data from at least two separate experiments pooled and are shown as mean ±SD. One-way ANOVA was used to establish significance. ns = not significant. *p<0.05, **p<0.01, ****p<0.0001.

**Figure 3. Pre-exposure to mRNA-LNPs has long-lasting effect on adaptive immune responses. A).** Experimental model. Animals at the indicated timepoints post-exposure to PBS or eGFP mRNA-LNP were injected into the same spot with PR8 HA mRNA-LNP and then anti-HA responses assessed as depicted two weeks later. B). Summary AUC graph on serum anti-HA antibody levels. C). Relative GC B cell responses from the same mice. Each dot represents a separate mouse. Data from at least two separate experiments pooled and are shown as mean ±SD, except for weeks 4 and 8 where data is from one experiment. One-way ANOVA was used to establish significance. ns = not significant. *p<0.05, ****p<0.0001.

**Figure 4. The inhibition of adaptive immune responses is specific to the mRNA-LNP platform. A).** Experimental model. Two weeks post-exposure to PBS or eGFP mRNA-LNP the animals were injected into the same spot with PR8 HA mRNA-LNP, or HA protein mixed with Alum or AddaVax. The anti-HA responses were assessed as depicted two weeks later. B). Summary AUC graph on serum anti-HA antibody levels.
C). Relative GC B cell responses from the same mice. Each dot represents a separate mouse. Data from at least two separate experiments pooled and are shown as mean ±SD, except for HA mixed with Alum and AddaVax where data is from one experiment. Welch’s t test was used to establish significance. ns = not significant. **p<0.005, ****p<0.0001.

Figure 5. Innate immune fitness increases with pre-exposure to mRNA-LNP. A). Experimental model. Two weeks post-exposure to PBS or eGFP mRNA-LNP the animals were infected with sublethal doses of Candida albicans or PR8 HA influenza. The weights and CFUs were monitored as depicted. B). Percent of body weight drop after Candida infection, and the corresponding AUC changes. C). Candida CFU numbers in the kidneys. D). Percent of body weight drop after PR8 HA influenza infection, and the corresponding AUC changes. Each dot represents a separate mouse. The data are from one experiment. Student’s t-test was used to establish significance. ns = not significant. *p<0.05.

Figure 6. Immune changes induced by the mRNA-LNP platform can be inherited. A). Experimental model. Adult WT B6 male and female mice were intradermally immunized with 10 μg of mRNA-LNP coding for influenza PR8 HA. Two weeks post-immunization the mice were mated. Non-immunized males mated to non-immunized females served as controls (DMN; dad-mom non-immunized). Immunized males with unimmunized (DI; dad immunized) or immunized females (DMI; dad-mom immunized), and immunized females with unimmunized males (MI; mom immunized). Offspring from 1st, 2nd, and 4th litters at eight-ten weeks of age were intranasally inoculated with a sublethal dose of PR8 influenza and weight monitored for 14 days. B). Percent of body
weight drop after influenza infection (upper), and the corresponding AUC changes (lower). The data are from one experiment using litters from 3 separate mating. The number of mice (female/male) from 1\textsuperscript{st} litters used for influenza challenge was 6/7 (DMN), 8/6 (DI), 3/9 (MI) and 8/7 (DMI); for the 2\textsuperscript{nd} litters 1/3 (DMN), 6/9 (DI), 7/5 (MI) and 6/2 (DMI); for the 4\textsuperscript{th} litters 2/9 (DMN), 8/13 (DI), 9/7 (MI) and 7/8 (DMI). One way ANOVA was used to establish significance. ns = not significant. ***p<0.0005, ****p<0.0001.

**Supplemental Figure 1. Gating strategy for HA-specific GC B cells**

**Supplemental Figure 2. Pre-exposure to mRNA-LNPs decreases antigen levels.**

\textbf{A).} Experimental model. Balb/c mice were pre-exposed to PBS, Luc mRNA-LNPs or PR8 HA mRNA-LNPs and imaged using IVIS 6 hours (0.25 day) post inoculation and then every day for 7 days. Two weeks later all the animals were injected in the same spot with Luc mRNA-LNPs and the luciferase signal monitored similarly to the first exposure. \textbf{B).} Relative total flux with time. \textbf{C).} Data from \textbf{B} presented as AUC. \textbf{D).} Total flux values (background subtracted) of each mouse at different time points are shown as log\textsubscript{10}. X marks mice where the signal was below detection. Data from two separate experiments pooled. One way ANOVA was used to establish significance. ns = not significant. ***p<0.0005, ****p<0.0001.

**Supplemental Figure 3. Anti-HA antibody levels in parents and litters.** \textbf{A).} Anti-HA antibody levels in parents 2 weeks post inoculation. \textbf{B).} Anti-HA antibody levels in parents 2 weeks and 28 weeks post inoculation. \textbf{C).} Anti-HA antibody levels in the 1\textsuperscript{st} litters prior- and 4 weeks post-infection.

**MATERIALS AND METHODS**
Ethics statement

Institutional Care and Use Committee at Thomas Jefferson University approved all mouse protocols. Protocol number: 02315.

Mice

WT C57BL/6J and Balb/c mice were purchased from Jax and bred in house. Balb/c mice were only used for the IVIS experiments. All experiments were performed with 8–12 weeks old female and male mice. Mice were housed in microisolator cages and fed autoclaved food.

mRNA-LNPs

For our studies, we used an LNP formulation proprietary to Acuitas Therapeutics described in US patent US10,221,127. These LNPs were previously carefully characterized and widely tested in preclinical vaccine studies in combination with nucleoside-modified mRNAs (Pardi et al., 2018b, 2018a). The following, previously published mRNA-LNP formulations were used: PR8 HA mRNA-LNP, eGFP mRNA-LNP and empty LNPs.

Infectious agents

A/Puerto Rico/8/1934 influenza stock was a generous gift from Dr. Scott Hensley (University of Pennsylvania). Wild type *C. albicans* used in this study was previously described (Igyártó et al., 2011). The work with the infectious agents was performed in a BSL2 laboratory and approved by the Institutional Biosafety Committee.

Intradermal immunization
Intradermal immunizations were performed as we previously described (Ndeupen et al., 2021, 2022). Briefly, the hair of the site of injections was wet shaved using Personna razor blades. The mice were then injected intradermally (upper left area on the back) with 2.5 μg/spot mRNA-LNPs or empty LNPs in 20 μl PBS or equivalent volume of PBS as 1st shot. The 2nd shot of 2.5 μg/spot mRNA-LNPs was administered 2, 4 or 8 weeks after the 1st shot at either the same or opposite site on the upper back area. To test for platform specificity, 5 μg HA protein combined with either Alum (InvivoGen) or AddaVax (InvivoGen) at 1:1 (v/v) ratio was intradermally injected at the same site two weeks after the 1st mRNA-LNP shot. For the inherited immunity experiment the parents were injected with 10 μg (2.5 μg/spot; 4 spots) of mRNA-LNP coding for PR8 HA or with the corresponding volume of PBS (Ndeupen et al., 2021, 2022).

**Recombinant hemagglutinin (HA) protein labeling**

Recombinant HA protein (rHA) was a kind gift from Drs. Barney Graham and Masaru Kanekiyo at NIH. Molecular Probes™ Alexa Fluor™ 647 Protein Labeling kit (A20173, Invitrogen) was used to label rHA following the product user guide.

**Characterization of B cell responses**

At day 14 post-injections, the mice were sacrificed and the skin draining lymph nodes (axillary and brachial) harvested. Single-cell suspensions were generated using mechanical disruption through cell strainers. The cells were stained with B cell panel consist of dump (fixable viability dye, F4/80, CD11b), CD38 (90), B220 (RA3-6B2), CD138 (281–2), GL-7 (GL-7), Sca-1 (D7), IgD (11-26c.2a), IgM (RMM-1) and AF647-labeled rHA. A gating strategy previously published ([Supplemental Figure 1](#)) was used to define GC B cell populations (Ndeupen et al., 2022). The GC B cell percentages were
normalized between experiments and are shown as relative values. The normalization was performed as follows. The mean GC % of all the samples from one experiment was used to divide each sample GC % value (relative value). All antibodies were purchased from BD Biosciences, Biolegend or Tonbo Biosciences. The stained samples were run on Fortessa (BD Biosciences) and the resulting data analyzed with FlowJo 10.

**In vivo challenge with PR8 influenza or Candida albicans**

The doses of influenza or *C. albicans* used in these studies were previously described (Kashem et al., 2015; Katzmarski et al., 2021; Willis et al., 2020). For viral infection, mice were anesthetized by intraperitoneal injection with a mixture of Xylazine/Ketamine and inoculated intranasally with 200 TCID$_{50}$ PR8 influenza virus. For fungal challenge, mice were intravenously injected with 3~4x10$^5$ CFU *C. albicans*. Subsequently the mice were monitored daily for distress and weight loss. The weight loss data are presented as percent of original body weight.

**Quantification of fungal burden**

On day 3 post *C. albicans* challenge, mice were sacrificed and liver and kidneys harvested. The organs were then homogenized in PBS, and the homogenates were diluted and used to inoculate YPAD agar plates. After overnight incubation at 30°C, the colonies were manually counted, and the CFU/ml organ calculated.

**ELISA**

Nunc Immuno 96 well plates (Fisher Scientific) were coated with 1μg/ml (50 μl/well) HA protein (Sino Biological) diluted in carbonate/bicarbonate buffer (Fisher Scientific) overnight at 4°C or 1 hour at 37 °C. After washing and blocking with TBS for 1 hour the
serum samples were diluted and added to the plate. Serially diluted HA-specific monoclonal antibody (Sino Biological) served as standard. Anti-mouse IgG-HRP (1:20,000; Fisher Scientific) in combination with TMB (Fisher Scientific) solution was used for detection. The signals were read at 450 nm using accuSkan FC microplate photometer (Fisher Scientific).

**In vivo bioluminescence imaging**

The imaging was performed with IVIS Lumina XR system (Caliper Life Sciences). To limit possible interference of melanin in B6 mouse skin with the signal generated by the luciferase activity, we used WT Balb/c mice for these experiments. Mice injected with PBS, mRNA-LNP coding for luciferase or PR8 HA, were intraperitoneally injected with D-Luciferin (Potassium Salt, Goldbio) at the dose of 150 mg/kg. Five minutes later, the mice were anesthetized in a chamber filled with 3 % isoflurane for 1 minute, then transferred to the imaging platform with maintained 2 % isoflurane via gas ports. With the Living Image Software provided by Caliper, the signal was acquired by measuring total flux (photons/sec) for 5 seconds exposure time. The total flux is the radiance (photons/sec/cm²/steradian) in each pixel summed or integrated over the region of interest (ROI) area (cm²) x 4π. The total flux values were normalized to ensure accurate quantitation and comparability. Briefly, the normalized value (Vn) in each independent experiment was calculated as following:

\[ V_n = V_o \div \overline{V_s} \]

Vo was original total flux value of a given sample at a given timepoint; \(\overline{V_s}\) was the mean of all values from all groups.
Statistical analyses

All data were analyzed with GraphPad Prism version 9.0.0. Statistical methods used to determine significance are listed under each figure.

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Rokade, S., Upadhya, M., Bhat, D.S., Subhedar, N., Yajnik, C.S., Ghose, A., Rath, S.,


A.  

1\textsuperscript{st} shot

- PBS  
- eGFP  
- eGFP

2 weeks

HA  
HA  
HA

2\textsuperscript{nd} shot

- anti-HA

C. ELISA

B. 

Graph showing OD450 against dilution for different samples:

- PBS(L)-HA(L)  
- eGFP(L)-HA(L)  
- eGFP(L)-HA(R)  
- Pos Ctrl  
- Neg Ctrl

C. Bar graph showing relative levels of anti-HA-specific CD8 T cells:

- PBS(L)-HA(L)  
- eGFP(L)-HA(L)  
- eGFP(L)-HA(R)
A. Sublethal influenza (intranasal) → 2 weeks → Sublethal Candida (intravenous) → Weight monitored for 14 days

B. Graph showing % of original body weight vs. days for PBS-CA and eGFP-CA.

C. Bar graph showing AUC of BW for PBS-CA and eGFP-CA.

D. Bar graph showing CFU/mL for kidney and liver for PBS-CA and eGFP-CA.

E. Graph showing % of original body weight vs. days for PBS-PR8 and eGFP-PR8.
A. Parents immunized with 10 µg of mRNA-LNP coding for PR8 Flu HA 2 weeks prior mating. Litters infected intranasally with sub-lethal dose of PR8 influenza, and weight monitored for 14 days.

B. 1st litters  2nd litters  4th litters

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AUC

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DMN  DI  MI  DMI