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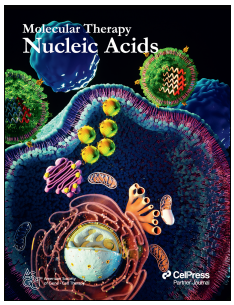

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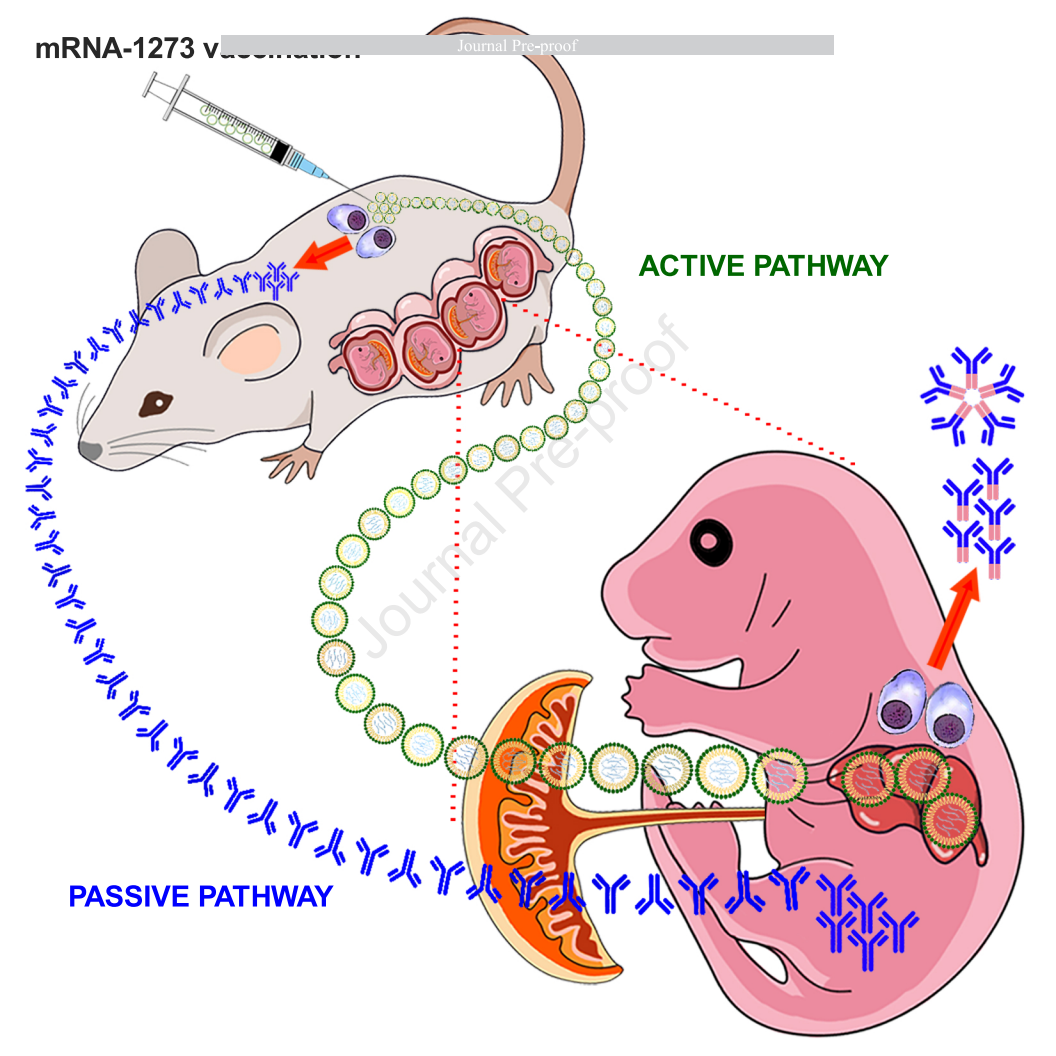

mRNA-1273 is placenta-permeable and immunogenic in the fetus

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Short Title: Transplacental mRNA-1273 transfer

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Abstract

COVID-19 mRNA vaccines are generally recognized as safe for gestational administration. However, their transplacental pharmacokinetics remains obscure. In this study, mRNA-1273 intramuscularly given to pregnant mice rapidly circulated in maternal blood and crossed the placenta within one hour to spread in fetal circulation. Although spike mRNA in fetal circulation faded away within 4-6 hours, it could accumulate in fetal tissues, mainly the liver and get translated into spike protein. Transplacental mRNA-1273 proved immunogenic in the fetuses, as postnatally equipped with anti-spike IgM, paternal allotypic anti-spike IgG_{2a} and heightened anti-spike cellular immunity. Gestationally administered, mRNA-1273 had a dose-dependent effect on its transplacental transfer and immunogenicity in the fetuses, with higher mRNA-1273 doses leading to increased transplacental mRNA-1273 passage and greater serum titers of endogenous anti-spike IgM/IgG generated by the fetuses. Thus, gestationally maternal mRNA-1273 vaccination might endow the newborns with not only passive but also active anti-spike immunity. Our results pose new insights into transplacental capacity of mRNA vaccines and their immunogenic potential in the fetuses, advancing our knowledge of mRNA medicine to protect the unborns against pathogens in perinatal life and broaden our horizons of prenatal mRNA molecular therapy.

Introduction

The two next-generation vaccines of Moderna mRNA-1273[1] and BioNTech BNT162b2,[2] based on SARS-CoV-2 spike protein-encoding mRNA strands packaged in lipid nanoparticles (LNPs),[3] have been widely used during and after COVID-19 pandemic. They conferred over 90% efficacy against COVID-19 with a favorable safety profile in adults.[1,2] However, heightened pharmacovigilance pertaining to potential or unexpected embryotoxic/fetotoxic effects of brand-new medical products administered during pregnancy precluded gravid women from mRNA-LNP vaccination at the outset[4] even though COVID-19 during pregnancy tended to pose a higher risk for maternal or neonatal complications.[5] Since accumulated clinical data and observations supported the safety of mRNA vaccines for the mother and fetus,[5] mRNA-LNP vaccination prior to[6] or during pregnancy[7] has been highly recommended. However, the pharmacokinetics of mRNA-LNPs in gravid females remains shrouded in clouds, especially as to their transplacental capacity. Although LNPs were reported to enable in vivo vascular endothelial growth factor mRNA delivery to the placenta accompanied by its vasodilation,[8] neither vaccine mRNA nor mRNA-decoded spike protein could be detected in the placenta[9] and cord blood[10] sampled 2 days at least and mostly over weeks or even months after final maternal BNT-162b2 or mRNA-1273 vaccination. It brought to the notion that the placenta acted as the natural barrier to mRNA-LNPs, providing additional reassurance about the safety of mRNA vaccines during pregnancy. However, it was reported that mRNA-LNPs were swiftly cleared from circulation during the first 24 hours with the time required for 50% decrement of mRNA-LNP concentration (T½) in a range of 2.7-3.8 hours,[11] implicating that transplacental mRNA-LNP transfer, if any, would most likely occur within 24 hours after maternal vaccination. Moreover, mRNA-LNPs administered intravenously in fetal[12] or adult animals[13] underwent rapid systemic spread with preferential LNP accumulation and peak mRNA functionality in the liver within 4 hours followed by decreasing protein levels at 24 hours after

injection or translation ceasing on day 2. Taken together, it seemed premature to negate transplacental mRNA-LNP transfer on the basis of undetectable vaccine mRNA or its products in belatedly-collected fetal blood or placenta that was even not favorable to harboring mRNA-LNPs or spike proteins. We conducted this murine study to reappraise the transplacental capacity of mRNA-1273 and scrutinize its immunogenicity in the fetuses.

Results

Detection of transplacental polyethylene glycol (PEG) lipid

Following a single-dose intramuscular injection of 4 μg mRNA-1273 into gestational day 14 (GD14) pregnant mice, the fetuses were delivered and sacrificed at selected time points to search fetal blood for transplacental LNPs, using anti-PEG antibodies. PEGylated LNPs swiftly moved into maternal bloodstream and efficiently crossed placenta to spread in fetal circulation within 30 minutes **(Figure 1A)**. However, they faded away in maternal circulation within 3-24 hours but lasted over time in fetal circulation for at least 7 days, indicating slower PEG breakdown in the fetuses than the dams. Further enzyme-linked immunosorbent assay (ELISA) confirmed that PEG levels stayed steady in fetal sera within 3 hours after maternal 4 μg mRNA-1273 vaccination, and dropped significantly at 6 hour and 1-3 day time points (**Figure 1B**). PEG in offspring's sera was barely found on days 7-11 post-maternal vaccination, and no longer detectable by days 14-18. There was no detectable PEG in fetal placenta, liver and soft tissues by ELISA at any of the time points examined (data not shown). Notably, a reduction of maternal mRNA-1273 doses caused a decline in serum PEG levels in the fetuses within 3 hours after maternal vaccination **(Figure 1C)**.

Detection of transplacental spike mRNA

We then examined whether transplacental LNP transfer was coupled with vaccine active substance of SARS-CoV-2 spike mRNA by real-time polymerase chain reaction (RT-PCR) (**Figure S1**). After maternal intramuscular vaccination of 4 μg mRNA-1273, spike mRNA entered maternal circulation and crossed the placenta to fetal blood within one hour, whereas transplacental spike mRNA shortly became undetectable in fetal circulation by 4-6 hours (**Table S1**). Thus, spike mRNA was more liable to degradation than PEG in fetal blood. Transplacental spike mRNA mainly accumulated in fetal livers, and also dwelt in fetal placentas and trunk soft tissues (**Figure 1D and Table S1**). Spike mRNA might persist in offspring's

liver and spleen at least till postnatal 3 weeks (**Table S1**). Notably, immunofluorescence staining demonstrated the lodging of PEGylated LNPs and spike protein in fetal liver cells (**Figure 1E**). Taken together, transplacental mRNA-1273 transfer came along with mRNA-decoded protein expression in the fetus. In dams vaccinated with 0.2 μg mRNA-1273, vaccine mRNA also rapidly spread to maternal circulation, accrued to the placenta and distributed to the fetus (**Table S2**). Although levels of spike mRNA in fetal placentas did not differ between 0.2 and 4.0 μg mRNA-1273 administered to the dams, low-dose mRNA-1273 gave rise to less spike mRNA accumulation in fetal livers than high-dose one (**Figure 1F**). Overall, transplacental mRNA-1273 transfer exhibited a maternally dose-dependent response, with higher maternal mRNA-1273 doses resulting in greater levels of PEG in the circulation and spike mRNA in the liver of the fetus. However, comparable levels of spike mRNA in fetal placentas between high and low-dose mRNA-1273 given to the dams might suggest placental trapping of mRNA-1273 before reaching the fetus.

Examination of anti-spike IgG_1/IgG_{2a} with their virus-blocking efficacy

To elucidate the immunological consequences of transplacental mRNA-1273 transfer in the fetus, we scrutinize the influence of mRNA-1273 doses given intramuscularly to pregnant mice on serum anti-spike immunoglobulin levels of dams and pups. After maternal mRNA-1273 vaccination at the same dose of 0.2, 1.0, 2.0 or 4.0 μg on GD14 and GD17, the dams and their pups were examined for serum anti-spike immunoglobulin levels 1 month after delivery. The vaccinated mothers significantly generated anti-spike IgG_1/IgG_{2a} in the absence of a dose-responsive fashion, ranging respectively around 100-200 ng/mL and 20-40 μg/mL with relatively steady levels over a postnatal period of 3 months (**Figure 2A**). However, mRNA-1273 given to pregnant mice exerted a dose-dependent effect on offspring's serum anti-spike IgG_1/IgG_{2a} levels, which showed a dwindling trend over time (**Figure 2B**). Virus-blocking efficacy of maternal sera was as high as 1024-2048-fold dilutions at least within postnatal 2-

3.5 months, whereas the pup's sera at 2 months old had lower neutralization activity, which even vanished by 3.5 months old (**Figure 2C**). It was essentially consistent with the distinct durability of serum anti-spike IgG between the dams (**Figure 2A**) and their offspring (**Figure 2B**). The discordance between the first month anti-spike IgG_1/IgG_{2a} levels of mothers and their offspring in response to mRNA-1273 doses used to vaccinate the dams might have relevance to transplacental mRNA-1273 transfer in a maternally dose-dependent manner (**Figures 1C&F & Tables S1-2**). It called into question whether transplacental mRNA-1273 transfer was not only maternally dose-dependent but also exerted a dose-dependent effect on triggering fetal immune system to generate endogenous anti-spike IgG.

Analyses of anti-spike IgG_{2a} allotypes in offspring

Investigations proceeded to assess the derivation of offspring's anti-spike IgG_{2a} after maternal mRNA-1273 vaccination, using two allelic forms (Igh-1a and Igh-1b) of the Igh-1 (IgG_{2a}, γ2a constant region). The mouse BALB/c strain possesses the IgG_{2a} of Igh-1a haplotype, whereas the C57BL/6 strain belongs to the Igh-1b haplotype. C57BL/6 females were mated to BALB/c males, and then given 4 μg mRNA-1273 vaccination respectively on GD14 and GD17. Postnatally, the offspring (BALB/c male x C57BL/6 female, F1) co-expressed both Igh-1a and Igh-1b haplotypes of anti-spike IgG_{2a} at their age of 4 weeks despite fading-out of Igh-1a haplotype by 8 weeks old (**Figures 3A&B and Table S3**). It provided the direct molecular evidence that the offspring were equipped with endogenous anti-spike IgG_{2a}, which could not originate from anything other than offspring's B-cell clones selectively expressing paternal Igh-1 allotype. Clearly, the offspring born to dams with gestational mRNA-1273 vaccination had been immunized by spike protein and additionally armed with endogenous anti-spike IgG. Incidentally, serum anti-spike IgG_{2a} of all C57BL/6 dams, mated to BALB/c males, was found to contain Igh-1a haplotype of fetal origin (**Table S3**), indicating a reverse direction of fetal-to-maternal anti-spike IgG_{2a} transfer. Thus, transplacental IgG transfer could be bidirectional.

When gestational C57BL/6 dams were vaccinated with 0.2 μg mRNA-1273 twice, their offspring barely generated anti-spike IgG_{2a} of Igh-1a haplotype despite high titers of Igh-1b allotypic anti-spike IgG_{2a} in sera (**Figure 3C and Table S4)**. These results pointed to a dose-responsive relationship between mRNA-1273 doses used to vaccinate the dams and serum titers of endogenous anti-spike IgG in the fetuses. As a consequence, mRNA-1273 had a maternally dose-dependent effect on not only its transplacental capacity but also its immunogenicity as to the productivity of endogenous anti-spike IgG in the fetuses.

Assessment of anti-spike IgM and cellular immunity

Being placenta-impermeable, anti-spike IgM in offspring was measured to reconfirm fetal immunization by transplacental mRNA-1273. Maternal vaccination with either 0.2 or 4.0 μg mRNA-1273 gave rise to heightened anti-spike IgM levels in offspring by their age of 4 weeks (**Figure 3D**). High-dose mRNA-1273 led to higher serum titers of fetal anti-spike IgM than low-dose one. Vaccinated dams and their offspring were further examined for cellular immunity to spike protein by the readout of incorporated tritium into lymphocytes. Both compared favorably in spike protein-specific lymphocyte proliferation (**Figures 4A&B**) with their respective saline control counterparts. Additionally, spike-reactive IFN-γ- and IL-2-secreting T-cells were enumerated by Elispot, proving at significantly heightened frequencies as opposed to their saline controls (**Figures 4C&D)**. Altogether, maternal mRNA-1273 vaccination during pregnancy might trigger adaptive immunity against spike protein in dams and their pups.

Immunological outcome of direct fetal exposure to mRNA-1273

To further validate the immunogenic effects of mRNA-1273 on pre-immune fetuses, we directly subjected GD14 murine fetuses to intraperitoneal mRNA-1273 injection. Postnatally, fetal recipients exhibited heightened titers of serum anti-spike IgG_1/IgG_{2a} (**Figures 5A&B**), which decreased gradually within postnatal 3 months. Their lymphocytes also proliferated

174 specifically in response to SARS-CoV-2 spike protein (**Figure 5C**) in association with a
175 heightened frequency of spike-reactive IFN-γ and IL-2-secreting T-cells (**Figure 5D**). These
176 results signified fetal immunoreactivity to mRNA-1273 administered in utero even before full
177 T-cell development.

Discussion

Maternal mRNA COVID-19 vaccination during pregnancy offered a "two-for-one" deal to protect mothers as well as their infants.[7] This added infant protection has long since been attributed to transplacental transfer of vaccine-elicited maternal anti-spike antibodies,[4,7] likened to maternal vaccination against influenza, tetanus, diphtheria and pertussis.[14] In this study, murine placentas proved permeable to mRNA-1273 along with mRNA-decoded spike protein translation in GD14 murine fetuses, which lacked functionally competent T-cells as murine T-cell receptors were not expressed until GD17.[15] However, vertical mRNA-1273 transmission made maternal mRNA-1273 vaccination immunogenic rather than tolerogenic in developing fetuses. It's in line with an immunization event arising after artificial fetal exposure to foreign peptides,[16,17] wherein fetal macrophages sequestered endocytosed antigens and differentiated towards dendritic cells to instruct well-developed T-cells later on in life.[16] Thus, developing immune system of fetuses born to mothers with gestational mRNA-1273 vaccination still had an active role in protecting against pathogens.

Mammalian placentas are categorized into epitheliochorial, endotheliochorial and hemochorial types on the basis of the cell layers intervening between maternal and fetal circulations,[18] known as interhemal barriers that influence placental permeability of animal species. Both humans and mice possess hemochorial placentas with similar cell types of placental trophoblasts[19,20] to mediate materno-fetal exchange. Hemochorial placentation lacks interhemal barriers of uterine endometrium (including its epithelia, stroma and vascular endothelia), leading to direct immersion of fetal trophoblast layers in maternal blood. It benefits bio-substance exchange between mothers and their fetuses by providing direct access of fetal trophoblasts to maternal blood.[21] In light of the identical interhemal barriers in the placenta, the mouse model should be appropriate for simulating placental exchange of bio-substances in humans. However, one should exercise caution in extrapolating the findings of transplacental

mRNA-1273 transmission from this murine study to human subjects since there is a distinction in histoarchitectures between murine and human placentas, evidenced by three-layered trophoblasts (hemotrichorial) at murine materno-fetal interface in contrast to one-layered syncytiotrophoblast (hemomonochorial) of human placenta.[20] It remained unclear whether the two extra trophoblast layers in murine placentas exerted negative or positive impacts on materno-fetal exchange of bio-substances, let alone mRNA-1273. This murine study demonstrated that mRNA-1273 possessed the dose-dependent transplacental capacity in gravid dams and exhibited immunogenic potential in the fetuses. These findings urge the need to reappraise transplacental capacity of mRNA-LNPs and clarify the status of immunoreactivity to spike protein in human fetuses or infants with gestational maternal mRNA COVID-19 vaccination. The information obtained will have a profound influence on the COVID-19 vaccination strategy in infants born to gestationally-vaccinated mothers.

mRNA vaccines exhibited good safety profiles in humans[1,2] and even pregnant individuals.[22-24] However, the positive safety outcomes in the clinical arena could not alleviate the apprehensions about the potential genotoxicity such as genome integration, oncogenesis or germline transmission,[25-27] which was fueled by enduring biodistribution of vaccine mRNA[28,29] or its product[30,31] in post-marketing studies. These inconvenient findings somewhat reflected the rapid marketing authorization of mRNA vaccines with incomplete preclinical studies due to the urgent health needs in the face of a public health crisis caused by the COVID-19 pandemic.[25] This murine study filled the void in transplacental pharmacokinetics of mRNA vaccines, which has been missing in preclinical studies even though mRNA vaccines themselves involved several new biotechnologies. In this research, mRNA-1273 did not pose discernible safety issues in pregnant mice and their pups. However, the proof of transplacental mRNA-1273 transmission with enduring mRNA retention in offspring's liver or spleen inevitably aroused an interest in the genotoxic effects of mRNA vaccines on the developing

fetus, where heightened activities of cell multiplication and specialization potentially created genomic instability[32,33] to render the fetus vulnerable to the integration of exogenous genetic elements.[34,35] Considering the occurrence of SARS-CoV-2 RNA retro-integration into human cell genome,[26] the risk of long-term genotoxicity in the offspring born to mRNA-vaccinated mothers cannot be overlooked.

Given the success of mRNA COVID-19 vaccines and today's biotech landscape, there is a prospect of extending mRNA-LNP technology to the genetic diseases with defective/missing proteins or enzymes such as cystic fibrosis, propionic acidemia, and phenylketonuria.[3] These candidate diseases if diagnosed prenatally can be managed by prenatal mRNA therapies before the onset or in the early stage of irreversible pathology to minimize disease morbidity and mortality, and achieve high therapeutic efficacy. In consideration of transplacental mRNA-1273 passage, the unmet need of fetal mRNA therapies may be fulfilled simply through maternal mRNA-LNP administration in case of no harm to the mothers, but the potential immunogenicity of mRNA-decoded peptides must be taken into consideration even in pre-immune fetuses. However, it's better to note that the ability of LNPs to deliver mRNA and accumulate within desired tissues or organs varied with changes in LNP chemistry.[12,36] Thus, modifications in lipid excipients used for mRNA-LNP formulations might affect their transplacental capacity and warrant an evaluation of their transplacental properties.

In this era of mRNA medicine, the new insights into transplacental pharmacokinetics of mRNA vaccines and immunogenic potential of mRNA-decoded protein in the fetuses may advance our knowledge to better protect the unborns against pathogens in perinatal life and broaden our horizons of prenatal mRNA therapeutics.

Methods

Mice

Inbred FVB/N, BALB/c (Igh-1a of IgG_{2a}, γ2a constant region) and C57BL/6 (Igh-1b of IgG_{2a}, γ2a constant region) mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) at the age of 6-8 weeks. Animals were housed in Animal Care Facility at Chang Gung Memorial Hospital (CGMH) under the standard guidelines from "Guide for the Care and Use of Laboratory Animals" and with the approval of CGMH Committee on Animal Research. Females were caged with males in the afternoon and checked for vaginal plugs the following morning. The day of the plug observed was designated as day 0 of the pregnancy.

Harvest of fetal tissues

Under anesthesia for pregnant mice, midline laparotomy was performed to expose the uteri. The fetuses were delivered through hysterotomy and immediately washed with saline. After decapitation, fetal blood was collected by pipetmans. Then, fetal placenta, liver and trunk soft tissues were obtained. Samples were stored in RNAlater solution at -80°C for downstream analyses, or subjected to homogenization in organic solvents of ethanol or dimethyl sulfoxide (DMSO) for PEG extraction.

mRNA-1273 vaccination in pregnant mice

Pregnant mice received intramuscular (thigh) injection of mRNA-1273 on their GD14 and GD17, each at the same dose of 0.2, 1, 2 or 4 μg, diluted in 100 μl saline. Postnatally, sera of the dams and their offspring were sampled periodically for downstream experimental analyses. For mRNA-1273 component tracking in the fetuses, a single-dose of 0.2 or 4 μg mRNA-1273 was intramuscularly given to the mothers on their GD14. The fetuses were then delivery by Caesarean section at indicated time points after maternal vaccination to harvest fetal tissues and placentas for downstream analyses.

In utero injection of mRNA-1273

Under anesthesia, the uteri of GD14 pregnant mice were exposed through a vertical laparotomy. A 60 μm glass micropipette with beveled tip was used to inject 0.05-0.1 μg of mRNA-1273 in 5 μl saline into the peritoneal cavities of all fetuses at a litter via trans-uterine approach. The control mice received in utero saline injection. Murine abdomen was closed in two layers by 5-0 silk suture. Then, mice were housed in an undisturbed room without bedding changes for 1 week. Pups were weaned at 3 weeks of age.

Immunodot blot assay to detect LNPs of mRNA-1273

This method was modified from the fat blot assay by Munnik et al.[37] to semiquantitatively detect mRNA-LNPs. Mouse sera and serially-diluted mRNA-1273 (1 μl for each sample) were spotted onto a nitrocellulose membrane (0.45 NC, Amersham Protran). The membrane was first blocked with 5% milk in Tri-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour on a rotating shaker and then incubated with anti-polyethylene glycol (PEG) antibody (1:3000, PEG-B-47, ab51257, Abcam, reacting only with conjugated forms) for 2 hours. After TBST washing for 3 times, the membrane was treated with peroxidase-conjugated goat anti-rabbit IgG (1:5000, AP132P, Sigma-Aldrich) for 2 hours, followed by Immobilon Western Chemiluminescent HRP Substrate (Millipore) for 2 minutes. Finally, the blots were subjected to chemiluminescence imaging detection (UVP Chemstudio). Positive controls were 2-fold serial dilutions of mRNA-1273, and negative controls included saline and maternal/pups' sera collected after maternal saline injection.

Real-time quantitative polymerase chain reaction (RT-PCR) to detect spike mRNA[10,38]

RNA was isolated from tissue samples of fetuses with maternal mRNA-1273 vaccination using GeneJET RNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. RNA concentration was determined using Nanodrop. RNA samples of 500 ng were reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (TaKaRa Bio). Primers used to detect target cDNA were as follows: Forward primer:

AACGCCACCAACGTGGTCATC. Reverse primer: GTTGTTGGCGCTGCTGTACAC. Bio-Rad iQ5 real-time PCR detection system and 2xSYBR qPCR Mix (BioTools) were used for PCR: 30s 95°C followed by 40 cycles of 5s 95°C and 20s 60°C. All samples (2 μL) were run in duplicate as 20 μL reactions. For setup of spike mRNA standard curves, cDNA reversely-transcribed from 100 ng/μL mRNA-1273 was serially diluted in 1:2 ratio. These 3-fold serial dilutions of 2 μL cDNA samples were further 10x diluted to 20 μL (corresponding to 10^4 - 0.0021 pg/μL of spike mRNA) in PCR amplification. Negative results were determined by the Ct values of tissue samples from the fetuses with maternal saline injection.

Determination of serum anti-spike IgG_1, IgG_{2a} and IgM levels

ELISA microtiter plates (Corning, Corning, NY, USA) are first coated with 25 ng/ml and 50 ng/ml SARS-CoV-2 spike protein (GTX02774-pro, GeneTex) respectively for the measurement of mouse anti-spike IgG_1 and IgG_{2a}/IgM levels. The wells are blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS, and incubated with 100 μl of diluted samples. In each well, biotinylated anti-mouse IgG_1 (Clone RMG1-1, BioLegend, San Diego, CA, USA) was used for IgG_1 detection, biotinylated anti-mouse IgG_{2a} (Clone RMG2a-62, BioLegend) for IgG_{2a} detection, and biotinylated anti-mouse IgM (Clone RMM-1, BioLegend) for IgM detection. Subsequently, streptavidin-horseradish peroxidase (HRP, Sigma-Aldrich) was added to the wells. Then, the reaction was developed by adding 100μl NeA-blue tetramethylbenzidine substrate (TMB) (Clinical Science Products, Mansfield, MA, USA) and stopped with 2M H_2SO_4. The optical density at 450 nm was read using an ELISA reader. Serum anti-spike IgG_1 and IgG_{2a} levels were determined by the standard curves of mouse monoclonal anti-SARS-CoV-2 spike IgG_1 (1A9, GTX632604, GeneTex) and mouse monoclonal anti-SARS-CoV-1/2 S Protein IgG_{2a} (clone 2B3E5, Sigma-Aldrich), respectively. Serum anti-spike IgM titers were recorded as the values of optical density.

Determination of anti-spike IgG_{2a} allotypes

Igh-1a and Igh-1b allotypes of IgG_{2a} were used to examine whether the pups (BALA/c (Igh-1a) male x C57BL/6 (Igh-1b) female, F1) generated endogenous anti-spike IgG_{2a} after maternal intramuscular vaccination of 0.2 or 4.0 μg mRNA-1273 respectively on GD14 and GD17 during pregnancy. ELISA was performed as described above, using 10x diluted serum samples treated with primary antibodies of biotinylated anti-mouse IgG2a (1:20000, Clone RMG2a-62, reacting with both Igh-1a and Igh-1b (Igh-1a/b) haplotypes), biotinylated anti-mouse Igh-1a (1:2000, clone 8.3, BD Pharmingen) or biotinylated anti-mouse Igh-1b (1:2000, clone 5.7, BD Pharmingen), respectively. The color developed was read at optic density of 450 nm. Controls included BALB/c (male) x BALB/c (female) and C57BL/6 (male) x C57BL/6 (female) F1 mice.

Quantification of PEG by sandwich ELISA

Sera or supernatants of tissue homogenates from murine fetuses were subjected to PEG quantification by ELISA kits (Life Diagnostics, Cat. #: MPEG) with the capture antibody specific to PEG backbone and the detection antibody to the terminal methoxy group of vaccine PEGylated lipid. This sandwich ELISA was conducted according to the manufacturer's instructions.

Lymphocyte proliferative responses to spike protein

Spleens were obtained from the mice (6-8 weeks old) with maternal mRNA-1273 vaccination during pregnancy. Splenic lymphocytes were enriched by density gradient centrifugation and then cultured in triplicate each with 2×10^5 cells in 200 μL RPMI 1640 medium containing 10% fetal calf serum in 96-well plates. Responder lymphocytes were grown in medium only as background controls, and stimulated with SARS-CoV-2 spike protein (1 μg/mL), third-party stimulator of BSA (100 ng/mL) or non-specific mitogen of Con-A (1 μg/mL). For the measurement of lymphocyte proliferation, day 5 cells were first subjected to 16-hour incubation with tritiated thymidine (ICN Biomedicals) at a final concentration of 1 μCi

per well and then harvested for counting incorporated tritium in a liquid scintillation counter (1450 Microbeta Plus counter). Lymphocyte proliferation was determined by the readout of incorporated tritium as counts per minute. Controls were the mice with maternal saline injection.

IFN-γ and IL-2 enzyme-linked immunospot assay (ELISPOT)

Murine IFN-γ/IL-2-secreting T-cells were quantified by mouse IFN-γ and IL-2 ELISpot Kits according to the manufacturer's instructions (R&D Systems). Briefly, splenic lymphocytes of each animal subject were enriched by density gradient centrifugation and then examined for their CD3 T-cell fractions by flow cytometry after the treatment of fluorescence-conjugated anti-CD3 antibodies (BioLegend). Wells in the microplates were first rinsed with culture media of RPMI 1640 containing 10% fetal calf serum for 20 minutes at room temperature. Then, cells were loaded into wells in triplicate at a dose of 10^6/100 μl culture media per well and incubated in a humidified 37 °C CO_2 incubator for 2 days under the stimulation of SARS-CoV-2 spike protein (1 μg/ml, GTX02774-pro, GeneTex). The plates were washed with Wash Buffer for 4 times and incubated with diluted Detection Antibody mixture (100 μl/well) for 2 hours at room temperature on a rocking platform. After wash, the plates were incubated with diluted Streptavidin-AP Concentrate A (100 μl/well) for 2 hours. The final wash was followed by the treatment of BCIP/NBT Substrate for 1 hour. After the chromogen was decanted, the plates were washed with deionized water and dried at room temperature. Plates were scanned and counted on an immunospot analyzer (Cellular Technologies Ltd). The readouts of spike-reactive IFN-γ/IL-2-secreting T-cells in each mouse were divided by its splenic $CD3^+$ T-cell fraction to estimate their frequencies per million T-cells.

Neutralization assay with SARS-CoV-2 spike pseudovirus[39]

HEK-293T cells stably expressing human ACE2 (293T-ACE2 cells) were grown in 96-well plates (6×10^4 cells/well) at 37°C with 5% CO_2 for 24 h. Serial 2-fold dilutions of mouse sera were mixed with SARS-CoV-2 wild-type spike pseudotyped lentivirus containing

luciferase gene (4,000 relative infection unit), provided by RNA Technology Plateform and Gene Manipulation Core, Academia Sinica, in DMEM with 1% fetal bovine serum (FBS) at 37°C for 1 h. Then, the serum-pseudovirus mixtures were added to 293T-ACE2 cells, which was incubated at 37°C with 5% CO_2 for 24 h and in DMEM with 10% FBS for another 24 h. The luciferase activity was determined by Bright-Glo Luciferase Assay kit (Promega, Madison, WI, USA) and the Synergy 2 (BioTek, Winooski, VT, USA) microplate reader. Neutralization titer was determined by the highest serum dilution that reduced the viral infectivity by at least 50%, compared to the corresponding control wells without sera added.

Histological examination of mRNA-LNPs and spike protein by immunofluorescence staining

The fetuses was fixed in 4% paraformaldehyde overnight and embedded in paraffin. Tissue sections were deparaffinized, rehydrated and then subjected to heat-induced antigen retrieval. After permeabilized with Tween-20 and blocked with 1% BSA, the sections were incubated with primary antibodies against PEG (1:100, PEG-B-47, ab51257, Abcam) and spike protein (1:100, chimeric mAb, D001, MBS8119537) for 1.5 hr, followed by fluorescence-conjugated donkey anti-rabbit IgG (1:100, Poly4064, BioLgend) and rat anti-human IgG Fc (1:100, M1310G05, BioLgend). Visualization of the nuclei was achieved by Hoechst 33342 staining (1: 20,000, Invitrogen). Sections were mounted with Dako fluorescence mounting medium. Images were taken using a confocal microscope.

Statistical Analyses

All error bar charts were shown as 95% confidence intervals (boxed areas) for the means (transverse lines crossing the boxes) along with superimposed data points of individual mice. The equality of means was examined by Student's t-test between two independent or paired groups, or by one-way analysis of variance (ANOVA) among three or more groups with post hoc Fisher's least significant difference (LSD) multiple comparisons. Differences were regarded as significant in all tests at $p < 0.05$.

Keywords： mRNA vaccine; lipid nanoparticle; transplacental transfer; maternal vaccination; in utero immunization; IgG allotype

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Author contributions: J.-C.C. conceptualized this study, acquired funding, performed in utero injection, analyzed the data, prepared the figures and wrote the manuscript. M.-H.H. conducted the experiments of all immunoassays and RT-PCR. R.-L.K. and L.-T.W. performed pseudovirus neutralization assay. M.-L.K. helped to design the experiments of anti-spike IgG_{2a} allotypes. L.-Y.T. and H.-L.C. performed immunostaining and assisted in experiments and animal surgery as well as care. C.-H.C. conceptualized this study, acquired funding, assisted in data analyses, supervised and coordinated the overall research and edited the manuscript.

Declaration of interests: The authors disclose no competing interests.

Data and code availability

Data reported in this paper will be shared by the correspondence author upon request.

Any additional information required to reanalyze the data reported in this paper is available from the correspondence author upon request.

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546 **Figure Legends**

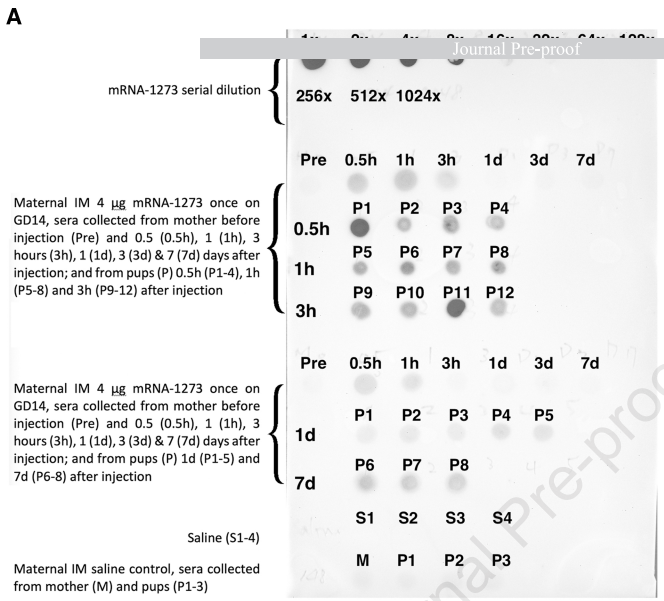
547 **Figure 1. Transplacental mRNA-1273 transfer after maternal mRNA-1273 vaccination**
548 **during pregnancy. (A)** GD14 FVB/N mothers, intramuscularly (IM) vaccinated with a single-
549 dose mRNA-1273 of 4.0 μg, were subjected to serum collection before vaccination (Pre), and
550 at indicated time points of 0.5-3 hours (h) and 1-7 days (d) after injection. Their pups were
551 delivered for serum sampling at the same time points. Immunodot blot assay demonstrated
552 transplacental PEGylated LNP transfer. **(B)** ELISA disclosed that fetal sera contained
553 significantly higher PEG levels at the time points of 1 h, 3 h, and 6 h after maternal mRNA-
554 1273 vaccination than those with maternal saline injection (Control, ANOVA with LSD
555 multiple comparison). A significant decrease of serum PEG levels occurred between 3 h and 6
556 h. Although PEG remained measurable in certain pups of groups 1-3 d and 7-11 d, their mean
557 levels did not differ from that of saline controls. On days 14-18, PEG was completely absent in
558 all neonatal sera, identical to saline controls. **(C)** At the time points of 1 h and 3 h following
559 maternal vaccination, 4.0 μg mRNA-1273 led to higher PEG levels in fetal sera than a dose of
560 0.2 μg. **(D)** Spike mRNA in fetal placenta, liver and soft tissue was quantified by RT-PCR after
561 maternal 4 μg mRNA-1273 vaccination (Dams 234, 235 and 236 in Table S1). Spike mRNA
562 levels of "(-)" and "< 0.021" were input as "0" and "0.021", respectively in building this chart.
563 Spike mRNA significantly dominated in fetal liver of groups 1, 4 and 6 hr. (ANOVA with LSD
564 multiple comparison) **(E)** Immunostaining disclosed intracellular PEGylated LNPs and spike
565 protein in fetal liver 6 hours after maternal 4.0 μg mRNA-1273 vaccination. DIC: differential
566 interference contrast. ZI: zoom-in. **(F**) At the time points of 1, 4 and 6 hours after maternal
567 mRNA-1273 vaccination, levels of spike mRNA in fetal placentas did not differ between 4.0
568 and 0.2 μg mRNA-1273 used to vaccinate the dams (Tables S1-2), whereas 4.0 μg mRNA-1273
569 led to significantly greater spike mRNA accumulation in fetal livers than 0.2 ug mRNA-1273.
570

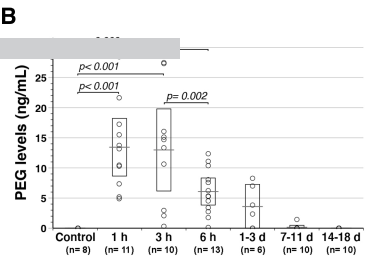
Figure 2. Anti-spike IgG_1/IgG_{2a} with virus-blocking efficacy in dams and their offspring after gestational mRNA-1273 administration. Pregnant FVB/N mice were intramuscularly vaccinated by the same doses of mRNA-1273 (0.2, 1, 2 or 4 μg) on GD14 and GD17. **(A)** One month after delivery (M1), all the 4 mRNA-1273 doses elicited significant levels of serum anti-spike IgG_1/IgG_{2a} in dams and (**B**) pups ($p< 0.001$, ANOVA), as compared with their saline controls. There were dose-responsive anti-spike IgG_1/IgG_{2a} levels in pups rather than dams (multiple comparisons by Fisher's LSD post hoc test). The dams (n=6) kept steady anti-spike IgG_1/IgG_{2a} titers in sera within postnatal 3 months (M1-M3) except for an initial drop of anti-spike IgG_{2a} levels ($p= 0.010$, pairwise comparison) at M2, whereas anti-spike IgG_1/IgG_{2a} in pups' sera (n=18) gradually faded away by M3-M4. The interconnected circles at different time points were the data of IgG_1/IgG_{2a} levels collected from an individual mouse. **(C)** Virus-blocking efficacy of maternal and offspring's sera was evaluated by pseudovirus neutralization assays, and shown in a representative mother and its offspring. Postnatal 2- and 3.5-month maternal sera had the neutralization titers of 2048- and 1024-fold dilutions respectively, whereas neutralization activity of offspring's sera was 64-fold at 2 months old but vanished by 3.5 months old.

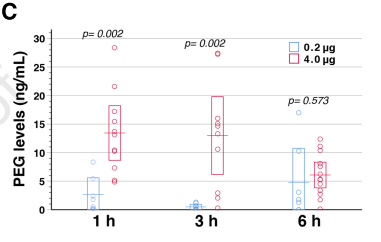
588 **Figure 3. Analyses of anti-spike IgG_{2a} allotypes and anti-spike IgM in offspring born to**
589 **the dams with gestational mRNA-1273 vaccination. (A)** After mRNA-1273 vaccination,
590 BALB/c (Igh-1a) x C57BL/6 (Igh-1b) F1 mice (n=9) significantly secreted anti-spike IgG_{2a}
591 (Igh-1a/b) in sera within 2-4 weeks (pairwise comparison). Igh-1a haplotype dominated the
592 allotypes of anti-spike IgG_{2a}. **(B)** C57BL/6 females (F) mated to BALB/c males (M) were
593 vaccinated with 4 μg mRNA-1273 twice on GD14 and GD17. Both paternal Igh-1a and
594 maternal Igh-1b allotypic anti-spike IgG_{2a} significantly showed up in BALB/c (M) x C57BL/6
595 (F) F1 mice at 4 weeks old ($p< 0.001$) despite undetectable paternal Igh-1a allotype by 8 weeks
596 old ($p= 0.508$). **(C)** In the case of 0.2 μg mRNA-1273 vaccination in C57BL/6 pregnant mice,
597 BALB/c x C57BL/6 F1 offspring (n=17) did not compare favorably in serum anti-spike IgG_{2a}
598 of Igh-1a ($p= 0.418$) with their saline controls (n=5), but owned significantly higher levels of
599 Igh-1b ($p< 0.001$) allotype than the controls by their age of 4 weeks. **(D)** After maternal
600 vaccination with either 0.2 or 4.0 μg mRNA-1273 twice, offspring showed significantly
601 heightened levels of serum anti-spike IgM by their age of 4 weeks, as compared to the controls
602 with maternal saline injection. Besides, 4.0 μg mRNA-1273 given to the dams elicited higher
603 serum titers of anti-spike IgM in offspring than 0.2 μg mRNA-1273 ($p< 0.001$). OD: optic
604 density at 450 nm.
605

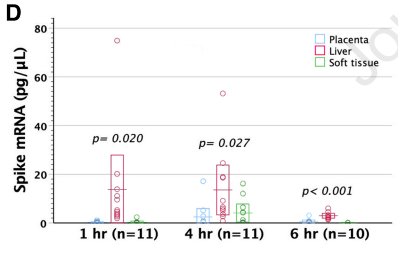
606 **Figure 4. Anti-spike cellular immunity in dams and their pups after maternal mRNA-**
607 **1273 vaccination during pregnancy. (A, B)** After maternal vaccination with 4 μg mRNA-
608 1273 twice, the dams and pups were examined for spike-specific lymphocyte proliferation by
609 the readout of incorporated tritium in vitro. Splenic lymphocytes of both dams ($p = 0.001$) and
610 pups ($p < 0.001$) proliferated specifically in response to spike, as opposed to those with maternal
611 saline injection. Besides, the dams ($p = 0.004$) and pups ($p < 0.001$) with maternal mRNA-1273
612 vaccination were superior in spike-specific lymphocyte proliferation to their respective saline
613 controls. **(C, D)** IFN-γ- and IL-2 Elispot images in triplicate shown were from a representative
614 dam and pup with maternal mRNA-1273 (4 μg) or saline vaccination during pregnancy. Both
615 groups exhibited heightened frequencies of IFN-γ- and IL-2-secreting T-cells, as compared
616 with their respective control counterparts.
617

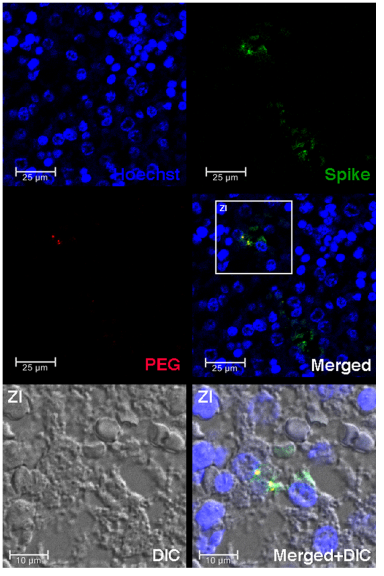
Figure 5. Immunological consequences of in utero mRNA-1273 injection. GD14 FVB/N fetuses were subjected to intraperitoneal injection of mRNA-1273 (IU mRNA-1273, n= 19). (**A, B**) Postnatally, serum anti-spike IgG_1/IgG_{2a} were examined at the age of 1 month. IU mRNA-1273 led to significantly higher titers of anti-spike IgG_1/IgG_{2a}, as compared with in utero saline injection (IU saline, n= 9). Serum anti-spike IgG_1/IgG_{2a} gradually decreased within postnatal 3 months. Circles interconnected by a line represented IgG_1/IgG_{2a} levels measured at 1 (M1), 2 (M2) and 3 (M3) months old from an individual mouse (n= 11). (**C**) Lymphocyte proliferation in response to spike protein was measured by the readout of incorporated tritium (n= 4) as counts per minute (cpm). Medium only was used as background controls, bovine serum albumin (BSA) as third-party stimulators and Con-A as a mitogen to stimulate T-cell population. IU mRNA-1273 significantly proliferated specifically in response to spike protein ($p< 0.027$), whereas IU saline (n= 4) failed to show lymphocyte proliferation under spike protein stimulation. There was a significant difference in lymphocyte proliferation under spike protein stimulation between IU mRNA-1273 and IU saline ($p< 0.006$). Rectangles within a dataset represented 95% confidence intervals for the means, which were shown as transverse lines crossing the rectangles. (**D**) Spike-reactive IFN-γ- and IL-2-secreting cells of splenic lymphocytes was enumerated by Elispot. Figures showed the spots with their counts from the representative mice of IU mRNA-1273 and IU saline. The frequency of spike-reactive IFN-γ- and IL-2-secreting T-cells was calculated by the mean of Elispot readouts (triplicates) divided by the $CD3^+$ cell ratio of splenic lymphocytes in each individual mouse.

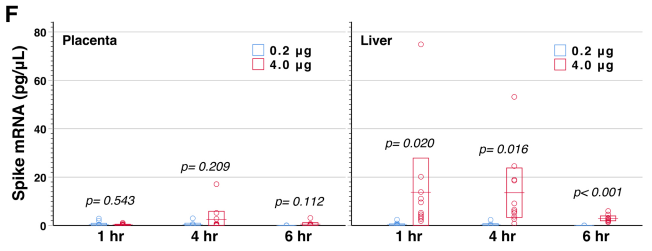


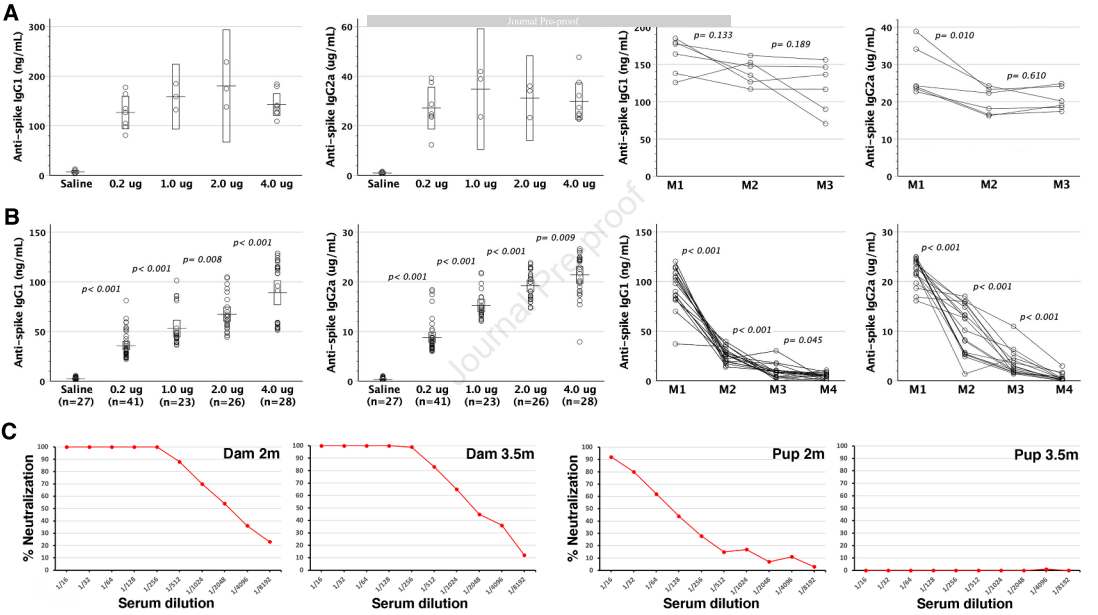


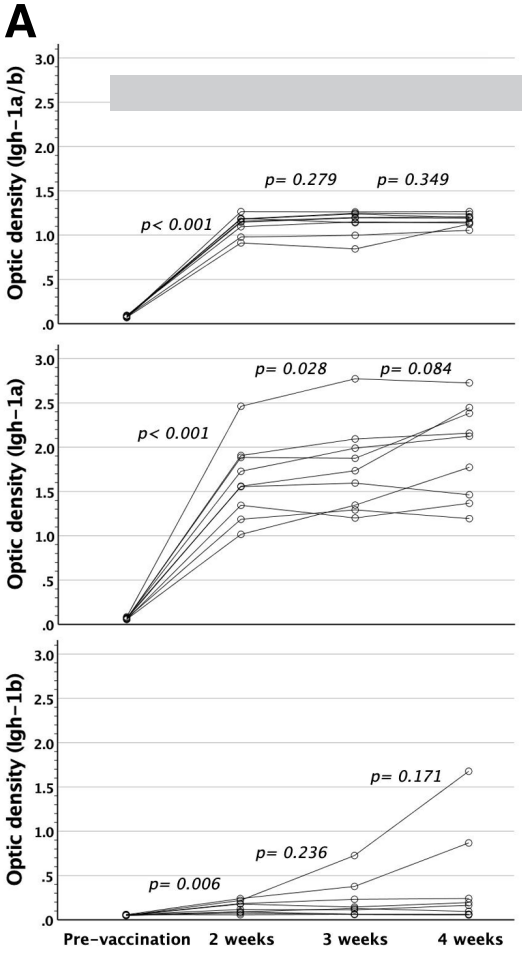


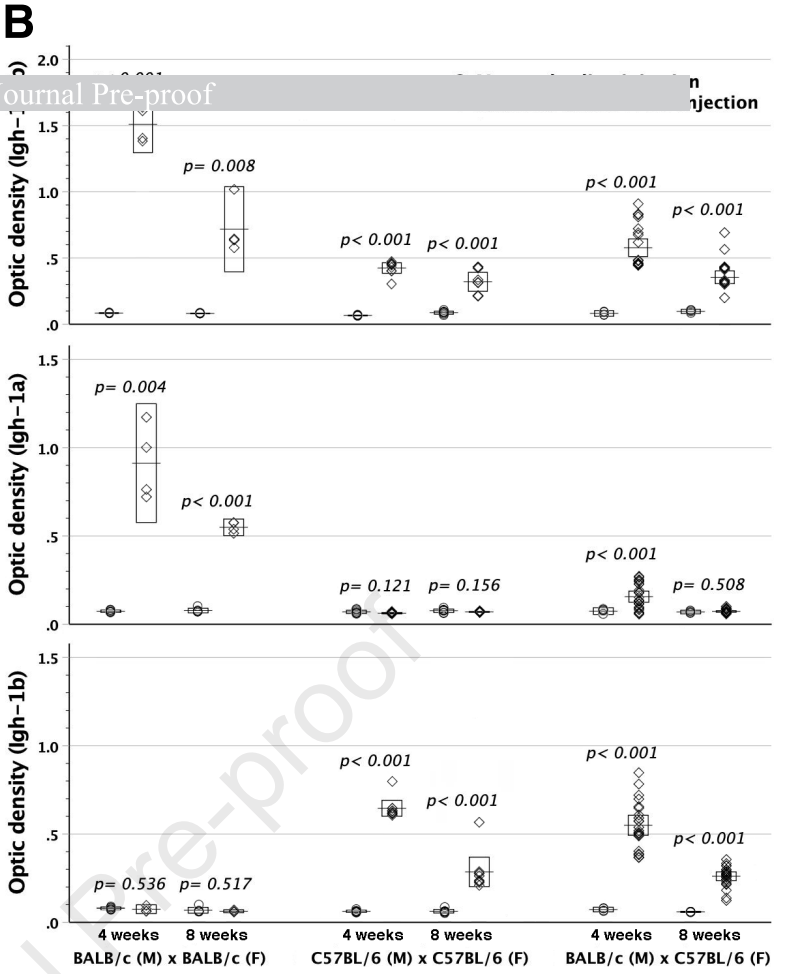


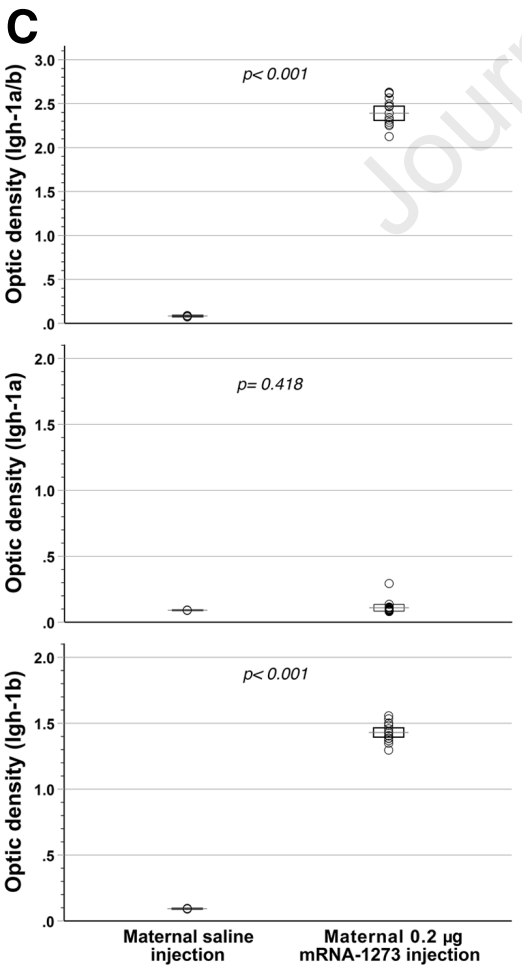


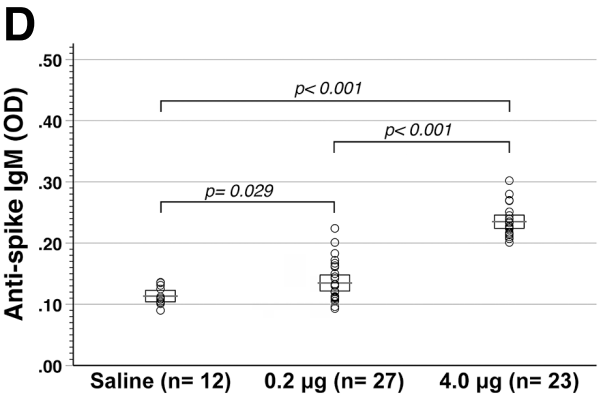


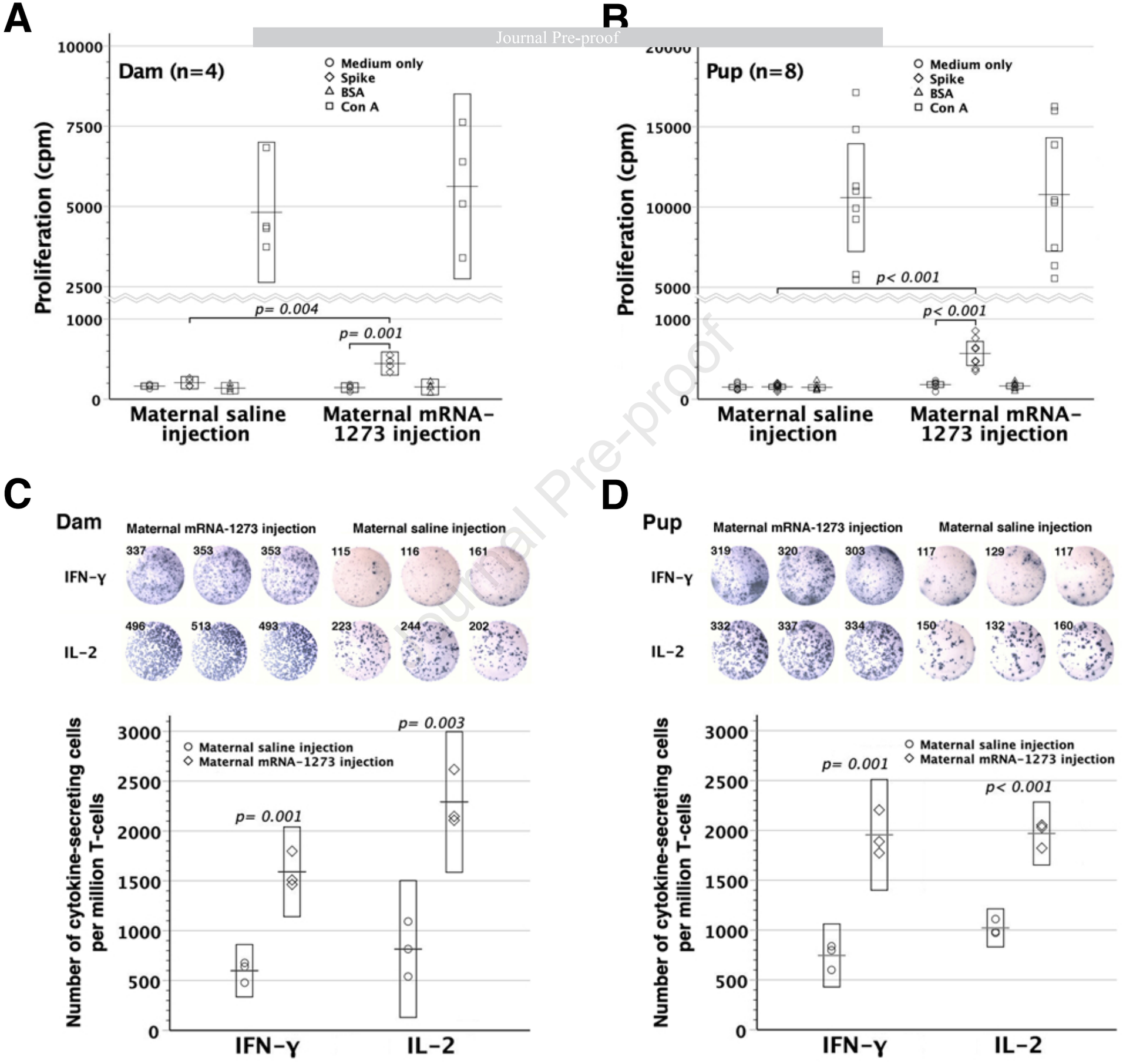


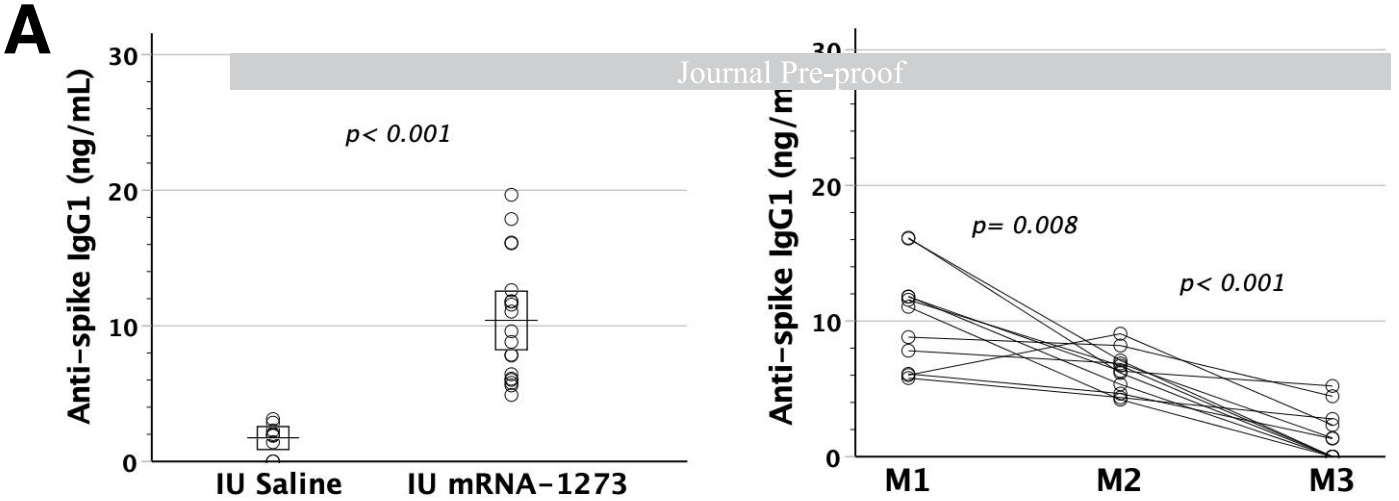


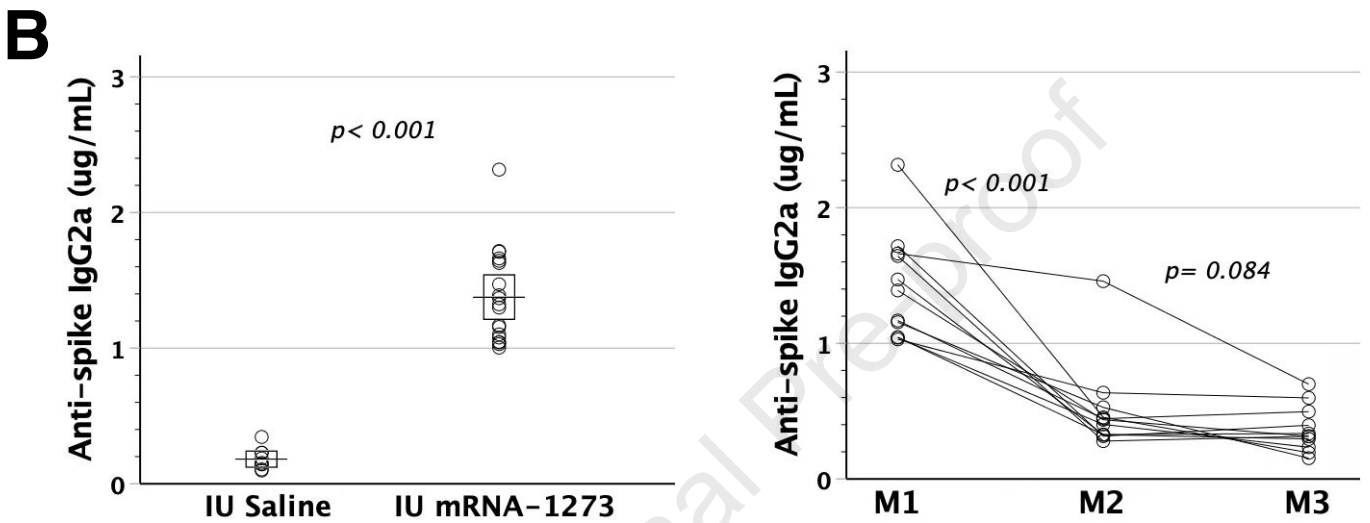


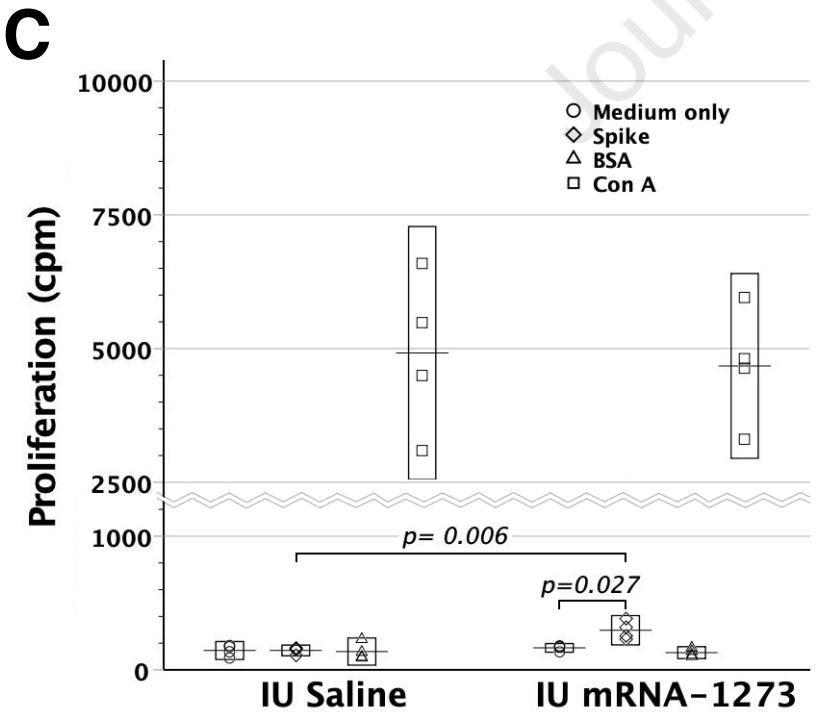


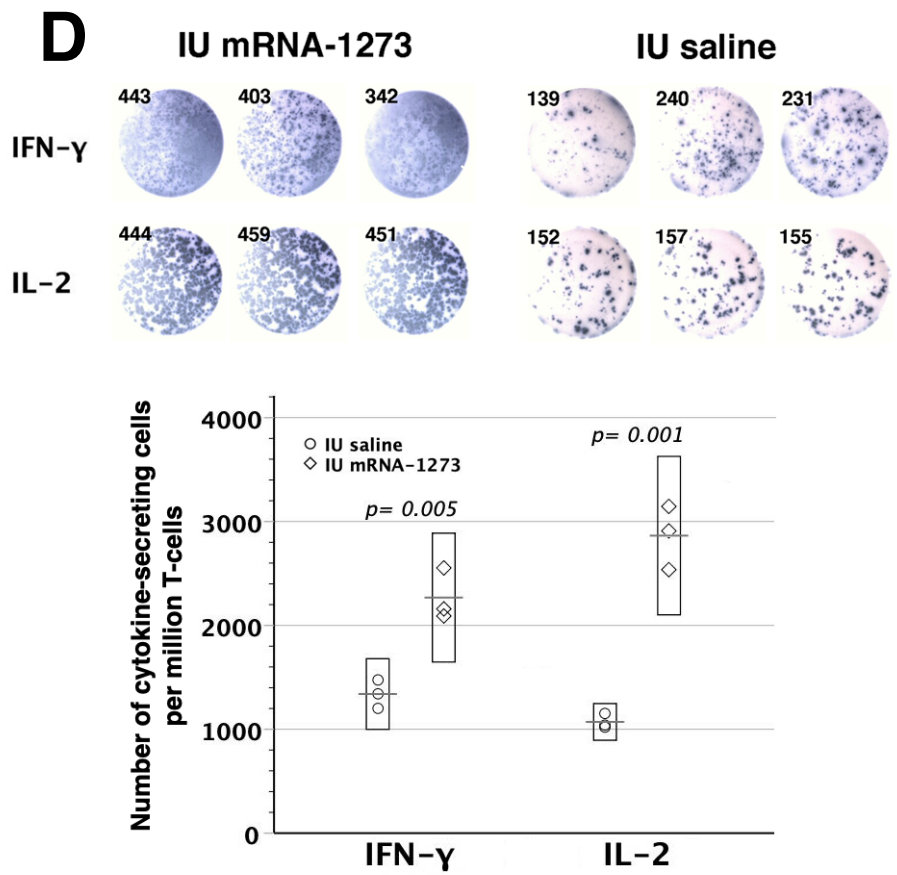












Chen and colleagues demonstrated that mRNA-1273 could cross the placenta and show immunogenicity in the fetus following its administration to pregnant mice. It provided the new insight that mRNA vaccine administration to expectant mothers might endow their newborns with not only passive but also active immunity against pathogens.