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In Vitro Safety and Immunotoxicity Assessment of a Novel mRNA-LNP Vaccine against Cytomegalovirus: Insights into Safety and Immunomodulatory Profiles

Somayeh Mami[1], Sajjad Shekarchian[2], Alireza Naderi Sohi[3], Jafar Kiani[4], Masoud Soleimani[5], and Mohammad Hossein Nicknam[1,2]

[1] Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] Molecular Immunology Research Center, Tehran University of Medical Sciences, Tehran, Iran
[3] Department of Stem Cells and Regenerative Medicine, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
[4] Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran
[5] Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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ABSTRACT

Predominantly a widespread beta herpesvirus, human cytomegalovirus (HCMV) triggers lifelong latent infection in most of the people, and HCMV vaccine development has been designated a high public health priority.

In the current study, the in vitro safety profile and potential immunotoxic effects of a novel messenger RNA (mRNA)-lipid nanoparticle (LNP) vaccine designed against human cytomegalovirus (HCMV) were assessed. The aim was to measure inflammation, allergic reactions, complement activation, cytotoxicity, and hemolytic effects of the mRNA-LNP vaccine. Proinflammatory cytokine secretion, evident in human peripheral blood mononuclear cells (hPBMCs) treated with unmodified mRNA-LNP, was markedly attenuated by incorporating modified nucleotides.

The vaccine appeared incapable of sparking allergic cytokine production or complement activation. Cell viability assays indicated no pronounced cytotoxicity, and hemolysis assays showed no notable hemolytic activity.

The findings suggest that the modified mRNA-LNP vaccine exhibits a promising in vitro safety profile, supporting further development of this vaccine candidate.

Keywords: Cytomegalovirus; CMV; Immunotoxicity; Lipid Nanoparticles; mRNA-LNP; Safety; Vaccine

Corresponding Author: Mohammad Hossein Nicknam, MD, PhD; Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+9821) 611 3207, Fax: (+9821) 642 2337, Email: mhnicknam@sina.tums.ac.ir

INTRODUCTION

Predominantly a widespread betaherpesvirus, human cytomegalovirus (HCMV) triggers lifelong latent infection in most people, and intermittent reactivation

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remains a potentiality.[1,2] While often clinically latent in immunocompetent individuals, HCMV poses a substantial risk to immunocompromised individuals, including solid organ transplant recipients and those with HIV/AIDS, in whom it has the potential to induce severe, potentially fatal disease.[3–5] Notably, congenital HCMV infection, acquired in utero, affects approximately 0.7% of live births worldwide and represents the foremost infectious agent responsible for sensorineural hearing loss and neurodevelopmental sequelae in children.[6,7] Despite this substantial clinical and socioeconomic burden, a licensed vaccine is still lacking, and current antiviral therapies, such as ganciclovir, are hampered by significant toxicities, limited oral bioavailability, and the emergence of drug resistance.[8–10] Recognizing this substantial deficit in therapeutic options, HCMV vaccine development has been designated a high public health priority, with modeling studies predicting that even a vaccine with modest efficacy could substantially reduce the incidence of congenital infection annually.[11,12] Recent advancements in messenger RNA (mRNA)-based vaccines formulated with lipid nanoparticles (LNPs) underpin a persuasive approach for the development of a protective HCMV vaccine.[13–15] LNPs optimize efficient delivery of mRNA to target cells, promoting robust expression of encoded antigens and eliciting potent and durable adaptive immune responses.[15] However, the inherent ability of mRNA-LNP formulations to influence the immune response necessitates thorough preclinical and clinical evaluation to ensure a favorable safety profile and mitigate potential risks of excessive inflammation or immunotoxicity, particularly in vulnerable populations, such as pregnant women.[16,17]

The emergence of mRNA-LNP-based vaccines has revolutionized vaccinology, but their specific molecular composition-a synthesis of lab-created mRNA and lipid-based delivery systems-poses important questions about immunotoxicity.[17–19] LNPs, in addition to augmenting mRNA stability and promoting cellular internalization, possess the capacity to likewise induce innate immune sensors, such as Toll-like receptors (TLRs), potentially triggering an unintended inflammatory response.[20,21] In vitro immunotoxicology assessments are essential to address these potential risks. For example, cytokine profiling assays enable the identification of inappropriate proinflammatory responses.[20,22] On the other hand, Complement-mediated Infusion Reactions (CMIR) and Complement Activation Related Pseudo Allergy (CARPA) are characterized by dose-dependent toxic effects in particular classes of therapeutic agents, including therapeutic oligonucleotides[23–25] and PEGylated liposomal formulations.[26–28] For example, liposomal formulations of doxorubicin (Doxil) and amphotericin (Ambisome), when administered into the systemic circulation, activate complement and result in complement-induced infusion reactions in the individual.[27] In contrast, PEG-based nanoparticles, when injected subcutaneously, can contribute to local complement activation. This activation can improve vaccine efficacy by increasing antigen uptake by dendritic cells and activating T cells, thereby completing the antigen-specific immune response. However, excessive complement activation has the potential to concurrently spark allergic effects (e.g., CARPA).[29–33]

One of the most important points in ensuring the safety and biocompatibility of mRNA-LNP vaccines is in vitro toxicological evaluations, such as MTT assays and hemolysis tests.[34–36] These assays provide crucial insights into the cytotoxicity and compatibility of vaccine components, which play a pivotal role in mitigating risks before progressing to clinical trials.[34–36] The MTT assay is commonly used to evaluate the cytotoxic effects of LNPs on various cell lines, including immune cells and primary human cells.[37,38] This approach provides a means for researchers to ascertain potential dose-dependent toxicity and modify LNP formulations to mitigate adverse reactions, such as inflammation or fever, that have been observed with some mRNA-LNP vaccines.[17,39] Similarly, hemolysis assays are paramount in evaluating the compatibility of LNPs with red blood cells, as some lipid components—such as cationic or ionizable lipids—can disrupt cell membranes and lead to hemolytic anemia.[40-42] By virtue of employing in vitro toxicological assessments, researchers can address overlooked defects that may only become apparent at later clinical stages or after approval, thereby reducing the risks of chronic inflammation, autoimmunity, or other adverse effects associated with mRNA-based therapies.[43,44]

In this study, after mRNA synthesis and encapsulation within the lipid nanoparticle coating, pro-inflammatory cytokines were measured to assess the inflammatory potential of the synthesized mRNA-LNP vaccine. Next, the allergenic potential of the vaccine was investigated by examining type 2 cytokines and the potential complement activation ability. Finally, the

toxic effects of the vaccine on the cell line and its potential hemolysis ability were investigated.

MATERIALS AND METHODS

Vector Construction

An appropriate genetic construct containing optimized 5'UTR and 3'UTR sequences intended for the target gene served to design the sequences of the three *PP65*, *PC* and *gB* genes.[45,46] The bacteriophage T7 promoter functioned to drive the synthesis of the RNA, and the complete sequence was inserted into the pUC57 vector. Upon completion of the design phase, Gene Universal Company synthesized a custom recombinant vector using our design as a guide. Sanger sequencing was used after synthesis to confirm the accuracy of the desired sequences. Transformation of *Escherichia coli* strain Stbl4 with the recombinant plasmids was performed using the heat shock method for cloning the plasmids. LB medium (Cat. No. L3397, Sigma-Aldrich, St. Louis, MO, USA) was deployed to culture the transformed bacteria with kanamycin (Cat. No. J60668.03, Thermo Scientific™, Waltham, MA, USA). FavorPrep™ Plasmid DNA Extraction Kit (Cat. No. FAPDE 000-Maxi, Favorgen Biotech Corp., Ping Tung, Taiwan) was harnessed for the isolation of the cloned plasmids after confirming the identity of the cloned sequences according to the protocol provided by the manufacturer. Then, EcoRI enzyme (Cat. No. FD0274, Thermo Scientific™, Waltham, MA, USA) was utilized for converting the purified circular plasmids into linear plasmids to prepare template cDNA for the IVT reaction.

In vitro Transcription and mRNA Purification

An In vitro Transcription (IVT) reaction served as the means for RNA synthesis. The IVT incorporated T7 RNA Polymerase (Cat. No. M0251S, New England Biolabs, Ipswich, MA, USA) at 100 U/µL and NTP mix (Cat. No. N0466S, New England Biolabs, Ipswich, MA, USA) at 5 mM and nuclease-free water.[47–49] The IVT reaction was then incubated for 90 minutes at 37 °C. The IVT reaction product (1 µL) was loaded onto a 1% agarose gel electrophoresis for analysis of synthesized RNA. Finally, DNase I (Cat. No. M0303S, New England Biolabs, Ipswich, MA, USA) was applied to the entire contents of the IVT microtube for 15 minutes to degrade template DNA and prepare the synthesized RNA for purification. mRNA was purified using the Monarch RNA Purification Kit (Cat. No. T2050L, New England Biolabs, Ipswich, MA, USA) according to the guidelines provided by the manufacturer. The concentration of the transcribed mRNA was then determined using a Nanodrop UV spectrophotometer (epoch2-Biotek, Winooski, VT, USA).

Lipid Nanoparticle Production

Following several adjustments, lipid nanoparticle (LNP) formulations were formulated upon established protocols for siRNA and mRNA delivery.[50–53] Briefly, lipids were dissolved in absolute ethanol at specific molar ratios: 50 (DLin-MC3-DMA, MedChenExpress), 10 (DSPC, Sigma, St. Louis, MO, USA), 38.5 (cholesterol, Sigma), and 1.5 (DMG-PEG2000). Simultaneously, mRNA was dispersed in a sodium citrate buffer (50 mM, pH 4) in an RNase-free tube. Both solutions were passed through a 0.22 µm filter and then mixed using the ethanol injection method at a volume ratio of 3:1 (water:ethanol). The N/P ratio was adjusted to 5.67. The entire procedure was performed under sterile conditions. After 30 minutes, the resulting nanoparticles were diluted in sterile PBS (10 mM, pH 7.2) and transferred to pre-sterilized Amicon Ultra-15 centrifuge filters (MWCO=100 kDa). To facilitate buffer exchange and product concentration, centrifugation was performed at 4000*g* for 15 to 30 minutes, which was repeated three times with the addition of fresh PBS. The final LNP formulation was then stored at 4 °C until required for further use.

Inflammatory and Allergic Cytokine Secretion Assay

First, each of the three mRNAs (encoding *gB*, *PC*, and *pp65* proteins) was synthesized individually with either modified nucleotides (i.e., N1-MethylpseudoUDP and 5-Methyl-CTP) or unmodified ones. Then, they were combined in equal mass ratios and encapsulated in LNPs as described. Human Peripheral Blood Mononuclear Cells (hPBMCs) were isolated from heparinized blood samples using Ficoll-Paque density gradient centrifugation (Cat. No. 002041600, inno-train Diagnostik GmbH, DE) and washed twice with sterile PBS (pH 7.4). The cells were then resuspended in RPMI-1640 medium (supplemented with 10% FBS and 100 µM penicillin-streptomycin) and seeded at 2×10^5 cells per well in a 96-well plate. The cells were subsequently treated with 100 ng/mL of LPS (Cat. No. L2880, Sigma-Aldrich, Germany) or LNPs containing 1, 3, or 5 µg encapsulated mRNA. After 6 to 8 hours, cytokine secretion of TNF-α (Cat. No. KPG-HTNF, KPG, IR), IL-

1β (Cat. No. KPG-HIL1β, KPG, IR), IL-6 (Cat. No. KPG-HIL6, KPG, IR), IL-4 (Cat. No. KPG-HIL4, KPG, IR), IL-5 (Cat. No. D5000B, R&D, Minneapolis, USA), and IL-13 (Cat. No. KPG-HIL13, KPG, IR) was measured according to the manufacturer's instructions.

Complement Activation Assay

To assess complement activation, the Human iC3b ELISA Kit (Cat. No. orb1146729, Biorbyt, Cambridge, UK) was applied in accordance with the manufacturer's instructions. Human blood samples were collected in Serum Separator Tubes. After the samples underwent a 2-hour clotting period at room temperature, centrifugation was performed at 1000*g* for 20 minutes, and the separated serum served as the test material. In order to investigate the effect of mRNA-LNPs on complement activation, serum samples were treated with three different concentrations of mRNA-LNP (1, 3, and 5 µg/mL) for each of the three antigens *PP65*, *PC*, and *gB*. As a positive control, cobra venom factor (CVF) (Cat. No. 233552-M, Sigma-Aldrich, St. Louis, MO, USA) was brought to a final concentration of 10 U/well. CVF is a well-known and potent complement activator and was used to ensure proper assay performance. Serum samples treated with PBS were used as negative controls. Initially, serum samples treated by PBS (negative control), three concentrations of mRNA-LNP (1, 3, and 5 µg/mL) for the three antigens *PP65*, *PC*, and *gB*, as well as CVF (positive control), and incubated for 30 minutes at 37°C. Then, iC3b factor assay was performed according to the manufacturer's instructions of the Human iC3b ELISA Kit (Biorbyt). The final absorbance of all samples was measured at 450 nm using an ELISA reader.

Cell Viability Assay

To assess cell viability, HEK 293 cells were cultured in adherent conditions. After seeding the cells in 96-well plates and allowing them to adhere for 24 hours, varying concentrations of mRNA-LNPs (containing 0.1, 0.5, 1, or 2 µg encapsulated mRNA) were added to the wells. Cell viability was then evaluated using the MTT assay (Abcam, Waltham, MA, USA) at 24-, 48-, and 72-hour post-mRNA-LNP addition, according to the manufacturer's instructions.

Hemolysis assay

Whole blood was procured in tubes containing Li-heparin. In this assay, three concentrations of mRNA-LNP (1, 3 and 5 µg/ml) for the three antigens *PP65*, *PC* and *gB* as well as PBS (as a negative control) and Triton x100 (as a positive control) were treated with whole blood for 4 hours. For hemoglobin assessment, a hemoglobin assay kit (Baharafshan, Tehran, Iran) was exercised as per the manufacturer's protocol. Toward this aim, initially, the concentration of free hemoglobin in plasma (PFH) separated from untreated whole blood was determined. Then, the concentration of hemoglobin in whole blood (at a 251-fold dilution) treated with mRNA-LNP, PBS and 1% Triton x100 was measured. The absorbance of all samples was ascertained at a wavelength of 540 nm. Finally, the percentage of hemolysis was calculated according to the following formula:

$$\%\ Hemolysis = \frac{(Absorbance\ of\ Sample) - (Absorbance\ of\ \text{PFH})}{(Absorbance\ Mean\ of\ Positive\ Control) - (Absorbance\ Mean\ of\ \text{PFH})}$$

Data Analysis Methods

Data analysis was performed using GraphPad Prism version 10 software. one-way ANOVA and Dunnett's post hoc test were used to statistically analyze these data.

RESULTS

Evaluation of the Effect of mRNA-LNP on Pro-inflammatory Factors

We previously undertook a comprehensive mRNA-LNP characterization assessment for another vaccine using the same formulation.[53] For the consequences of the novel mRNA-LNP vaccine upon the triggering of proinflammatory responses, we evaluated the secretion of IL-1β, IL-6, and TNF-α using hPBMCs isolated from healthy donors after treatment with mRNA-LNPs containing unmodified or modified nucleotides. The results evidenced that subjecting hPBMCs to unmodified mRNA-LNPs culminated in a dose-dependent escalation in the secretion of proinflammatory cytokines (Figure 1a–c). To be more precise, at the 5 µg/mL level, a statistically significant elevation in IL-1β levels was discernible (p<0.001) compared to the PBS-treated control group. Correspondingly, at the 5 µg/mL of unmodified mRNA-LNPs, IL-6 release was significantly increased (p<0.05). Concerning TNF-α, exposure of hPBMCs to unmodified mRNA-LNP at 5 µg/mL culminated in a significant escalation of secretion (p<0.01), while the concentration of 3 µg/mL exhibited an upward trajectory in TNF-α levels, although this was not statistically significant. Importantly, the lowest concentration of unmodified

mRNA-LNP (1 μg/mL) failed to spark any significant changes in IL-1β, IL-6, or TNF-α secretion compared to the control group. Unlike mRNA-LNP incorporating unmodified nucleotides, the use of modified nucleotides in the mRNA-LNP formulation significantly mitigated the elicitation of proinflammatory cytokines (1d–f). Treatment of hPBMCs with modified mRNA-LNPs at all tested concentrations (1, 3, and 5 μg/mL) did not culminated in a statistically significant elevation in IL-1β, IL-6, or TNF-α secretion compared to the PBS control group. These findings indicate that the incorporation of modified nucleotides into the mRNA sequence considerably attenuates the in vitro inflammatory potential of LNP-encapsulated mRNA.

Evaluation of Allergic Cytokine Production and Complement Activation

To investigate the capacity of mRNA-LNP for eliciting allergic responses, we measured the concentrations of core T_H2 cytokines (IL-4, IL-5, and IL-13) in the medium containing mRNA-LNP-treated hPBMCs with different concentrations. hPBMCs treated with mRNA-LNP (1, 3, and 5 μg/mL) did not demonstrate any statistically significant shifts in the secretion of IL-4, IL-5, or IL-13 compared with the PBS-treated control group (Figure 2a–c). This suggests that the novel mRNA-LNP vaccine does not stimulate the production of these allergic cytokines in vitro. We also investigated the potential of mRNA-LNP in the serum of healthy donors with the aim of its ability to activate the complement system by measuring iC3b (a marker of complement activation). Evidently from Figure 2d–f, mRNA-LNP treatment at concentrations of 1, 3, and 5 μg/mL did not culminate in a significant rise in iC3b compared to the PBS control group. In contrast, the positive control (CVF), unsurprisingly, triggered a robust and highly significant elevation in iC3b (p<0.0001), confirming the sensitivity of the assay for detecting complement activation.

Assessment of In vitro Toxic Effect

The assessment of the vaccine's toxic effect, which is initially conducted at the cellular level, is one of the fundamental aspects in evaluating the safety of vaccines. It ensures that the vaccine maintains the desired balance between effectiveness and the absence of complex disease and undesirable side effects. To study the toxic effects of the vaccine, the cell viability of the mRNA-LNP-treated HEK293 cell line was investigated. After synthesizing mRNAs encoding all three antigens and their encapsulation into lipid nanoparticles, the cultured cells were incubated with them as shown (Figure 3), cell viability was evaluated at 24, 48, and 73 hours after start of incubation using the MTT assay. Regarding the results, no significant change in cell viability was detected after 24 hours of continuous exposure to mRNA-LNPs. Although a decreasing trend in viability was observed after 48 and 72 hours for cells treated with LNPs harboring 1 and 2 μg mRNA, it was not statistically significant except for cells treated with 2 μg *gB* encoding mRNA at 48 h (p<0.05, Figure 3h).

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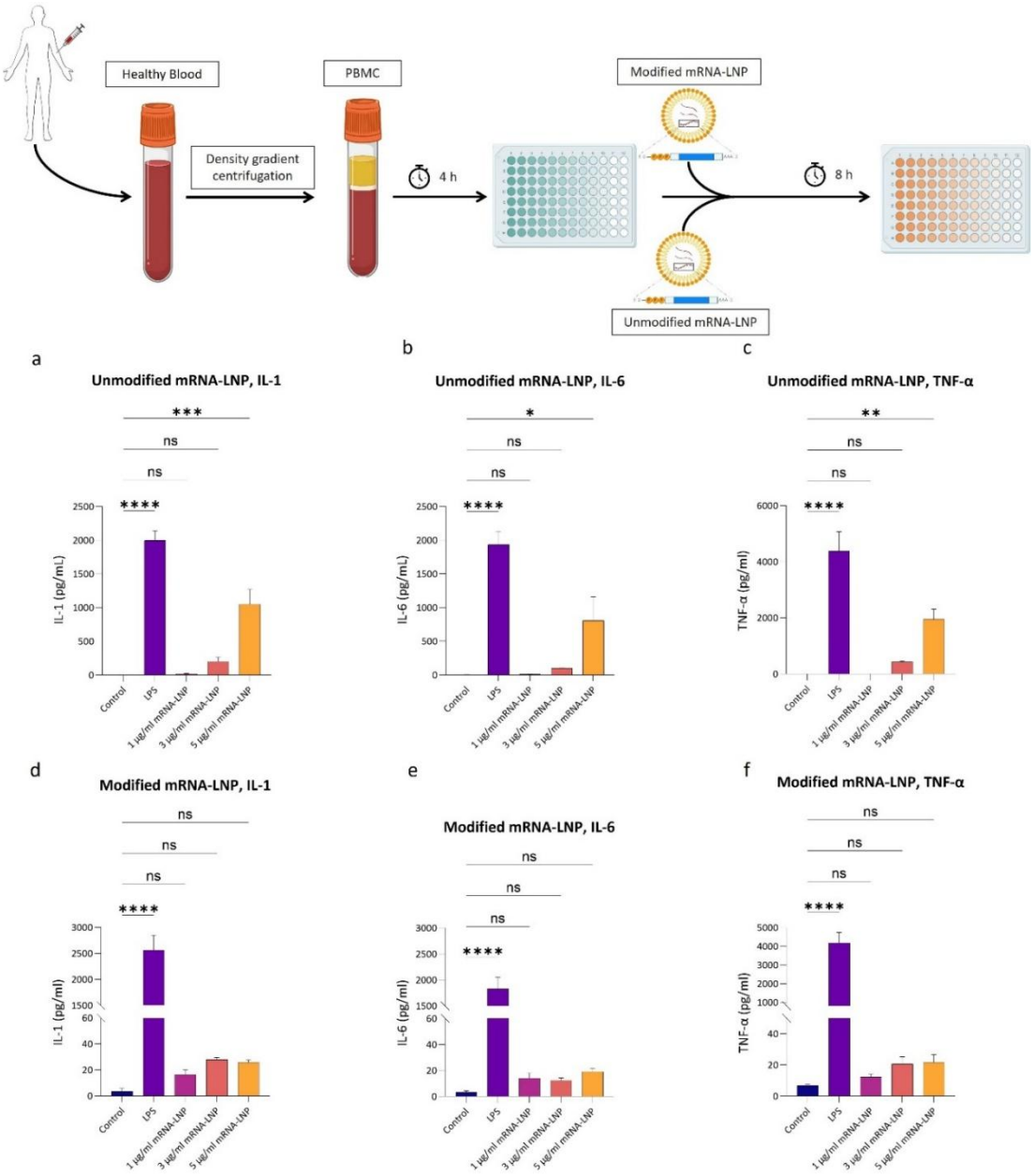

Figure 1. Effect of messenger RNA (mRNA)-lipid nanoparticle (LNP) on Pro-Inflammatory Cytokine Secretion in human peripheral blood mononuclear cells (hPBMCs). a. Secretion of TNF-α in response to unmodified mRNA-LNPs at various concentrations (1 µg/mL, 3 µg/mL, and 5 µg/mL) compared to PBS and LPS controls.b. Secretion of IL-6 in response to unmodified mRNA-LNPs at various concentrations (1 µg/mL, 3 µg/mL, and 5 µg/mL) compared to PBS and LPS controls. c. Secretion of IL-1β in response to unmodified mRNA-LNPs at various concentrations (1 µg/mL, 3 µg/mL, and 5 µg/mL) compared to PBS and LPS controls. d. Secretion of TNF-α in response to modified mRNA-LNPs at various concentrations (1 µg/mL, 3 µg/mL, and 5 µg/mL) compared to PBS and LPS controls. e. Secretion of IL-6 in response to modified mRNA-LNPs at various concentrations (1 µg/mL, 3 µg/mL, and 5 µg/mL) compared to PBS and LPS controls. f. Secretion of IL-1β in response to modified mRNA-LNPs at various concentrations (1 µg/mL, 3 µg/mL, and 5 µg/mL) compared to PBS and LPS controls. Error bars represent mean±SEM. Statistical significance was determined using one-way ANOVA followed by Dunnett's post hoc test (*p<0.05, **p<0.01, ***p<0.001).

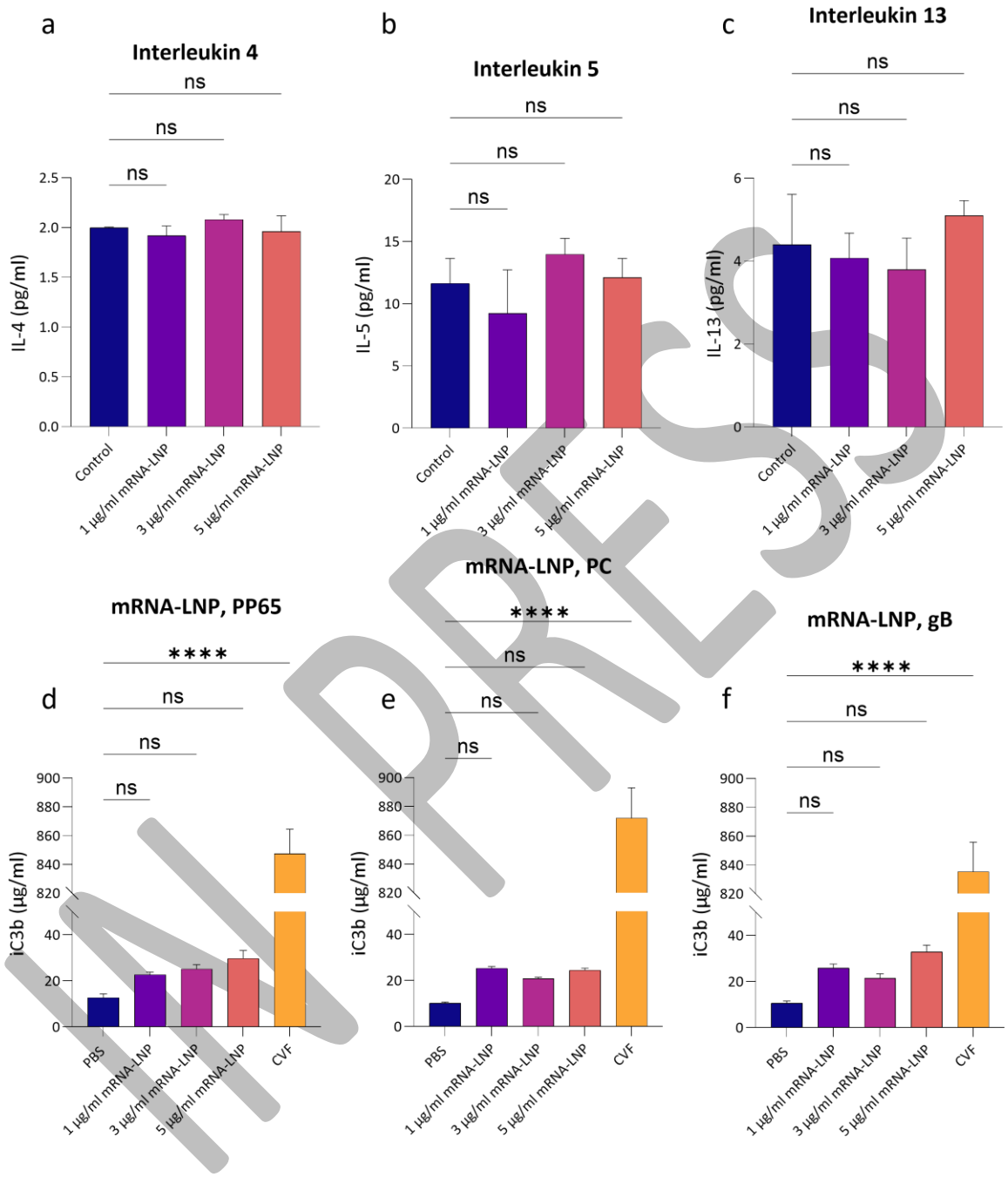

Figure 2. Effect of messenger RNA (mRNA)-lipid nanoparticle (LNP) on allergic cytokine production and complement activation. Human peripheral blood mononuclear cells (hPBMCs) were treated with PBS (Control), mRNA-LNP (1, 3, and 5 µg/ml) for 8 hours. Supernatants were collected for ELISA to measure the levels of (a) IL-4, (b) IL-5, and (c) IL-13. Blood serums were used to measure (d, e, f) iC3b treated by *PP65*, *PC*, and *gB* mRNA-LNP. Data are presented as mean±SEM. Statistical significance was determined by one-way ANOVA with Dunnett's post-hoc test compared to the control group (ns: not significant, **** $p<0.0001$).

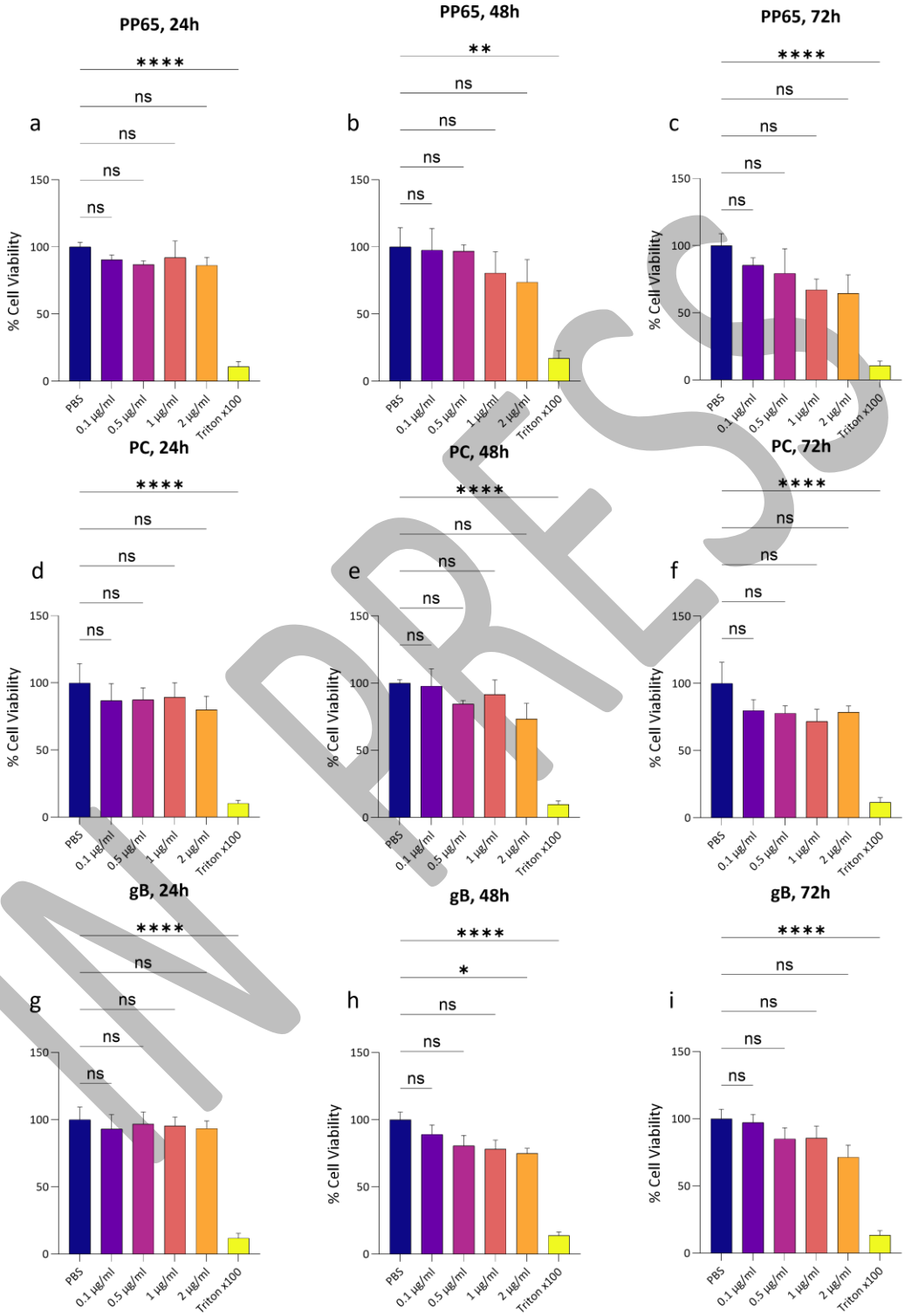

Figure 3. Viability evaluation of messenger RNA (mRNA)-lipid nanoparticle (LNP) using MTT assay. HEK293 cells were treated by different amounts of *gB*, *PC* and *pp65* mRNA-LNPs for 24, 48 and 72 h. Statistical significance was determined by one-way ANOVA and Dunnett's posthoc test. (ns: non-significant, ** p<0.01 and **** p<0.0001).

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Hemolytic Activity of mRNA-LNP

Aiming to evaluate the immediate consequence of mRNA-LNP on blood cells, we conducted an assay to measure hemolysis using whole blood. In this study, the percentage of hemolysis induced by different concentrations of mRNA-LNP targeting three different antigens (*PP65*, *PC*, and *gB*) was measured and compared with the results derived from the PBS control group (as a negative control) and the Triton X-100 control group (as a positive control for hemolysis). Evidently (Figure 4a–c), treatment with mRNA-LNPs targeting *PP65*, *PC*, or *gB* at 1, 3, and 5 µg/mL failed to culminate in significant hemolysis compared to the PBS control group. The percentage of hemolysis remained below 2% for all tested mRNA-LNP concentrations, irrespective of the specific antigen (*PP65*, *PC*, and *gB*), and these values were statistically non-significant (NS) compared to the PBS control group, which exhibited hemolysis levels below 1%. In contrast, Triton X-100 elicited near-total hemolysis, reaching approximately 100% in all cases (p<0.0001). These results suggest that the mRNA-LNP vaccine formulation is devoid of significant hemolytic activity in vitro at the range of concentrations evaluated.

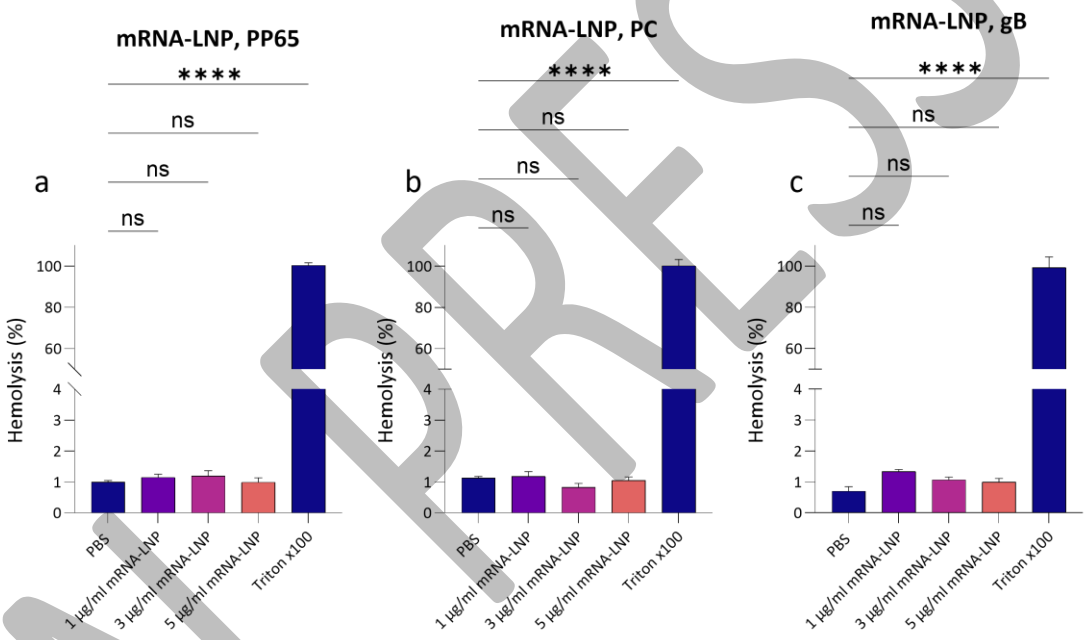

Figure 4. Hemolytic activity of messenger RNA (mRNA)-lipid nanoparticle (LNP). Whole blood was treated with PBS (negative control), mRNA-LNP targeting *PP65* (A), *PC* (B), or *gB* (C) at 1, 3, and 5 µg/ml, or Triton X-100 (positive control) for 4 hours. The percentage of hemolysis was determined by measuring free hemoglobin in the plasma and whole blood incubated with mRNA-LNP. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Dunnett's post-hoc test compared to the PBS control group (ns: not significant, **** p<0.0001).

DISCUSSION

The in vitro immunotoxicity characteristics and safety considerations pertinent to a novel mRNA-LNP-based HCMV vaccine candidate are investigated herein. Given that HCMV poses a chief threat, especially to immunocompromised people and newborns, the development of such a vaccine is strongly warranted, but there is currently no approved vaccine. Offering the potential for robust antigen expression and durable immunity, mRNA-LNP technology represents a highly promising approach. However, owing to the intrinsic immunomodulatory properties ofmRNA-LNP formulations' intrinsic immunomodulatory qualities, rigorous risk profiling is indispensable, the provision of which is detailed herein. The study findings indicate that peripheral blood mononuclear cells (hPBMCs) exhibited a dose-dependent, enhanced pro-inflammatory cytokine response (IL-1β, IL-6, TNF-α) to unmodified mRNA-LNPs. However, this pro-inflammatory

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response was markedly attenuated, and no appreciable elevation in these cytokines was observed when modified nucleotides were incorporated into the mRNA-LNP formulation. Unchanged secretion profiles for key T_H2-associated cytokines (IL-4, IL-5, IL-13) indicated the mRNA-LNP vaccine's negligible capacity to elicit notable allergic reactions. Complement activation, assessed by measuring iC3b, resulted in negligible activation by the mRNA-LNP treatment. HEK293 cell viability measurements revealed no significant alterations after 24 hours of in vitro exposure to mRNA-LNPs, exhibiting only a slight, predominantly statistically insignificant, reduction during extended 48- and 72-hour incubation periods. Based on hemolysis assays, the mRNA-LNP formulation did not significantly induce hemolysis in whole blood.

The observed decrease in pro-inflammatory cytokine release with modified mRNA-LNP formulations is consistent with growing evidence that nucleoside modifications are crucial for reducing the innate immunogenicity of synthetic mRNA. Unmodified exogenous mRNA can be recognized by pattern recognition receptors (PRRs), leading to antiviral and pro-inflammatory responses that impede translation and cause adverse effects.[54–56] Incorporating modified nucleosides, such as pseudouridine (Ψ) or N1-methylpseudouridine (m1Ψ), as used in successful COVID-19 mRNA vaccines, mitigates this by altering mRNA's interaction with key innate immune sensors.[57,58] Specifically, these modifications reduce recognition by endosomal Toll-Like Receptors (TLRs) like TLR3 and TLR7/8, thereby decreasing the production of type I interferons and pro-inflammatory cytokines.[59] They also may dampen the activation of cytosolic sensors like Retinoic Acid-Inducible Gene I (RIG-I) by preventing the necessary conformational changes for downstream signaling. Furthermore, modifications could reduce the activation of other sensors such as protein kinase R (PKR) and 2'-5'-oligoadenylate synthetase (OAS), preventing translational arrest and RNA degradation.[60–62] By mitigating these innate immune pathways, modified mRNA not only reduces pro-inflammatory cytokine secretion but also enhances mRNA stability and translational efficiency, contributing to a better safety profile and potentially more robust adaptive immune responses due to sustained antigen expression without excessive inflammation.

The findings demonstrated that lipid nanoparticles, incorporating Dlin-MC3-DMA as the ionizable lipid, did not trigger an inflammatory response in peripheral blood mononuclear cells (hPBMCs). This lack of stimulation was observed even at high concentrations of modified mRNAs, which naturally involved an increase in amounts of lipid components, including the ionizable lipid, due to the constant N/P ratio. These results align with the study by S. Tahtinen et al, which also showed that Dlin-MC3-DMA induces a lower systemic inflammatory response compared to SM-102, the ionizable lipid used in the Moderna COVID-19 vaccine (i.e., mRNA-1273 or Spikevax).[63] One of the challenges in the field of mRNA vaccines is the dual debate over the use of modified and unmodified nucleotides. While the results of this study and previous research indicate that the use of unmodified nucleotides stimulates the secretion of pro-inflammatory factors from hPBMCs[63], in contrast, T. E. Mulroney and colleagues have shown that the use of modified nucleotides leads to a +1 ribosomal frameshifting, which can lead to the production of unfavorable protein products.[64] Although no adverse consequences of mistranslation of approved SARS-CoV-2 mRNA-based vaccines have been reported, we need to have rational strategies for developing and optimizing mRNA-based vaccines in the future.

The research does not extend outside in vitro evaluations. While fundamental at early safety screening, they lack the fidelity to accurately mirror the multifaceted in vivo circumstances. Therefore, the results need to be extrapolated cautiously, and further in vivo studies are necessary for confirming the safety profile of the vaccine. Measuring a particular set of cytokines was the executable way to assess allergic and inflammatory reactions. A more exhaustive analysis of immune mediators could yield a more comprehensive understanding of the vaccine's immunomodulatory effects. The cell viability assay was performed on a single cell line (HEK293). Assessing the vaccine's toxicity in different cell lines—specifically, immune cells—would increase the robustness of the safety evaluation's validity. Focusing exclusively on short-term outcomes, the study omitted evaluation of the mRNA-LNP vaccine's extended biological sequelae.

Therefore, subsequent in vivo investigations using appropriate animal models are imperative in order to confirm initial results and comprehensively evaluate vaccine safety and efficacy. Further research is also needed to delve deeper into how modified nucleotides decrease the inflammatory reaction, possibly by examining the activation of specific immune system

receptors and communication pathways. Evaluating delayed toxicological manifestations and the durability of the induced immune response (immunogenicity) necessitates comprehensive long-term follow-up studies. The determination of fitting animal models is essential to assess the vaccine's capacity in order that prevent infection or disease, preferably ones that closely resemble the effects of HCMV on humans. Successful preclinical animal testing mandates subsequent human clinical trials designed to confirm the vaccine candidate's safety and immunogenicity for human application.

In conclusion, this study provides a valuable foundation for the further development of the HCMV mRNA-LNP vaccine candidate. The in vitro safety data are encouraging, particularly the finding that modified nucleotides can mitigate the inflammatory potential of the vaccine. However, it is crucial to acknowledge the limitations of the study and emphasize the need for further in vivo and clinical investigations to fully evaluate the safety and efficacy of this promising vaccine.

STATEMENT OF ETHICS

The present study has been granted approval by the ethics committee of Tehran University of Medical Science, bearing the code of ethics IR.TUMS.MEDICINE.REC.1401.750.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

Available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

If applicable, list any artificial intelligence (AI) tools used in preparing this manuscript.

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