The Possible Role of Sex As an Important Factor in Development and Administration of Lipid Nanomedicine-Based COVID-19 Vaccine

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ABSTRACT: Nanomedicine has demonstrated a substantial role in vaccine development against severe acute respiratory syndrome coronavirus (SARS-CoV-2 and COVID-19). Although nanomedicine-based vaccines have now been validated in millions of individuals worldwide in phase 4 and tracking of sex-disaggregated data on COVID-19 is ongoing, immune responses that underlie COVID-19 disease outcomes have not been clarified yet. A full understanding of sex-role effects on the response to nanomedicine products is essential to building an effective and unbiased response to the pandemic. Here, we exposed model lipid nanoparticles (LNPs) to whole blood of 18 healthy donors (10 females and 8 males) and used flow cytometry to measure cellular uptake by circulating leukocytes. Our results demonstrated significant differences in the uptake of LNP between male and female natural killer (NK) cells. The results of this proof-of-concept study show the importance of recipient sex as a critical factor which enables researchers to better consider sex in the development and administration of vaccines for safer and more-efficient sex-specific outcomes.

KEYWORDS: COVID-19, vaccine, sex-specific response, nanomedicine

Sex-disaggregated reports of COVID-19 mortality rate by 38 countries revealed the existence of a male bias mortality in almost all of them (37 out of 38).1 This male dominated mortality against the novel corona virus may be, at least in part, due to the sex specific immune system responses, both to the virus and therapeutics.1 The existence of sex differences in therapeutics have been well documented; for example, it has been described by multiple investigators that sex differences can influence pharmacodynamics and pharmacokinetics of therapeutics and subsequently affect their efficacy and toxicity.2-5 However, there is no robust and consistent sex-specific dosing recommendations for therapeutics, even for the ones that demonstrated clear and validated differences in pharmacokinetics between sexes.6

Due to sex differences in physiology, genetic background, Toll-like receptor (TLR) pathway response,7 and microbiome, males and females show different response to vaccines.8 Females show a higher level of humoral immune response and experience more severe side effects and autoimmune diseases (in both children and the adult population) compared to males. For example, they showed a more severe immune response to vaccines developed for protection against hepatitis, herpes, influenza, pertussis, and tetanus.9,10 Sex differences have been observed in vaccine uptake and subsequent adverse/therapeutic effects, and therefore, it should be considered in vaccine formulation and dosage prescribed for men and women.

Despite the huge concern raised by several scientists in considering the sex for vaccine development and administration for COVID-19,11,12 the current practice of vaccine administration which started in the many countries lacks sex-specific instructions/dosage.13,14 More importantly, the vaccine response (e.g., immune response and the extent of the possible adverse events) are both affected by sex.15 As the two of the leading companies (i.e., Pfizer/BioNTech and Moderna) used nanoparticle-based vaccines in the fight against the COVID-19 pandemic, the role of sex for these vaccines would be more complicated and important than non-nanoparticle-based vaccines. The main reason for this claim is that the role of sex in nanomedicine (although revealed to be of a significant importance) has been poorly investigated.16,17

According to very recent reports, both vaccines performed slightly better in men than in women. More specifically, the Pfizer/BioNTech Vaccine BNT162b2 mRNA and the mRNA-1273 from Moderna were 96.4% and 95.4% effective in men and 93.7% and 93.1% effective in women, respectively.18,19 More in-depth and mechanistic research should be conducted to robustly identify the nature and mechanism of sex-specific protective immune responses to COVID-19 and to understand why males are better protected than females.

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RESULTS AND DISCUSSION

Nanoparticle-based vaccines developed by Pfizer/BioNTech and Moderna are based on the use of various lipids which all have different roles in stabilization, encapsulation, cellular entry, and mRNA release. Among these lipids, ionizable lipid has a key role as it binds messenger RNA by electrostatic interactions and also plays a role in endosomal escape. Following internalization into a cell and endosome, the ionizable lipids will be protonated and bind to the endogenous phospholipid of the endosome, which results in disruption of its bilayer and release of its cargo, the mRNA which will be translated into the immunogenic spike SARS-CoV-2 protein by ribosomes.20

Upon injection of the COVID-19 vaccine in the deltoid muscle, it will be captured by dendritic cells, front-line immune cells which are particularly present under the skin and in or near muscle tissue; the dendritic cells will then activate the adaptive immune system through producing and presenting the antigen to T cells.21 This prompted us to explore the capture of cationic lipid nanoparticles by immune cells and evaluate in vitro if the patient’s sex has any effect on its uptake and cellular behavior. In this case, DOTAP liposomes were

Figure 1. (a) Representative intensity size distributions of cationic lipid nanoparticles before (dark gray) and after (light gray) 1-h incubation with human plasma (HP) at 37 °C (i.e., DOTAP and DOTAP+HP respectively). (b) Size of nanoparticles before and after exposure to HP. Results are given as mean ± standard deviation (SD) of three replicates. (c) Representative zeta potential distributions of cationic lipid nanoparticles before and after 1-h incubation with HP at 37 °C. (d) Zeta potential of cationic lipid nanoparticles before and after 1-h incubation with HP at 37 °C. Statistical significance was evaluated by Student’s t test: *P < 0.05, **P < 0.001.

Figure 2. Leukocyte uptake of cationic lipid nanoparticles in whole blood of healthy donors measured as a percentage of FITC positive cells. Each value is the average ± standard deviation (n = 8 males and n = 9 females). Statistical significance of the difference in cellular uptake was evaluated using the Student’s t test: *p values < 0.05.
chosen as the model system of cationic lipid nanoparticles, which were exposed to whole blood of 18 healthy donors (10 females and 8 males), and cellular uptake by circulating leukocytes was measured by flow cytometry. An exposure time of 30 min was chosen according to previous findings. Size and zeta potential of DOTAP liposomes before and after incubation with human plasma (HP) are displayed in Figure 1.

Figure 1 showing that precoated lipid NPs are larger than their pristine counterpart. This finding unambiguously demonstrates that the observed reduction in capture by immune cells is not due to a nonspecific effect of the biomolecular corona and is not due to a nonspecific size effect. Protein precoating could be considered as a potential strategy in developing a delivery platform able to reduce undesired immune responses (e.g., severe allergic reactions) irrespective of the waning of antibody titers may result in some immunity. Several years after infection, T cell responses to SARS-CoV-1 and MERS-CoV can still be detected.

The performance of both the humoral and cell-mediated immune responses is known to be affected by sex. For example, the stronger immune system of younger adult females may be related to a higher population of the T and B cells as well as their more robust response, while aging and menopause may affect females more, which results in lower numbers of NK cells and their activity toward pathogens. Here, our results may also indicate that that difference in NK cells’ uptake of DOTAP liposomes between males and females may ultimately result in different immune responses. But whether this generates different antibody responses and long-term responses by T cells is still unclear and needs further investigation.

When NPs are exposed to bodily fluids, they are coated by a personalized protein corona that may influence their biological identity in a patient-dependent manner, thus posing serious concerns in the clinical application of these materials. As an alternative strategy, precoating NPs with plasma proteins and antibodies is a promising approach to enable controlled immune interactions. To test this suggestion, DOTAP liposomes were pre-exposed in human plasma and subsequently exposed to patients’ whole blood. Preincubating cationic lipid nanoparticles to human plasma drastically reduced the uptake by immune cells and eliminated any gender difference (Figure 3). According to the literature, larger size NPs are more avidly internalized than smaller ones. This is in apparent disagreement with size results reported in Figure 1 showing that precoated lipid NPs are larger than their pristine counterpart. This finding unambiguously demonstrates that the observed reduction in capture by immune cells is caused by the biomolecular corona and is not due to a nonspecific size effect. Protein precoating could be considered as a potential strategy in developing a delivery platform able to reduce undesired immune responses (e.g., severe allergic reactions).
reaction) and produce therapeutic effects that are not influenced by the patient’s sex.

In summary, based on the outcomes of this proof-of-concept study, we emphasize that the role of sex needs to be carefully considered in nanoparticle-based vaccine development and administration. The sex-specific outcomes of the clinical trials by the leading companies have yet to be reported in detail (e.g., comorbidity and the demographic information on the participants). The very recent meta-analysis report of a huge number of confirmed global COVID-19 cases showed that males and females have almost the same prevalence but different mortalities; men are more at risk of severe disease outcome and death compared to females. In the case of COVID-19, sex bias in the disease severity could be linked to sex differences in physiology, sex chromosomes (XX and XY), immune response, habits, culture, behavior, and ethnicity. In addition, sex differences in the prevalence of chronic disease (e.g., respiratory diseases, cardiovascular diseases, and diabetes) could also potentially associate with sex bias in severity and mortality. These critical concerns raise the question of whether potential sex-dependent therapeutic/toxic effects of the COVID-19 vaccine have been fully considered through vaccine development and administration.

**MATERIALS AND METHODS**

**Preparation of Cationic Lipid Nanoparticles.** DOTAP was bought from Avanti Polar Lipids (Alabaster, AL) and dissolved in chloroform. After solvent evaporation, the lipid film was hydrated with water to obtain a 1 mg/mL lipid solution. Then, a mechanical extrusion was performed 20 times through a 0.1 μm polycarbonate filter using the Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Biocoronated cationic lipid nanoparticles were prepared by exposure to human plasma (HP = 50%) for 1 h at 37 °C.

**Size and Zeta-Potential Experiments.** Dynamic light scattering (DLS) and microelectrophoresis (ME) measurements were performed with a Zetasizer Nano ZS90 (Malvern, UK) at room temperature. Positioning and attenuation were employed under automatic default conditions. Results are reported as the mean ± standard deviation of three replicates.

**Ethic Statement.** The Ethics Committee of the Sapienza University of Rome conferred the approval for the study. All healthy donors were informed and gave written consent in accordance with the Declaration of Helsinki.

**Particle Sequestration from Circulating Leukocytes.** A total of 120 μL of peripheral whole blood was incubated with 50 μg/mL of either pristine or biocoronated NBD-labeled cationic lipid nanoparticles for 30 min at 37 °C and then washed with 2 mL of physiological solution by centrifugation. A buffer of 12 mM NaHCO₃, 155 mM NH₄Cl, and 0.1 mM EDTA was used to lyse red blood cells. Then, leukocytes were labeled for 25 min at 4 °C with the following diluted antibodies: anti-CD3/PerCP (cat. 347344, dilution 1:50), CD56/PE (cat. 340363, dilution 1:50), anti-CD4/APC (cat. 555349, dilution 1:10), anti-CD14/APC-H7 (cat. 560180, dilution 1:50), anti-CD45/PE-Cy7 (cat. 557748, dilution 1:100), and anti-CD19/VS05 (cat. 561121, dilution 1:50), all from BD Biosciences. The fluorescence of internalized nanoparticles was evaluated by fluorescence-activated cell sorting (FACS) analysis with a FACS Canto (BD Biosciences, San Jose, CA). The data analysis was performed using the FlowJo program as reported elsewhere. Data were generated from a total of 17 samples contributed by 8 male and 9 female healthy donors.

**Statistical Information.** Graphs show the experimental data as a mean ± standard deviation. Sample size: n = 7 for pristine cationic lipid nanoparticles, males; n = 10 for pristine cationic lipid nanoparticles, females; n = 8 for biocoronated cationic lipid nanoparticles, males; n = 9 for biocoronated cationic lipid nanoparticles, females. Student’s t-test was employed.

**Data Availability.** Data sets are available from the corresponding authors on reasonable request.

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**Notes**

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**REFERENCES**

(1) Scully, E. P.; Haverfield, J.; Ursin, R. L.; Tannenbaum, C.; Klein, S. L. Considering how biological sex impacts immune responses and COVID-19 outcomes. *Nat. Rev. Immunol.* 2020, 20, 442.

(2) Meibohm, B.; Beierle, I.; Derendorf, H. How important are gender differences in pharmacokinetics? *Clin. Pharmacokinet.* 2002, 41 (5), 329–42.

(3) Sakuma, T.; Kawasaki, Y.; Jarukamjorn, K.; Nemoto, N. Sex Differences of Drug-metabolizing Enzyme: Female Predominant Expression of Human and Mouse Cytochrome P450 3A Isoforms. *J. Health Sci.* 2009, 55 (3), 325–337.
(4) Schwartz, J. B. The Influence of Sex on Pharmacokinetics. Clin. Pharmacokinet. 2003, 42 (2), 107–121.

(5) Soldin, O. P.; Mattison, D. R. Sex differences in pharmacokinetics and pharmacodynamics. Clin. Pharmacokinet. 2009, 48 (3), 143–57.

(6) Anderson, G. D. Sex and racial differences in pharmacological response: where is the evidence? Pharmacogenetics, pharmacokinetics, and pharmacodynamics. Journal of Women’s Health 2005, 14 (1), 19–29.

(7) Khan, N.; Summers, C. W.; Helbert, M. R.; Arkwright, P. D. Effects of age, gender, and immunosuppressive agents on in vivo toll-like receptor pathway responses. Hum. Immunol. 2010, 71 (4), 372–376.

(8) Fischinger, S.; Boudureau, C. M.; Butler, A. L.; Streeck, H.; Alter, G. In Sex differences in vaccine-induced humoral immunity. Seminars in Immunopathology. Springer, 2019; pp 239–249.

(9) Flanagan, K. L.; Fink, A. L.; Plebanski, M.; Klein, S. L. Sex and gender differences in the outcomes of vaccination over the life course. Annu. Rev. Cell Dev. Biol. 2017, 33, 577–599.

(10) Fink, A. L.; Klein, S. L. Sex and gender impact immune responses to vaccines among the elderly. Physiology 2015, 30 (6), 408–416.

(11) Bischof, E.; Wolfe, J.; Klein, S. L. Clinical trials for COVID-19 should include sex as a variable. J. Clin. Invest. 2020, 130 (7), 3350.

(12) McCartney, P. R. Sex-Based Vaccine Response in the Context of COVID-19. Journal of Obstetric, Gynecologic & Neonatal Nursing 2020, 49 (5), 405–408.

(13) Everything you need to know about the Pfizer/BioNTech covid-19 vaccine. https://www.newsscientist.com/article/2261805-everything-you-need-to-know-about-the-pfizer-biontech-covid-19-vaccine/.

(14) Pfizer and BioNTech Announce Vaccine Candidate against Covid-19 Achieved Success in First Interim Analysis from Phase 3 Study. https://www.pfizer.com/news/press-release/press-release-detail/pfizer-and-biontech-announce-vaccine-candidate-against.

(15) McCartney, P. R. Sex-Based Vaccine Response in the Context of COVID-19. J. Obstet Gynecol Neonatal Nurs 2020, 49 (5), 405–408.

(16) Hajipour, M.; Aghaverdi, H.; Serpooshan, V.; Vali, H.; Sheibani, S.; Mahmoudi, M. Sex as an important factor in nanomedicine. Nat. Commun. 2021, 12.

(17) Sharifi, S.; Caracciolo, G.; Pozzi, D.; Digiacomo, L.; Swann, J.; Daldrup-Link, H. E.; Mahmoudi, M. The role of sex as a biological variable in the efficacy and toxicity of therapeutic nanomedicine. Adv. Drug Delivery Rev. 2021, DOI: 10.1016/j.addr.2021.04.028.

(18) Polack, F. P.; Thomas, S. J.; Kitchin, N.; Absalon, J.; Gurtman, A.; Lockhart, S.; Perez, J. L.; Perez Marc, G.; Moreira, E. D.; Zerbini, C.; Bailey, R.; Swanson, K. A.; Roychoudhry, S.; Li, P.; Kalina, W. V.; Cooper, D.; Klin, S. L. Clinical trials for COVID-19 should include sex as a variable. J. Clin. Invest. 2020, 130 (7), 3350.

(19) Sheibani, S.; Mahmoudi, M.; Daldrup-Link, H. E. Can the biomolecular corona induce an allergic reaction? Biomolecules 2018, 8 (4), 197–207.

(20) Szewczyk, S.; Krzak, A.; Knop, D.; Trumpf, H.; Heinold, A.; Heinemann, F. M.; Thümmler, L.; Temme, C.; Breyer, M.; Witzke, O.; Dittmer, U.; Lenz, V.; Horn, P. A.; Lindemann, M. Cellular Immunity in COVID-19 Convalescents with PCR-Confirmed Infection but with Undetectable SARS-CoV-2-Specific IgG. Emerging Infect. Dis. 2021, 27 (1), 122.

(21) Zuo, J.; Dowell, A. C.; Pearce, H.; Verma, K.; Long, H. M.; Begun, J.; Aiano, F.; Amin-Chowdhury, Z.; Hallis, B.; Stapley, L.; Borrow, R.; Linley, E.; Ahmad, S.; Parker, B.; Horsley, A.; Amirthalingam, G.; Brown, K.; Ramsay, M. E.; Ladhani, S.; Moss, P. Robust SARS-CoV-2-specific T cell immunity is maintained at 6 months following primary infection. Nat. Immunol. 2021, 22, 620.

(22) Zuo, J.; Dowell, A. C.; Pearce, H.; Verma, K.; Long, H. M.; Begun, J.; Aiano, F.; Amin-Chowdhury, Z.; Hallis, B.; Stapley, L.; Borrow, R.; Linley, E.; Ahmad, S.; Parker, B.; Horsley, A.; Amirthalingam, G.; Brown, K.; Ramsay, M. E.; Ladhani, S.; Moss, P. Robust SARS-CoV-2-specific T cell immunity is maintained at 6 months following primary infection. Nat. Immunol. 2021, 22, 620.

(23) Zuo, J.; Dowell, A. C.; Pearce, H.; Verma, K.; Long, H. M.; Begun, J.; Aiano, F.; Amin-Chowdhury, Z.; Hallis, B.; Stapley, L.; Borrow, R.; Linley, E.; Ahmad, S.; Parker, B.; Horsley, A.; Amirthalingam, G.; Brown, K.; Ramsay, M. E.; Ladhani, S.; Moss, P. Robust SARS-CoV-2-specific T cell immunity is maintained at 6 months following primary infection. Nat. Immunol. 2021, 22, 620.

(24) Sariol, A.; Perlman, S. Lessons for COVID-19 Immunity from Other Coronavirus Infections. Immunity 2020, 53 (2), 248–263.

(25) Gubbels Bupp, M. R.; Potluri, T.; Fink, A. L.; Klein, S. L. The Confluence of Sex Hormones and Aging on Immunity. Front. Immunol. 2018, 9, 1269.

(26) Yovel, G.; Shakkar, K.; Ben-Eliyahu, S. The effects of sex, menstrual cycle, and oral contraceptives on the number and activity of natural killer cells. Gynecol. Oncol. 2001, 81 (2), 254–62.

(27) Sheibani, S.; Basu, K.; Farnudi, A.; Ashkarran, A.; Ichikawa, M.; Presley, F. F.; Bui, K. H.; Eitjeabi, M. R.; Vali, H.; Mahmoudi, M. Nanoscale characterization of the biomolecular corona by cryo-electron microscopy, cryo-electron tomography, and image simulation. Nat. Commun. 2021, 12 (1), 1–9.

(28) Dawson, K. A.; Yang, Y. Current understanding of biological identity at the nanoscale and future prospects. Nat. Nanotechnol. 2021, 16 (3), 229–242.

(29) Simon, J.; Müller, L. K.; Kokkinopoulou, M.; Lieberwirth, I.; Morsbach, S.; Landfester, K.; Mailänder, V. Exploiting the biomolecular corona: pre-coating of nanoparticles enables controlled cellular interactions. Nanoscale 2018, 10 (22), 10731–10739.

(30) Yu, S. S.; Lau, C. M.; Thomas, S. N.; Jerome, W. G.; Maron, D. J.; Dickerson, J. H.; Hubbell, J. A.; Giorgio, T. D. Size- and charge-dependent non-specific uptake of PEGylated nanoparticles by macrophages. Int. J. Nanomed. 2012, 7, 799–813.

(31) List, Y.; Jerkic, M.; Slutsky, A. S.; Zhang, H. Molecular mechanisms of sex bias differences in COVID-19 mortality. Critical Care 2020, 24 (1), 1–6.

(32) Humphries, K.H.; Izadnejadgar, M.; Sedlak, T.; Saw, J.; Johnston, N.; Schenck-Gustafsson, K.; Shah, R.U.; Regitz-Zagrosek, V.; Grewal, J.; Vaccarino, V.; Wei, J.; Bairey Merz, C.N. Sex
differences in cardiovascular disease—impact on care and outcomes. 
*Front. Neuroendocrinol.* **2017**, *46*, 46.

(41) Mauvais-Jarvis, F. Sex differences in metabolic homeostasis, diabetes, and obesity. *Biol. Sex Differ.* **2015**, *6* (1), 1−9.

(42) Pinkerton, K. E.; Harbaugh, M.; Han, M. K.; Jourdan Le Saux, C.; Van Winkle, L. S.; Martin, W. J.; Kosgei, R. J.; Carter, E. J.; Sitkin, N.; Smiley-Jewell, S. M.; George, M. Women and lung disease. Sex differences and global health disparities. *Am. J. Respir. Crit. Care Med.* **2015**, *192* (1), 11−16.

(43) Vulpis, E.; Cecere, F.; Molfetta, R.; Soriani, A.; Fionda, C.; Peruzzi, G.; Caracciolo, G.; Palchetti, S.; Masuelli, L.; Simonelli, L.; D’Oro, U.; Abruzzese, M. P.; Petrucci, M. T.; Ricciardi, M. R.; Paolini, R.; Cippitelli, M.; Santoni, A.; Zingoni, A. Genotoxic stress modulates the release of exosomes from multiple myeloma cells capable of activating NK cell cytokine production: Role of HSP70/TLR2/NF-κB axis. *OncoImmunology* **2017**, *6* (3), No. e1279372.