approved by the Institutional Review Boards (IRB) at St. Joseph's Hospital (IRB number PHXB16-0027-10-18).

Exosome isolation and nanoparticle tracking analysis

Exosomes were isolated from 500 µl of plasma using Invitrogen Exosome Isolation Kit followed by 0.22-micron filtration (7). All exosomes were analyzed for size by NanoSight NS300 (Malvern Panalytical, Great Malvern, U.K.), and the mean size of the particles used in our experiments was <200 nm (8).

Detection of Abs to SARS-CoV-2 spike protein and nucleocapsid protein from human plasma samples

Development of Abs to SARS-CoV-2 spike Ag was determined using an ELISA developed in our laboratory. In brief, 1 µg/ml SARS-CoV-2 spike protein (Sino Biological) suspended in PBS was coated on an ELISA plate and incubated overnight at 4°C. Human plasma was added to these plates at 1:750 dilution. Detection was performed using secondary anti-human IgG-HRP (1:10,000) and developed using tetramethylbenzidine substrate and read at 450 nm. Plasma samples from individuals infected with SARS-CoV-2 ($n = 10$) and healthy individuals immunized with both doses of vaccine ($n = 20$) were used as positive controls. Healthy individuals with no history of SARS-CoV-2 infection and no vaccination for SARS-CoV-2 were used as negative controls ($n = 20$). Ab concentration was calculated using a standard curve from known concentrations of respective Abs (Thermo Fisher Scientific).

Characterization of exosomes using Western blot

Total exosome protein (15 µg) was resolved by PAGE, and the proteins were transferred onto a polyvinylidene difluoride membrane. Western blots were performed as described in (9). The band intensity of the target protein was quantified using ImageJ software, and all the blots were normalized with CD9.

Transmission electron microscopy of isolated exosomes for SARS-CoV-2 spike protein

Exosomes were labeled with immunogold and mouse anti–SARS-CoV-2 spike Ab, and coronavirus FIPV3-70 Ab (1:100) was added to the grids. Grids were washed and stained with uranyl acetate and viewed by transmission electron microscopy (JEOL USA, Peabody, MA) (10).

ELISPOT for human T cell responses to SARS-CoV-2 spike protein Ag

Blood was obtained from individuals after obtaining informed consent, and the study was approved by the IRB (IRB number PHXB16-0027-10-18). PBMC was isolated by Ficoll-isopaque gradient separation (ICN Biomedicals, Aurora, OH) and cryopreserved. Later, these PBMCs were processed for ELISPOT assay as described in previous publications (9, 10).

Immunization of mice with exosomes with SARS-CoV-2 Ag

C57BL/6 mice were immunized s.c. with exosomes isolated from vaccinated individuals positive for SARS-CoV-2 spike protein (exosomes from day 14 after dose 2 of vaccine). Three groups of animals were immunized without any adjuvants with 100 µg on days 1, 7, and 21: 1) control group of animals ($n = 5$), 2) exosomes isolated from one healthy individual following vaccination ($n = 5$), 3) exosomes isolated from second healthy individual following vaccination ($n = 5$), and 4) mice immunized with SARS-CoV-2 spike protein ($n = 5$). Animals were sacrificed at day 30, blood was collected for ELISA, and spleens were harvested for T cell responses.

Detection of Abs to SARS-CoV-2 spike protein in mice serum

Development of Abs to SARS-CoV-2 spike Ag was determined using an ELISA, as described in previous section.

ELISPOT for murine splenocyte responses to SARS-CoV-2 spike protein Ags

Mice spleens were harvested at day 30 postimmunization, and splenocytes were isolated by Ficoll-Hypaque gradient centrifugation and analyzed by ELISPOT as described previously (9, 10).

Statistical analysis

Data were analyzed using Prism 8.0 software (GraphPad). The Ab levels for SARS-CoV-2 spike protein and the OD of exosomes containing SARS-CoV-2 spike protein were compared using Wilcoxon rank test. Results from animal experiments were analyzed using two-way ANOVA. Data were expressed as mean and SD. The p values <0.05 were considered statistically significant.

Results and Discussion

Exosome isolation

The mean size of the particles used in our study were <200 nm, in agreement with the exosome size described by the International Society for Extracellular Vesicles. Representative images for exosomes are given in Fig. 1A. There was no significant difference in the number of exosomes from different individuals.

Transmission electron microscopy demonstrated the surface expression of SARS-CoV-2 spike protein in isolated exosomes from vaccinated individuals

We performed transmission electron microscopy using Abs specific for SARS-CoV-2 spike to demonstrate the presence of SARS-CoV-2 Ags on the surface of exosomes from control and healthy vaccinated individuals. Exosomes from vaccinated individuals are positive for SARS-CoV-2 Ag (Fig. 1B). We have also stained both the exosome samples with coronavirus FIPV3-70 Ab as negative control and did not observe any positive reaction in exosomes (Fig. 1B).

Kinetics of development of Abs for SARS-CoV-2 spike protein in vaccinated people

Abs to SARS-CoV-2 spike protein were detected in all the healthy vaccinated individuals after day 14 of the second dose of the vaccine (mean concentration, 2401.25 ± 773.45 ng/ml) with p values <0.0001 when compared with no vaccination and day 14 of the first dose of the vaccine. The Ab levels after 4 mo of vaccination had decreased to 1107.38 ± 681.63ng/ml. This decrease is significantly lower compared with the Abs developed at day 14 following the second shot of vaccine ($p = 0.0313$) (Fig. 2A).

Circulating exosomes isolated from vaccinated individuals contained SARS-CoV-2 spike protein Ag S2

We analyzed plasma from vaccinated healthy individuals at days 0, 7, and 14 after the first dose of the vaccine and day 14 of the second dose for the presence of exosomes carrying the SARS-CoV-2 spike protein (Fig. 2B, 2C). The results demonstrated the presence of SARS-CoV-2 spike Ag S2 on exosomes at day 14 of dose 1. There is a significant increase in the concentration of the spike protein at day 14 of dose 2, with a p value of 0.0299. The amount of SARS-CoV-2 spike protein in exosomes after 4 mo of both doses of vaccine was significantly decreased compared with day 14 after the second dose, with the p value of 0.0078. Kinetics of Ab development to the spike protein and exosomes with spike protein for each healthy individual at different time points (day 14 after first and second dose and 4 mo after second dose) are shown in Fig. 2D. Both the kinetics of Ab development and the amount of SARS-CoV-2 spike protein exosomes are in agreement with each other, as both are increased following the second booster dose at day 14 (Fig. 2E). There is a decrease in Ab levels to the SARS-CoV-2 spike protein and the amount of SARS-CoV-2 spike protein in

nach 14 Tagen = 0,0299
nach 4 Monaten = 0,0078 (= 26,1% der Spike-Proteine noch vorhanden)