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Letter to the Editors-in-Chief

Circulating immune cells with megakaryocyte signature in response to COVID-19 mRNA vaccination

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Coronavirus disease 2019 (COVID-19) is a life-threatening systemic disease caused by the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). Faced with this public health emergency, the scientific community developed several vaccines. Among them, BNT162b2 (Pfizer/BioNTec) and mRNA-1273 (Moderna) are the first broad-spectrum application of mRNA-based vaccine technology, and Ad26.COV2.S (Johnson and Johnson) is an Adenoviral vector-based vaccine, which have been granted emergency use authorization by Food and Drug Administration (FDA). While these vaccines provide a key to control the pandemic, very rare thrombocytopenic side effects such as vaccine-induced immune thrombotic thrombocytopenia (VITT) [1] and immune thrombocytopenic purpura (ITP) [2] have been reported. Cases of VITT have been mostly linked to Adenoviral vector-based vaccines, and patients with ITP have been reported following mRNA vaccines. In addition, Flego et al. recently reported enhanced platelet turnover and a transient platelet decrease in a group of subjects after BNT162b2 administration [3]. Their study provided evidence for the role of platelets in the immune response to an mRNA-based vaccine. Despite intensive studies and advances in understanding the underlying pathology, the mechanism by which COVID-19 vaccination leads to aberrant immune responses targeting platelets and how platelets are involved in mediating the immune responses are not fully understood. It is generally believed that local and recruited antigen presenting cells (APCs) uptake lipid nanoparticles (LNPs) containing mRNA at the injection site and transiently express spike protein, leading to priming of T cell responses in draining lymph nodes [4]. It is not known, however, if activation of APCs impacts platelet function and/or generation, or whether cells expressing spike protein are present in circulation after COVID-19 vaccination. In this study, we studied peripheral blood of four healthy subjects before and after each dose of mRNA-1273 vaccination. Here, we report that plasmacytoid dendritic cell (pDC)-like cells with megakaryocyte signature expressing spike protein appeared in circulation after the third dose of mRNA-1273 vaccination.

Blood samples were collected from subjects who received mRNA-1273 vaccines. All the four subjects received the second dose of mRNA-1273 vaccines 4–5 weeks after the first dose, and three of them received the third dose mRNA-1273 vaccines 9–11 months after the second dose. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of our institution. Complete blood count (CBC) analysis showed a transient drop in platelets in all subjects tested 3 days after receiving each dose of vaccination (Fig. 1A). The severity of platelet count decrease varied among individuals but was the most severe after the second dose. In most cases, platelets recovered to slightly higher than baseline 7 days after vaccination. Hematocrit tended to be increased 3 days after each dose but returned to pre-vaccination levels on day 7 (Fig. 1B). It has been reported that SARS-CoV-2 spike protein by itself disrupts endothelial cell barrier and increases vascular permeability [5]. Thus, we speculate that such endothelial cell dysfunction may, at least partly, contribute to vascular leakage and hemoconcentration, resulting in hematocrit increase. There was no clear trend in the total number of leukocytes (Fig. 1C). Plasma samples were tested for the levels of D-dimer, and results showed a mild increase 3 days after each dose of the vaccine (Fig. 1D).

Among APCs which take up mRNA encapsulated in LNPs, pDCs specifically express C-type lectin domain family 4 member C (CLEC4, also known as CD303) that recognize desialylated sugar chains [6]. Involvement of pDC-like cells in the recognition of glycosylation status on megakaryocyte surface, leading to the regulation of thrombopoiesis, has also been reported [7]. Thus, we studied cells with pDC signature in circulation after mRNA-1273 vaccines. Interestingly, the number of $CD303^+$ cells in peripheral blood was increased 2 days after the third dose in all three subjects tested (Fig. 2A-B). Hoechst-positive cells (excluding red blood cells and platelets) were also stained for spike protein, and positively stained cells were identified in $CD303^+$ fraction (Fig. 2C). $CD303^+$ cells and $CD303^-$ cells in the blood collected 2 days after the third dose (blue box and gray box respectively, in Fig. 2C) were then, separately analyzed for the expression of other markers. Interestingly, a substantial fraction of $CD303^+$ cells expressed CD123 and GPIbα, the former is a marker for pDCs and the latter is specifically expressed in megakaryocytes and platelets. Moreover, these triple-positive (CD303, CD123, and GPIbα) cells concordantly expressed spike protein indicated by color scale, with red representing high expression and blue indicating low (Fig. 2D). Cells expressing CD303, CD123, and GPIbα (defined as $GPIb\alpha^+$ pDC-like cells) were quantitatively analyzed before and after the third dose of vaccination. $GPIb\alpha^+$

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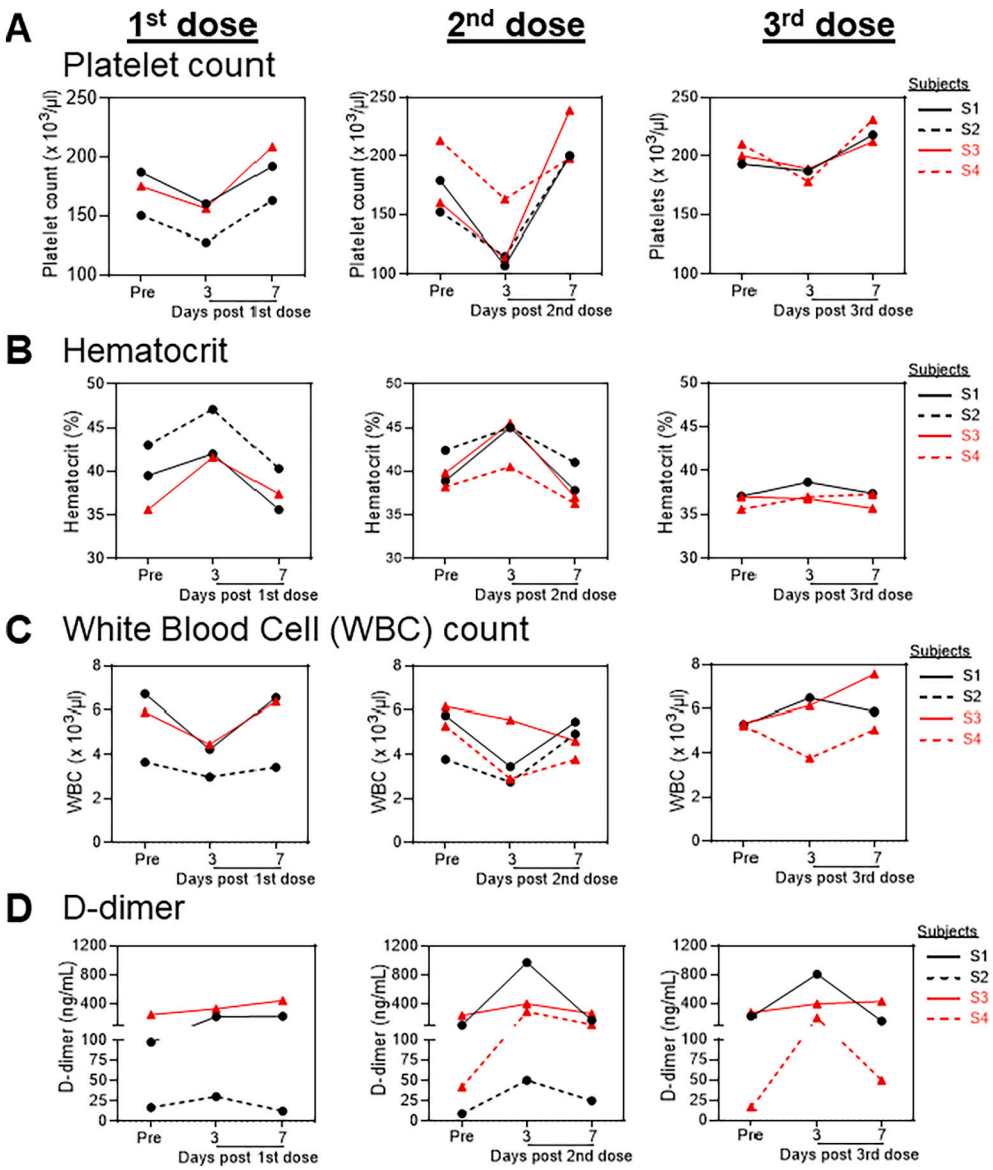

Fig. 1. Blood cell counts and plasma D-dimer measurement. (A-C) Blood samples were collected before and after each dose of mRNA-1273 vaccination, and complete blood cell count (CBC) was measured using the IDEXX ProCyte Dx Hematology Analyzer (IDEXX Laboratories Inc., Westbrook, ME). (D) D-dimer in the plasma was measured by ELISA using monoclonal antibodies (DD189cc and biotinylated DD255cc; HyTest, Turku, Finland) [2]. Among subjects tested, two (S1 and S2, black solid and broken line) were males and two (S3 and S4, red solid and broken line) were females. The age of the subjects S1-S4 at time of first dose administration was 57, 41, 49 and 48, respectively. Blood samples of S4 at the first dose and S2 at the third dose were not collected for analysis.

pDC-like cells transiently increased, among which, those expressing spike protein peaked 2 days after the third dose (Fig. 2E). These results show that pDC-like cells with megakaryocyte markers that took up the COVID-19 mRNA vaccine transiently appeared in the peripheral blood. To confirm these results, pDC-enriched cells were prepared by negative selection using magnetic beads using whole blood collected from the two subjects 3 days after the third dose of vaccination, and RNA was extracted for reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Using a pair of primers which specifically amplifies mRNA-1273 encoded spike mRNA but not viral genomes, we confirmed the vaccine-derived spike mRNA in the two subjects (S1 and S2) 3 days after the third dose of vaccination (Fig. 2G). These signals were not observed in the pre-vaccination samples or in samples >40 days post vaccination. The amplified band was extracted and confirmed by the direct sequencing of the PCR products (data not shown).

Expression of c-Mpl, the thrombopoietin (TPO) receptor, in DCs have been previously shown [8]. However, the expression of other megakaryocytic genes, such as GPIbα, in pDCs has not been reported, and concern remains regarding platelet-bound pDCs as the cause of GPIbα expression on pDCs observed by flow cytometry. Thus, cytospins were prepared using pDC-enriched cells prepared from S1 and S2 (Fig. 2F) and stained with the antibodies against CD303 and GPIbα followed by confocal microscopy analysis. The result showed that the signal of anti-GPIbα antibody staining was not on the adhered platelets, but on the cell surface and inside of the CD303^{+} cells (Fig. 2G).

The origin of GPIbα^{+} pDC-like cells is not clear at the moment, and one possibility is pDCs taking up the megakaryocyte/platelet or vice versa. It has been reported in a *Listeria monocytogenes* infection model that complement C3-bound bacteria adhere to platelets, which are taken up by splenic DCs, resulting in intracellular detection of platelet-specific marker integrin αIIb (CD41) in DCs [9]. This is considered as a mechanism by which platelets deliver antigens to DCs, activating immune responses to facilitate clearance of bloodborne pathogens. More recently, Lee-Sundlov et al. demonstrated that immune cells with pDC-like signature recognize megakaryocyte O-glycan sialylation status and regulate thrombopoiesis using a mouse model with targeted deletion of O-glycan sialyltransferase (St3gal1) specifically in megakaryocytes. These reports support the possibility that pDCs take up megakaryocytes/platelets. Conversely, Cunin et al. demonstrated that megakaryocyte emperipolesis mediates membrane transfer from intracytoplasmic neutrophils to megakaryocyte and daughter platelets [10]. Their study indicates emperipolesis as a mechanism for megakaryocyte to ingest other

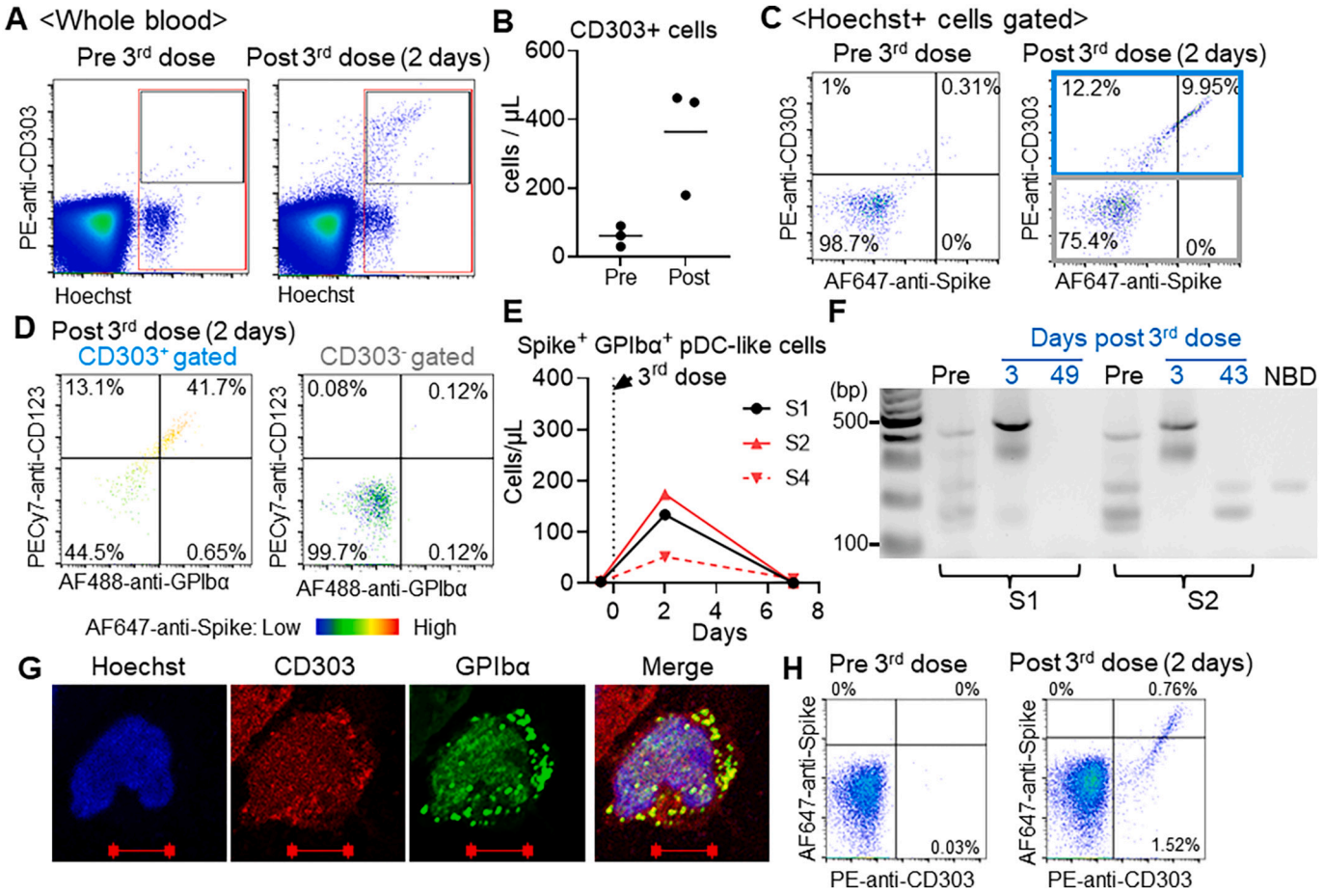

Fig. 2. Characterization of cells with pDC signature in peripheral blood after the third dose of mRNA-1273 vaccination. Whole blood samples were diluted in phosphate buffered saline (PBS, pH 7.4) containing 2 mM EDTA. After blocking Fc receptors with Human TruStain FcX (BioLegend), samples were stained with AlexaFluor 488 anti-human GPIbα (LJ-P3, MERU VasImmune), PE anti-human CD303 (BDCA-2) (clone 201A, BioLegend), AlexaFluor 647 SARS-CoV-2 Spike S1 subunit AlexaFluor 647-conjugated antibody (R&D Systems), and PE/Cy7 anti-human CD123 (clone 6H6, BioLegend). Hoechst 33342 was used for nuclear DNA staining. Samples were analyzed using a Novocyte flow cytometer (Agilent Technologies) and data was analyzed with FlowJo v.10.7.1. (A, B) Cells positively stained with Hoechst and anti-CD303 antibodies (black box) were counted before and 2 days after the third dose vaccination. (C) Blood cells positively stained with Hoechst (excluding red blood cells and platelets, red box in (A)), were analyzed for the expression of CD303 and spike protein. (D) CD303-positive and -negative cells were gated in the blood sample collected 2 days after the third dose (blue box and gray box, respectively in (C)) and were further analyzed for the expression of CD123 and GPIbα. The expression of spike protein is indicated by color code. (E) The cells positively stained by the antibodies against CD303, CD123, GPIbα, and spike protein were counted in the three subjects before and after the third dose of vaccination as in (D) and plotted. (F) pDC-enriched cells were prepared using the Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec) by negative selection starting from 20 mL of citrated whole blood. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) and Monarch Total RNA Miniprep Kit (New England BioLabs). cDNAs were synthesized using Proto Script II (New England BioLabs) and PCR was performed using Phusion Hot Start II DNA polymerase (Thermofisher Scientific). (G) pDC-enriched cells prepared in (F) were used to prepare cytospins after fixation with 2 % paraformaldehyde for 1 h, and permeabilization using 0.2 % Triton X-100. After blocking Fc receptors with Human TruStain FcX, samples were stained with AlexaFluor 488 anti-human GPIbα (LJ-P3), PE anti-human CD303. Nuclei were counterstained with Hoechst 33342. Samples were analyzed using a Zeiss LSM 880 Confocal Laser Microscopy (Carl Zeiss). Scale bars = 10 μm. (H) Platelets in whole blood were gated by forward side scatter and anti-GPIbα staining, and further analyzed by the antibodies against CD303 and spike protein.

cells and generate platelets with membrane contents derived from non-megakaryocytic cells. The function of emperipolesis is unknown, but it is known to be increasingly observed in patients with pathologic conditions or in the animal models under inflammatory stress conditions. In support of this idea, we found that a small fraction of platelets expressing CD303 and spike protein 2 days after the third dose of vaccination (Fig. 2H). A minor population of CD303-positive platelets (S1; 1.15 %, S2; 0.76 %, S4; 0.069 %) expressing spike protein was identified. This result indicates the possibility of megakaryocytes engulfing spike protein-expressing pDC-like cells, leading to membrane transfer and generation of CD303$^+$ spike protein$^+$ platelets. Determination of the origin of these cells needs further investigation. CD303-negative platelets expressing spike protein were not observed in the current study, but it is possible that CD303-negative spike protein-positive platelets are generated by other mechanisms and promptly cleared from circulation.

Antibodies generated against spike protein may bind to spike protein$^+$ platelets in circulation which can lead to Fc receptor-mediated platelet activation and platelet aggregate formation. This may provide a feasible explanation for a transient drop of platelet count and increase of D-dimer levels observed in subjects studied after the second dose of vaccination. In our study, alterations in the number of platelets and D-dimer levels were transient and modest. A similar trend was observed after the third dose, but the effect on the platelet count was minimal (see Fig. 1A and D, compare right panels with middle panels). We speculate that the third dose of the mRNA-1273 vaccine is given at half the dose (50 μg) of the original, thereby leading to minimal effects on platelets. Current study analyzed only four subjects, and the blood profiles observed in response to vaccination varied among individuals. Larger studies are needed to establish the relevance of our observation and to

elucidate the mechanism by which cells with immune signature and megakaryocyte markers are generated.

CRediT authorship contribution statement

Y.M. and S.K. performed plasma analyses, interpreted the data, and helped with manuscript preparation. T.K. designed experiments, directed the study, performed experiments, and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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