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ORIGINAL ARTICLE

Distinct platelet crosstalk with adaptive and innate immune cells after adenoviral and mRNA vaccination against SARS-CoV-2

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Abstract

Background: Genetic-based COVID-19 vaccines have proved to be highly effective in reducing the risk of hospitalization and death. Because they were first distributed in a large-scale population, the adenoviral-based vaccines were linked to a very rare thrombosis with thrombocytopenia syndrome, and the interplay between platelets and vaccinations increasingly gained attention.

Objectives: The objective of this article was to study the crosstalk between platelets and the vaccine-induced immune response.

Methods: We prospectively enrolled young healthy volunteers who received the mRNA-based vaccine, BNT162b2 (n = 15), or the adenovirus-based vaccine, AZD1222 (n = 25) and studied their short-term platelet and immune response before and after vaccine injections. In a separate cohort, we retrospectively analyzed the effect of aspirin on the antibody response 1 and 5 months after BNT162b2 vaccination.

Results: Here, we show that a faster antibody response to either vaccine is associated with the formation of platelet aggregates with marginal zone-like B cells, a subset geared to bridge the temporal gap between innate and adaptive immunities. However, although the mRNA-based vaccine is associated with a more gradual and tolerogenic response that fosters the crosstalk between platelets and adaptive immunity, the adenovirus-based vaccine, the less immunogenic of the 2, evokes an antiviral-like response during which the platelets are cleared and less likely to cooperate with B cells. Moreover, subjects taking aspirin (n = 56) display lower antibody levels after BNT162b2 vaccination compared with matched individuals.

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Conclusion: Platelets are a component of the innate immune pathways that promote the B-cell response after vaccination. Future studies on the platelet-immune crosstalk post-immunization will improve the safety, efficacy, and strategic administration of next-generation vaccines.

KEYWORDS
aspirin, B-lymphocytes, immunothrombosis, platelet count, vaccine

1 | INTRODUCTION

Vaccination against SARS-CoV-2 has changed the course of the COVID-19 pandemic worldwide. Through an unprecedented effort in medical history, vaccines with 95% efficacy against symptomatic infections and severe disease have been developed within less than 12 months. Since then, more than 13 billion vaccine doses have been administered worldwide (https://covid19.who.int/), and millions of lives have been saved [1]. Because SARS-CoV-2 evolves into diverging lineages, maintaining high vaccination coverage across all communities and population groups remains the most effective strategy for lowering the risk of infection and death (https://covid.cdc.gov/covid-data-tracker/#rates-by-vaccine-status) [2–4]. Thus, optimizing vaccine design and administration is the key to controlling the current and future diseases.

The first anti-SARS-CoV-2 vaccines approved by the European Medicines Agency and administered in Italy since January 2021 were genetic-based vaccines that use novel antigen-delivery platforms [5]. BNT162b2, also known as Comirnaty (developed by BioNTech/Pfizer), is a mRNA-based vaccine encoding for the receptor binding domain (RBD) of the SARS-CoV-2 spike protein encapsulated in a lipid nanoparticle (LNP). AZD1222, also known as ChAdOx1nCov-19 (developed by Oxford University and AstraZeneca), is a replication-deficient simian adenoviral vector vaccine containing DNA encoding for the full-length spike protein [6]. Genetic-based vaccines are designed to act as "self-adjuvants" by stimulating innate immune signaling pathways, which can augment the immunogenicity of the encoded antigen. However, there is still an incomplete understanding of which the innate pathways are activated by these new vaccines and on how they cooperate in the development of protective immunity *in vivo* [7,8].

Platelets, best known for their role in hemostasis, actively participate in the innate immune response in a process called immunothrombosis [9–11]. Moreover, several studies have demonstrated that platelets also enhance the adaptive immune response by modulating T and B-cell recruitment, activation, and differentiation [12–16]. Recently, we have observed that healthy adults who respond rapidly to the BNT162b2 mRNA-based vaccine (ie, develop high levels of neutralizing antibodies against the SARS-CoV-2 RBD after just 1 vaccine dose) have higher levels of platelet-leukocyte aggregates and display circulating platelet-bound B cells as early as 3 days after the first vaccine injection [17], suggesting that the platelets could be involved in mounting a protective response after vaccination.

Essentials

- How platelets and antiplatelet therapy modulate immunity is critical to optimize vaccine designs.
- Platelet-marginal zone B-cell interactions associate with faster antibody responses to vaccines.
- Platelet-consuming reactions against adenoviral-based vaccines dampen the antibody response.
- Taking aspirin for cardiovascular prevention interferes with vaccine efficacy.

Recent reports of thrombosis and/or thrombocytopenia after COVID-19 vaccination have shed a negative light on the relationship between platelets and vaccination and increased vaccine hesitancy. mRNA-based vaccines have been associated with rare cases of immune thrombocytopenia (ITP) [18]. Similar autoimmune reactions have been described before in association with vaccines based on different antigen-delivery platforms, but proof of a causal relationship between vaccinations and these very cases of ITP is still lacking [19–21]. Episodes of thrombosis combined with thrombocytopenia have been reported mainly 4 to 20 days after the first dose of adenoviral-based vaccines [22–24]. These reactions have been linked to the presence of platelet-activating antibodies anti-platelet factor 4 (PF4) in the blood and have defined vaccine-induced thrombosis and thrombocytopenia (VITT) by analogy with heparin-induced thrombocytopenia, another drug-induced thrombotic disorder. The occurrence of VITT is very rare (13-39 cases per 1 million vaccinated persons) [25], and it can be avoided because it seems to be related to vaccine contaminants [26] and/or unwanted intravenous (i.v.) injection [27]. Understanding how different vaccine platforms may trigger protective platelet-immune crosstalk, without inducing thrombosis or excessive platelet consumption, is critical to design safer vaccines for the future.

Here, we present the results of 2 longitudinal cohort studies that looked at the short-term and long-term effects of mRNA-based and adenovirus-based anti-SARS-Cov-2 vaccines to investigate the interplay between platelets and vaccination and identify the features of the platelet-immune crosstalk that facilitate protective immunity or increase the risk of adverse effects such as thrombosis and thrombocytopenia.

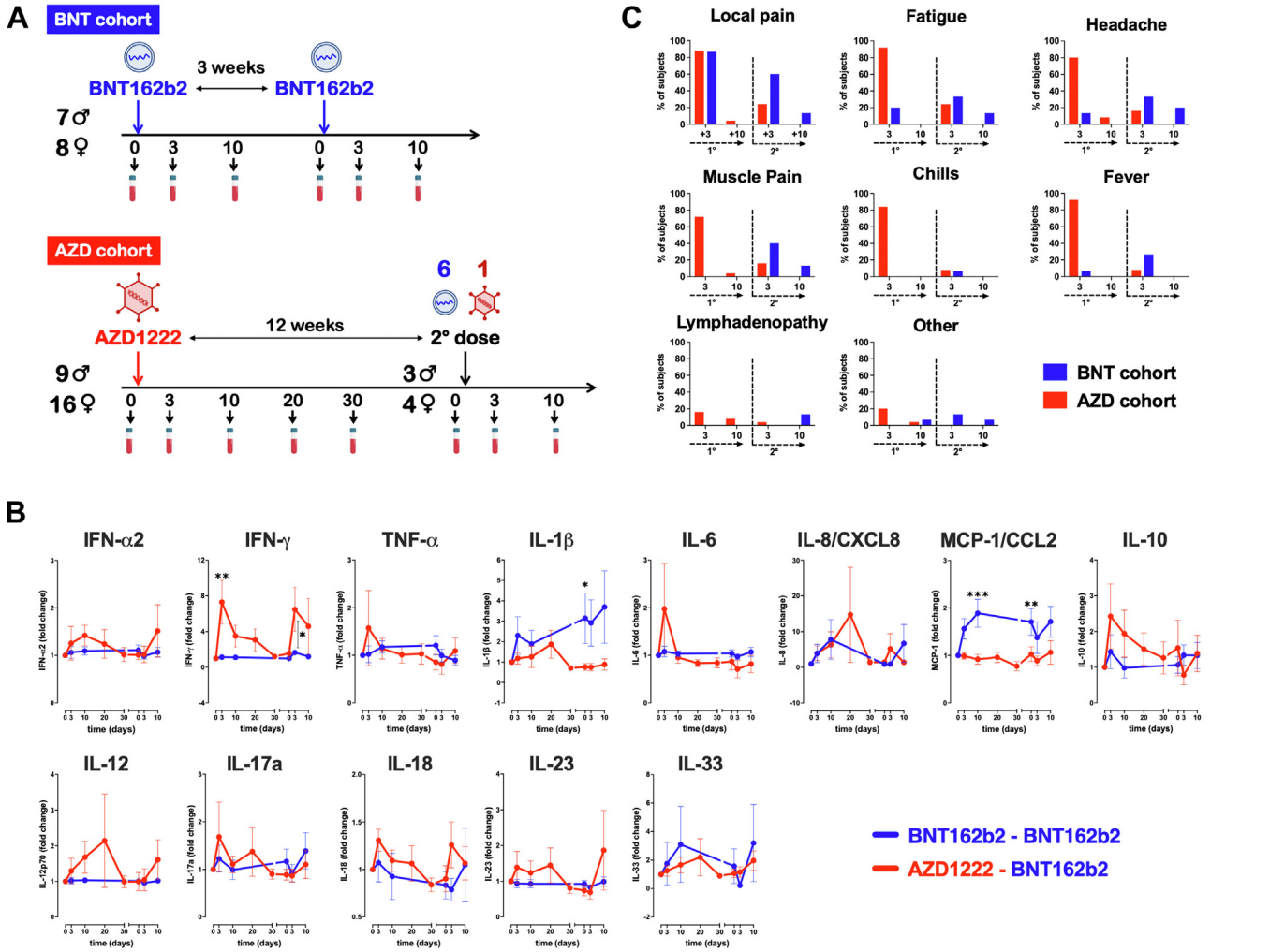

FIGURE 1 Comparison of the inflammatory response and side effects induced by the adenovirus-based and the mRNA-based anti-SARS-CoV-2 vaccines. (A) Study design schematic showing the timeline of blood withdrawals (tube symbol) from male (♂) and female (♀) subjects vaccinated with 2 doses of BNT162b2 (BNT cohort) and subjects vaccinated with one dose of AZD1222 followed by a second dose of either BNT162b2 or AZD1222 (AZD cohort). (B) Plasmatic concentration of cytokines and chemokines of subjects receiving 2 doses of BNT162B2 (blue continuous line) or one dose of AZD1222 and one of BNT162B2 (red continuous line). Graphs show mean ± SD of the fold change relative to the concentration before vaccination. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (C) Self-reported postvaccine adverse effects. Bar graphs show the percentage of subjects in each cohort that reported to have experienced the adverse effect 3 or 10 days after the first (1°) or the second (2°) injection. IFN, interferon; IL, interleukin; TNF-α, tumor necrosis factor α

2 | METHODS

2.1 | Study populations

We conducted 2 observational studies that were approved by the Ethics Committee of the Policlinico Umberto I of Rome. In one study, we prospectively enrolled healthy adults aged >18 years, without signs of infection or significant comorbidities, scheduled for anti-Sars-CoV-2 vaccination at our institution (Sapienza University Hospital, Policlinico Umberto I) in 2021 (EC identifier 6305 - Prot. 0470/2021; ClinicalTrials.gov Identifier: NCT05171959). We excluded individuals with severe immunosuppression, pregnancy, or breastfeeding and subjects who underwent recent transfusions of platelets or plasma and who used antiplatelet or anticoagulant medications in the 10 days before enrollment. Among the enrolled subjects, we compared 2 cohorts (Figure 1A). The BNT cohort included 15 subjects who received 2 doses, 21 days apart, of the mRNA-based BNT162b2 vaccine, also known as Comirnaty (developed by Pfizer-BioNTech). Nine months later, 6 of them agreed to donate blood after the third BNT162b2 dose to perform selected experiments on platelet-B cell aggregates. The AZD cohort included 25 subjects who received one dose of the adenoviral vector AZD1222 vaccine, also known as ChAdOx1 nCoV-19 (developed by University of Oxford-AstraZeneca), and 12 weeks later, either one dose of AZD1222 or one dose of the mRNA-based BNT162b2 vaccine. In the time between the 2 doses, the AZD1222 vaccinations were suspended in Italy for subjects aged younger than 60 years because of the concerns raised by the first reports of VITT. Only 7 subjects of the second cohort agreed to donate blood after the second dose of the vaccine, one of whom received AZD1222 and six received BNT162b2. Two subjects of the

AZD cohort had been infected with Sars-CoV-2 before vaccination. As controls, we recruited 6 subjects who had received no vaccination in the 3 months before the study.

In a second study (SapienzaVax), we enrolled 2065 health care workers (age: 45.9 ± 13.3 years) of the Policlinico Umberto I of Rome immunized with 2 doses, 21 days apart, of the BNT162b2 vaccine (EC identifier 6020—Prot. 0486/2021; ClinicalTrials.gov Identifier: NCT04844632) [28]. Within this cohort, we retrospectively analyzed the antibody response of all the participants (n = 56) taking a low dose of aspirin (100 mg/die) and the first 56 participants not taking aspirin, matched for age, sex, body mass index, and comorbidities (hypertension, type 2 diabetes, coronary artery disease, and the history of stroke).

All study participants gave written informed consent on the day of enrollment.

2.2 | Biological sample collection

In subjects of the BNT and the AZD cohort, venous blood samples were collected 0 to 24 hours before and 3 and 10 ± 2 days after each vaccine injection. In the AZD cohort, we also collected samples 20 ± 2 and 30 ± 2 days after the first AZD1222 dose (Figure 1A). Sodium-citrate anticoagulated whole blood was used within 30 minutes from blood withdrawal for platelet functional analysis, immunophenotyping, and platelet-leukocyte aggregate quantification by flow cytometry. EDTA-anticoagulated blood was used to determine the cell counts and the platelet morphologic parameters with a Sysmex KX-21N (Sysmex Corp). Serum and plasma samples were stored at −80°C until batch analysis.

Within the SapienzaVAX study, the venous blood samples were collected 1 or 5 months after the 2 BNT162b2 doses. Serum samples stored at −80°C were used in batch to determine the antibody response.

2.3 | Clinical data collection

At baseline and any further time point, each participant completed a short questionnaire on relevant medical history, smoking, the history of exposure to SARS-CoV-2, and drugs used to account for potential confounders.

2.4 | Cytokine array

Plasmatic cytokine concentrations were assessed in double using the LEGENDplex bead-based immunoassay (Inflammatory cytokines panel I, Biolegend), as described previously [17].

2.5 | Immunophenotyping and platelet-leukocyte aggregate quantification

Within 15 minutes from blood withdrawal, 50 μL of whole blood was incubated with fluorochrome-conjugated monoclonal antibodies. After 15 minutes of incubation, red cell lysis was performed with BD FACS lysing solution (BD Biosciences). At least 50 000 events in the singlet gate were acquired. Flow cytometric analyses were conducted with BD LSR Fortessa, and the data were analyzed with FlowJo LLC software, ver.10.8. The antibody panel to identify leukocyte subsets included α-CD66b-PE, α-CD56-PEDazzle594, α-CD19-PerCPCy5.5, α-CD16-PECy7, α-CD14-APC, α-CD4-Alexa700, α-CD25-Bv421, α-CD3 Bv510, α-CD8 Bv605 (SONY Biotechnology), and α-CD41-BB515 (BD Bioscience) to detect platelets binding to each subset. The antibody panel to identify B-cell subsets and interacting platelets included α-CD19-PerCPCy5.5, α-CD27-PE, α-IgD-FITC, and α-CD41-APC (SONY Biotechnology). The antibody panel to identify $CD21^{low}$B-cells and interacting platelets included α-CD19-PerCPCy5.5, α-CD21-PE, α-CD38-APC (SONY Biotechnology), and α-CD41-BB515 (BD Bioscience). Fluorescence-minus-one controls were performed to ensure proper gating. The complete gating strategy to identify leukocyte subsets is illustrated in the supplement (Supplementary Figure S1). Granulocyte activation was determined by quantifying the median fluorescence intensity of α-CD66b-PE and α-CD16-PECy7 in the granulocyte gate. Platelet-leukocyte aggregates (PLA) were identified based on the expression of CD41a in the individual leukocyte subpopulations and expressed as a fold increase over baseline before vaccine administration.

2.6 | Platelet functional analysis

To monitor the activation status and responsiveness of circulating platelets, sodium-citrated blood diluted 1:10 with Tyrode's buffer, containing mmol/L (137 NaCl, 0.34 Na_2HPO_4, 2.9 KCl, 12 $NaHCO_3$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 D-glucose, and 0.35% bovine serum albumin) pH 7.3, was labeled for 10 minutes with PAC1-FITC and α-CD62P-PE (BD Bioscience) in the absence or presence of agonists. Samples fixed with 1% formaldehyde were acquired on a BD Accuri C6 Plus flow cytometer and analyzed with FlowJo LLC software, ver.10.8.

2.7 | Antibody response quantification

Serum SARS-CoV-2-neutralizing antibodies were detected using COVID-19 Immunorank MICRO- enzyme-linked immunosorbent assay (ELISA) (Leinco Technologies, Inc), and the neutralization index, expressed as percent, was calculated following the manufacturer's specifications. We defined fast responders and slow responders as the subjects who reached, 20 days after the first dose, a neutralization index above or below the mean of their cohort, respectively.

IgG to the trimeric SARS-CoV-2-Spike glycoprotein were quantified in the serum samples using an indirect chemiluminescence immunoassay (SARS-CoV-2 Trimeric S IgG-LIAISON- DiaSorin Inc) according to the manufacturer's assay specifications [28]. The test measures values between 1.85 and 800 Arbitrary Units per milliliter (AU/mL), corresponding to values between 4.8 and 2080 binding antibody units (BAU/mL) (1 AU/mL = 2.6 BAU/mL). Positive anti-Spike IgG levels (TrimericS IgG) were defined as equal to or more than 13 AU/mL. The experiments were performed in double for each subject.

2.8 | Statistics

The categorical variables are expressed as count and percentages. Continuous variables were reported as mean ± SD or median and interquartile ranges (IQR). Continuous variables were compared using Student *t*-tests for normally distributed continuous variables and with Mann–Whitney U tests for non-normally distributed ones. The differences between percentages were assessed by chi-squared tests. Appropriate two-tailed nonparametric tests were used to evaluate the differences between individual time points of the study (two-way analysis of variance (ANOVA) test with Šidák multiple comparisons tests for intergroup analyses and with Tukey multiple comparisons tests for intragroup analyses). Correlations between the measured parameters were assessed by conducting Spearman rank correlations. A two-sided p value <.05 was considered statistically significant. All analyses were performed using computer software packages (GraphPad Prism 9 and IBM SPSS ver. 27.0).

3 | RESULTS

3.1 | The adenovirus-based vaccine evokes a stronger inflammatory response compared with the mRNA-based vaccine

We first compared the short-term cytokine response induced by the adenovirus-based and the mRNA-based vaccine (Figure 1B). BNT162b2 rapidly doubled the plasmatic concentration of the chemokine MCP-1/CCL2 and induced a gradual increase of IL-1β that incremented further after the second dose, as we reported previously [17]. AZD1222 induced a greater increase in the overall plasmatic concentration of inflammatory cytokines, compared with BNT162b2. Notably, IFN-γ, IFN-α2, TNF-α, IL-6, and IL-10 increased acutely 3 days after the injection, with IFN-γ increasing up to 5-fold to 10-fold, whereas IL-1β, IL-8, IL-12p70, and IL-33 peaked 20 days after the injection. The second dose of BNT162b2 after AZD1222 induced a second wave of IFN-γ and IFN-α2, which was not detected after the homologous BNT162b2 vaccination.

In line with the different cytokine profiles, AZD1222 recipients, who had an acute inflammatory response, experienced more than one systemic side effect, which resolved within 24 to 72 hours, following the first injection. Compared with AZD1222 recipients, BNT162b2 recipients reported fewer symptoms after the first injection, but more after the second injection (Figure 1C).

3.2 | The adenovirus-based but not the mRNA-based vaccine induces a transient decrease in the platelet and the granulocyte count in healthy volunteers

The most striking difference we detected between the 2 vaccines was a distinct effect on the platelet and the granulocyte count. As we reported previously [17], BNT162b2 was not associated with a significant change in the platelet count (Figure 2A). On the contrary, the subjects injected with AZD1222 experienced a significant 20% decrease in the platelet count 3 days after the injection. All the observed subjects experienced the same decline. However, the decline was transient, and only one subject (with 165 000 platelets/μL at baseline) reached a count lower than 150 000 platelets/μL, ie, the lower limit of the normal platelet range. As the platelet count normalized, 10 days post-AZD1222, we detected a non-significant increase in the mean platelet volume and the platelet-large cell ratio and a significant increase in the platelet distribution width (Supplementary Figure S2) that could be indicative of the increased production of new platelets, which are generally larger in size.

Concomitantly to the decline in the platelet count, AZD1222 but not BNT162b2 recipients experienced a 40% decrease in the granulocyte count (Figure 2B) and a 5% decrease in the lymphocyte count (Figure 2C). The monocyte counts increased 3 days after both vaccines but to a greater extent after AZD1222 (Figure 2D). Based on a Spearman rank correlation analysis, we identified a significant negative correlation between the fold change of the platelet and granulocyte counts and the change of IFN-γ 3 days after AZD1222, and a negative correlation between the fold change of IL-6 and the platelet count (Figure 2E).

Multiparametric flow cytometry analyses of the circulating leukocyte subtypes (Figure 3) revealed that neutrophils declined notably after a single AZD1222 injection but were not affected by a single BNT162b2 injection and were marginally reduced after the second injection of BNT162b2 in both heterologous and homologous vaccinations (Supplementary Figure S3). Eosinophils decreased incrementally after each dose of the AZD1222/BNT162b2 heterologous vaccination but were not affected by the homologous BNT162b2 vaccination. Among monocytes, we detected in both cohorts gradual shrinkage of the classical monocytes ($CD14^{+}CD16^{-}$) and a rapid expansion of intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{dim}CD16^{+}$) monocytes 3 days after each injection. However, CD16+ monocytes increased to a greater extent after AZD1222, than after BNT162b2.

Among lymphocytes, B cells were the ones affected the most, displaying a rapid 35% reduction 3 days after AZD1222 and a gradual reduction after BNT162b2, that became significant after the second dose in both heterologous and homologous vaccinations. Regulatory T cells increased after each BNT162b2 injection and were notably higher 10 days after the completion of the homologous BNT162b2 vaccination but were unaffected after one dose of AZD1222. Natural killer T (NKT) cells increased significant after AZD1222, but not after BNT162b2. Circulating natural killer (NK) cells levels decreased after one AZD1222 injection, but not after one BNT162b2 injection. A second BNT162b2 injection induced a significant decline of the NK cell count in both heterologous and homologous vaccinations. Based on the Spearman rank correlation analysis, NK cells were the only leukocyte population whose count positively correlated with the changes in IFN-γ concentration after AZD1222 (Figure 2E).

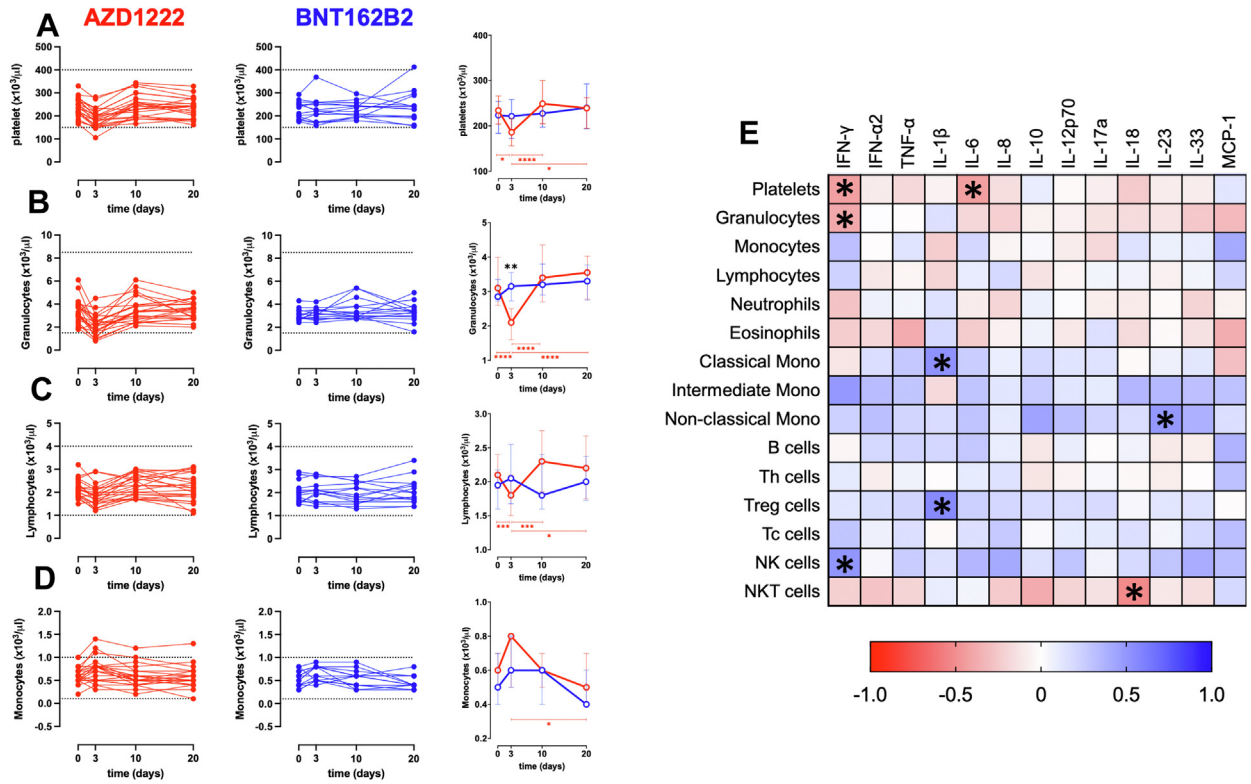

FIGURE 2 Comparison of the platelet and leukocyte count changes after a single dose of the adenovirus-based and the mRNA-based anti-SARS-CoV-2 vaccines. Changes over time of the (A) platelet, (B) granulocyte, (C) lymphocyte, and (D) monocyte counts in individual subjects vaccinated with either one dose of AZD1222 (red) or one dose of BNT162b2 (blue), determined in EDTA-anticoagulated blood with a Sysmex KX-21N. Graphs on the left show the count of individual study participants. Graphs on the right show the median ± IQR of the 2 cohorts in comparison. Two-way ANOVA test with Šidák multiple comparisons tests for intergroup analyses (in black) and with Tukey multiple comparisons tests for intragroup analyses (in blue or red). $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$. (E) Spearman rank correlation between the fold change of cytokines and the fold change of platelet and leukocyte counts 3 days after AZD1222 relative to before vaccination. The color of each box refers to the correlation coefficient r, that ranges from +1 (positive correlation, blue) to −1 (negative correlation, red), and the * indicate significant ($p < .05$) correlations

3.3 | The adenovirus-based but not the mRNA-based vaccine induces a transient activation of platelets and granulocytes in healthy volunteers

To assess whether the decline in the platelet count was accompanied by changes in the activation state of circulating platelets, we stained platelets with antibodies that detect active integrin αIIbβ3 (PAC1-FITC) and α-granule-released P-selectin (α-CD62P-PE) on the platelet surface. Integrin activation of unstimulated platelets was notably increased 3 days after AZD1222, compared with baseline (before vaccination) and controls, but remained within the normal ranges after BNT162b2 (Figure 4A). P-selectin surface expression was not notably different from baseline (Figure 4B). Platelet activation in response to exogenous agonists stayed within the normal ranges after a single dose of either vaccine (Supplementary Figure S4).

Like platelets, circulating granulocytes expressed higher levels of surface activation markers, namely CD16 (Figure 4C) and CD66b (Figure 4D), 3 days after AZD1222, but not after BNT162b12.

3.4 | The adenovirus-based and mRNA-based vaccines induce the formation of different platelet-leukocyte aggregates

To study the platelet-leukocyte crosstalk, we quantified the circulating PLA (Figure 5). Consistent with the higher platelet and granulocyte activation, we detected notably more platelet-neutrophil and platelet-eosinophil aggregates 3 days after the AZD1222 injection. Moreover, AZD1222, but not BNT162b2, induced a significant increase over baseline of the interactions between platelets and NK cells, ie, the innate arm of the lymphoid lineage and major producers of IFN-γ. After BNT162b2, we identified more platelet aggregates compared with baseline with adaptive immune cells such as B cells, regulatory T cells, and NKT cells. AZD1222 recipients also experienced a significant increase in platelet-B cell interactions compared with baseline, although to a lesser extent compared with BNT162b2 recipients.

To understand how platelets may be affecting B-cell function, we investigated which B-cell subtypes they were binding shortly

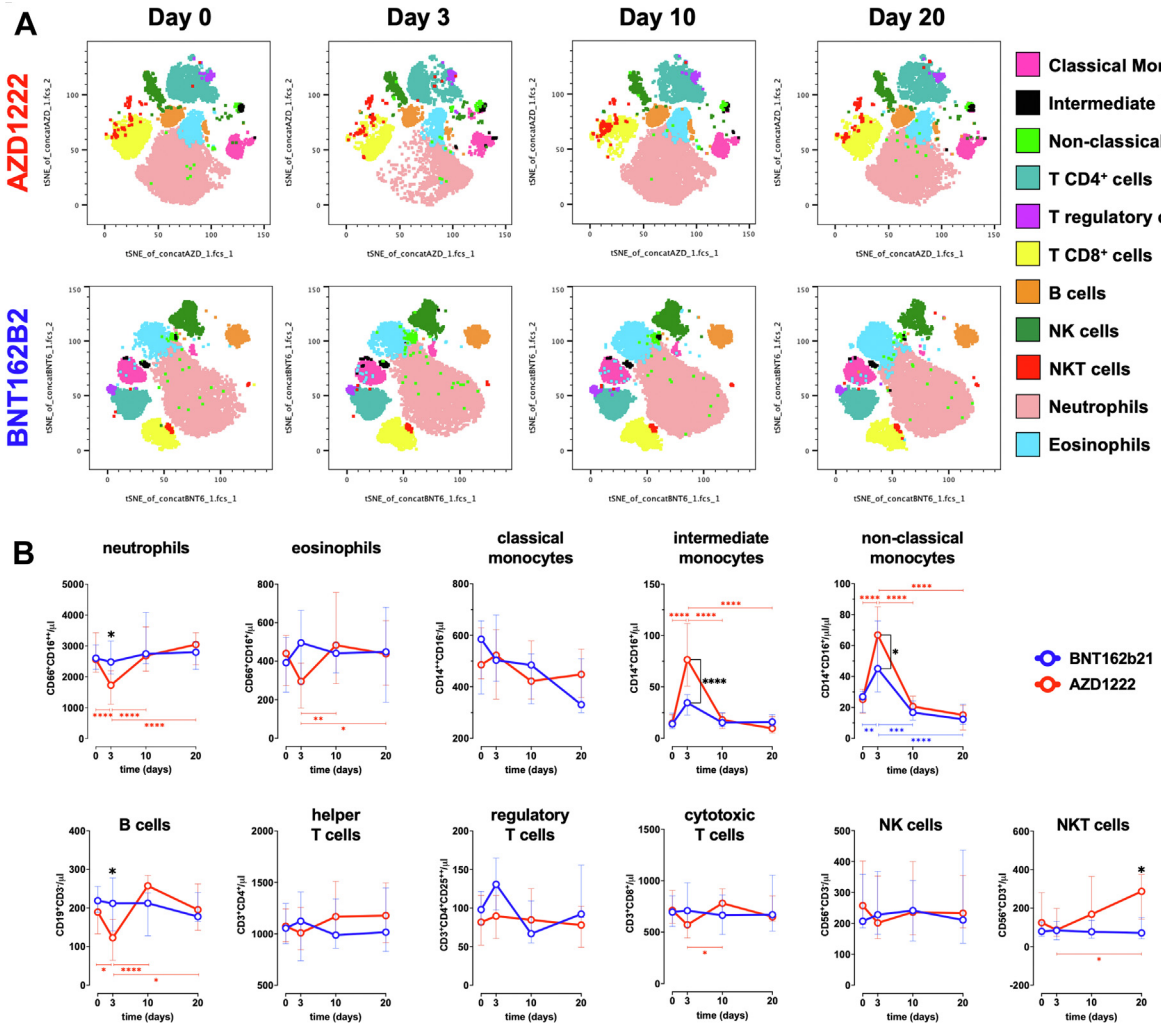

FIGURE 3 Comparison of the circulating leukocyte subtypes changes after a single dose of the adenovirus-based and the mRNA-based anti-SARS-CoV-2 vaccines. (A) Representative t-distributed stochastic neighbor embedding (t-SNE) dot plot of leukocyte subpopulations before (0) and 3, 10, and 20 days after AZD1222 (top panels) or BNT162b2 (bottom panels) immunization. (B) Changes over time of the absolute count of the leukocyte subpopulations in subjects vaccinated with either one dose of AZD1222 (red) or one dose of BNT162b2 (blue). Leukocyte subpopulations were identified by multiparameter flow cytometry on a BD LSR Fortessa and analyzed with FlowJo LLC software, version 10.8.1, based on the expression of surface markers: classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and non-classical ($CD14^{dim}CD16^+$) monocytes, neutrophils ($CD66^+CD16^+$), eosinophils ($CD66^+CD16^-$), B cells ($CD3^-CD19^+$), $CD4^+$ ($CD3^+CD4^+$), regulatory ($CD3^+CD4^+CD25^{high}$) and $CD8^+$ ($CD3^+CD8^+$) T cells, natural killer (NK, $CD3^-CD56^+$), and natural killer T cells (NKT, $CD56^+CD3^+$). The absolute count of each leukocyte subtype was determined by multiplying the total white blood cell count by the percentage of the specific cell type based on the flow cytometry analyses. Shown is the median ± IQR. Two-way ANOVA tests with Šidák multiple comparisons tests for the intergroup analysis (black) and with Tukey multiple comparisons tests for the intragroup analysis (in blue or red). *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$

after vaccination. After AZD1222, circulating levels of naïve-B cells ($IgD^+CD27^-CD19^+$) were steady and switched-B cells ($IgD^-CD27^+CD19^+$) decreased, whereas $CD21^{low}$B cells ($CD21^{low}CD38^-CD19^+$) and marginal zone-like (MZL)-B cells ($IgD^+CD27^+CD19^+$) increased significantly (Figure 6A). Although $CD21^{low}$-B cells were the subtype that expanded the most, platelets bound mainly to MZL-B cells, reaching a maximum 10 days after vaccination (Figure 6B). Notably, MZL-B cells were the B-cell subtype that bound more frequently to platelets also in recipients of the third dose of BNT162b2. In these subjects, we detected MZL-platelet aggregates in circulation 3 days after vaccination (Supplementary Figure S5), but we do not know if the faster kinetics is owing to the type of vaccine or to the number of boosters received.

3.5 | Platelet and immune features that correlate with a faster humoral response after anti-SARS-CoV-2 vaccines

To evaluate how the platelet-immune crosstalk evoked by the 2 vaccines related to their efficacy, we measured the change over time

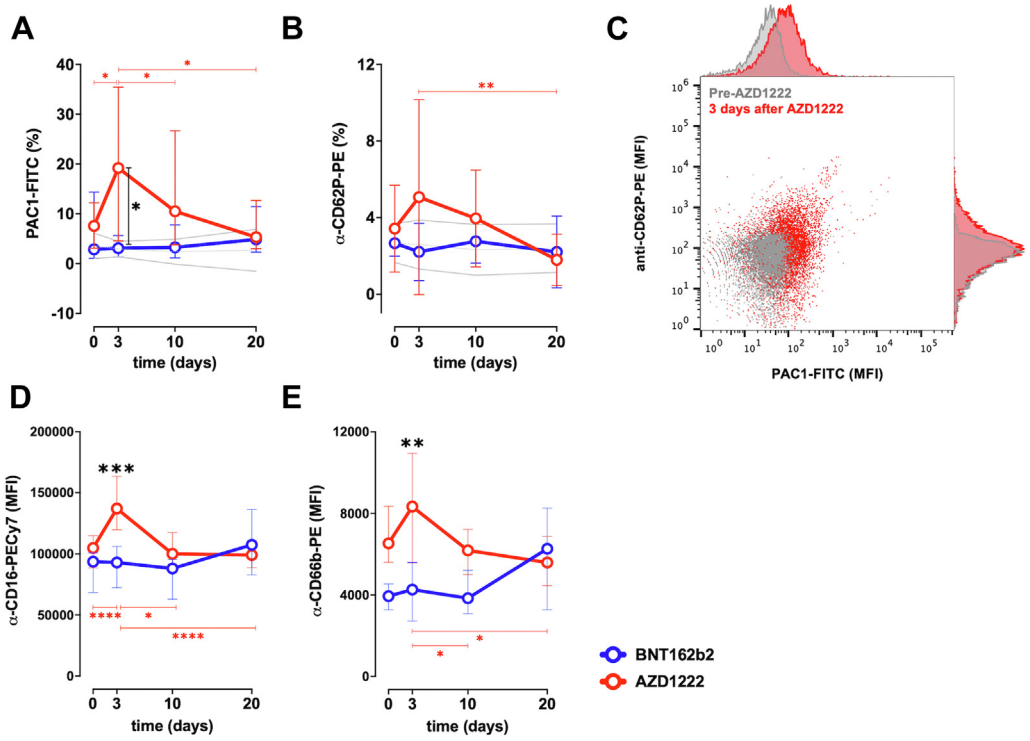

FIGURE 4 Comparison of the activation status of circulating platelets and granulocytes after a single dose of the adenovirus-based and the mRNA-based anti-SARS-CoV-2 vaccines. (A, B) Activation of circulating platelets determined by multicolor flow cytometry of nonstimulated citrated blood by quantifying the (A) percentage of platelets bound to PAC1-FITC (that recognizes the active conformation of integrin αIIbβ3) and (B) the percentage of platelets bound to α-CD62P-PE (that is stored in α granules in resting platelets). Gray lines show the median (dashed line) ± IQR (straight lines) of the % of PAC1-FITC+ or α-CD62P-PE+ of a group of $n = 6$ controls that had not received any vaccination in the previous 3 months. (C) Representative plot showing the shift of median fluorescence intensity (MFI) of platelets co-stained with PAC1-FITC and α-CD62P-PE before (gray) and 3 days after (red) AZD1222 vaccination. (D, E) Activation of circulating granulocytes determined by multicolor flow cytometry of nonstimulated citrated blood by quantifying the median fluorescence intensity (MFI) of the activation markers (D) CD16 (FcγRIII) and (E) CD66b (CEACAM8, CGM6, and NCA-95). Samples were acquired on a BD LSR Fortessa and analyzed with FlowJo LLC software, version 10.8.1. Two-way ANOVA tests with Šidák multiple comparisons tests for the intergroup analysis (in black) and with Tukey multiple comparisons tests for the intragroup analysis (in blue or red). $^{*}p < .05$; $^{**}p > .01$; $^{***}p < .001$

of the serum levels of neutralizing antibodies against the RBD of the SARS-CoV-2 spike protein (Figure 7A). Ten days after completing a 2-dose vaccine regimen (either homologous or heterologous), all subjects mounted a strong humoral response, reaching a median neutralization index of 96% [IQR: 93-98], with no significant difference between the 2 cohorts. However, the kinetics of the antibody response was notably different depending on the type of vaccine received at the first injection. BNT162b2 recipients reached a median neutralization index of 50% [IQR: 28%-62%] 20 days after the first dose, whereas AZD1222 recipients reached a median neutralization index of 21% [IQR: 12%-44%] at the same time point, except for 2 subjects of the AZD cohort who had COVID-19 before the study and had a 24% neutralization index at baseline and a 98% neutralization index just 10 days after the first injection.

To assess which early parameters correlated the most with a fast antibody response, we performed 2 analyses. First, we conducted Spearman rank correlations between the fold change of PLA and cytokines (3 days postvaccination) and the median neutralization index 20 days post-vaccination (Figure 7B). Second, we stratified vaccine recipients among fast and slow responders, based on their ability to reach a neutralization index above the mean of their cohort after one vaccine dose (Figure 7C-F). The antibody response induced by BNT162b2 positively correlated with the frequency of platelet-B cell aggregates (r = 0.575, $p = .027$) and the fold change of IL-1β (r = 0.597, $p = .05$) (Figure 7B). Consistently, fast BNT162b2-responders had notably more platelet-B cell aggregates (Figure 7D) and reached higher IL-1β levels (Figure 7E) compared with baseline, whereas slow BNT162b2-responders displayed fewer platelet-B cell aggregates that formed later. In the AZD cohort, we detected a similar trend (Figure 7D), and fast AZD1222-responders formed notably more platelet aggregates with MZL-B cells (Figure 7F). On the contrary, the frequency of platelet-neutrophil aggregates was unrelated to the antibody response (r = 0.057, $p = .8$), and the frequency of platelet-eosinophil aggregates negatively correlated with the antibody response (r = −0.665, $p = .006$) (Figure 7B). Based on the Spearman rank correlation analyses, the acute change in the IFN-γ concentration 3 days post-AZD1222 did not correlate with a rapid neutralizing antibody response against SARS-CoV-2 (r = 0.25, $p = .2$) (Figure 7B). Indeed, both fast and slow AZD1222-responders had higher plasmatic concentrations of IFN-γ compared with BNT162b2 recipients, but the

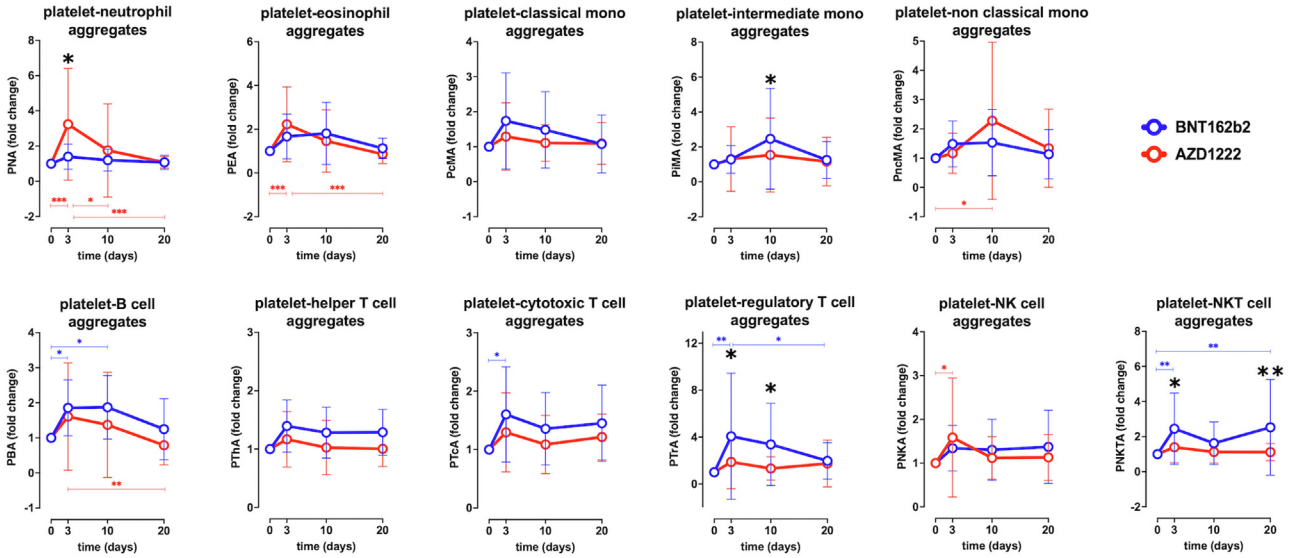

FIGURE 5 Comparison of the platelet-leukocyte interactions induced by the adenovirus-based and the mRNA-based anti-SARS-CoV-2 vaccines. Relative frequencies of the circulating platelet aggregates with neutrophils ($CD66^+CD16^+$), eosinophils ($CD66^+CD16^-$), classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and nonclassical ($CD14^{dim}CD16^+$) monocytes, B cells ($CD3^-CD19^+$), helper ($CD3^+CD4^+$), cytotoxic ($CD3^+CD8^+$) and regulatory ($CD3^+CD4^+CD25^{high}$) T cells, natural killer (NK, $CD3^-CD56^+$), and natural killer T cells (NKT, $CD56^+CD3^+$). Shown is the median ± IQR of the fold change relative to baseline (before vaccine administration) of the $CD41a^+$ events (normalized to the platelet count) in subjects vaccinated with either one dose of AZD1222 (red) or one dose of BNT162b2 (blue). Flow cytometry acquisition was performed on a BD LSR Fortessa and analyzed with FlowJo LLC software, version 10.8.1. Platelet-leukocyte aggregates (PLA) were identified based on the expression of CD41a in the individual leukocyte subpopulations, normalized to the platelet count, and expressed as fold increase over baseline before vaccine administration. Two-way ANOVA test with Šidák multiple comparisons tests for the intergroup analysis (in black) and with Tukey multiple comparisons tests for the intragroup analysis (colored). *$p < .05$; **$p < .01$; ***$p < .001$

slow AZD1222-responders experienced an acute increase of IFN-γ, whereas fast AZD1222 responders experienced a gradual IFN-γ increase (Figure 7E). In sum, the speed and intensity of the humoral response negatively correlated with excessive innate immune reactions but positively correlated with the formation of platelet B cell interactions (Figure 7G).

In line with these findings, a retrospective subset analysis (Table 1) of a separate longitudinal study showed that subjects taking the antiplatelet drug aspirin daily had a notably lower antibody titer 1 and 5 months after a 2-dose regimen of the BNT162b2 vaccine, compared with a group of vaccinated subjects not taking aspirin, matched for age, body mass index, sex, and comorbidities, thus

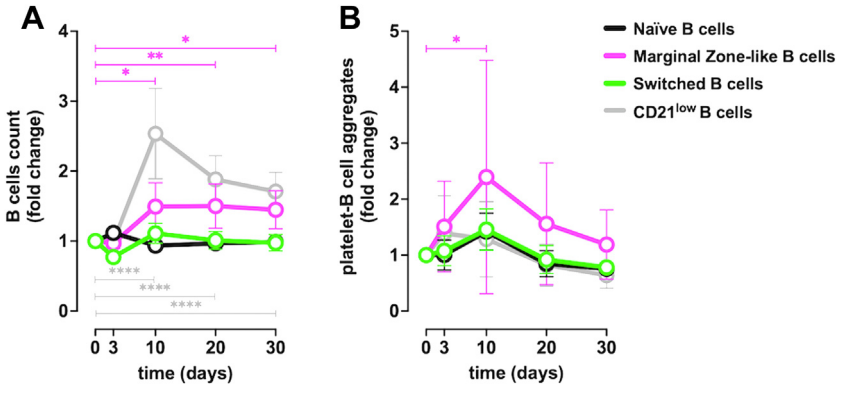

FIGURE 6 Platelet interactions with specific B-cell subtypes after the first dose of the adenovirus-based anti-SARS-CoV-2 vaccination. (A) Relative frequency of the circulating naïve ($IgD^+CD27^-CD19^+$), marginal zone-like ($IgD^+CD27^+CD19^+$), switched ($IgD^-CD27^+CD19^+$), and $CD21^{low}$ B cells ($CD21^{low}CD38^-CD19^+$) after one dose of AZD1222. (B) Relative frequency of the platelet binding to specific B-cell subtypes. Shown is median ± IQR of the fold change relative to baseline (before vaccination). Flow cytometry acquisition was performed on a BD Accuri C6 Plus and analyzed with FlowJo LLC software, version 10.8.1. Two-way ANOVA tests with Šidák multiple comparisons tests for intergroup analyses (in black) and with Tukey multiple comparisons tests for intragroup analyses (colored). *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$

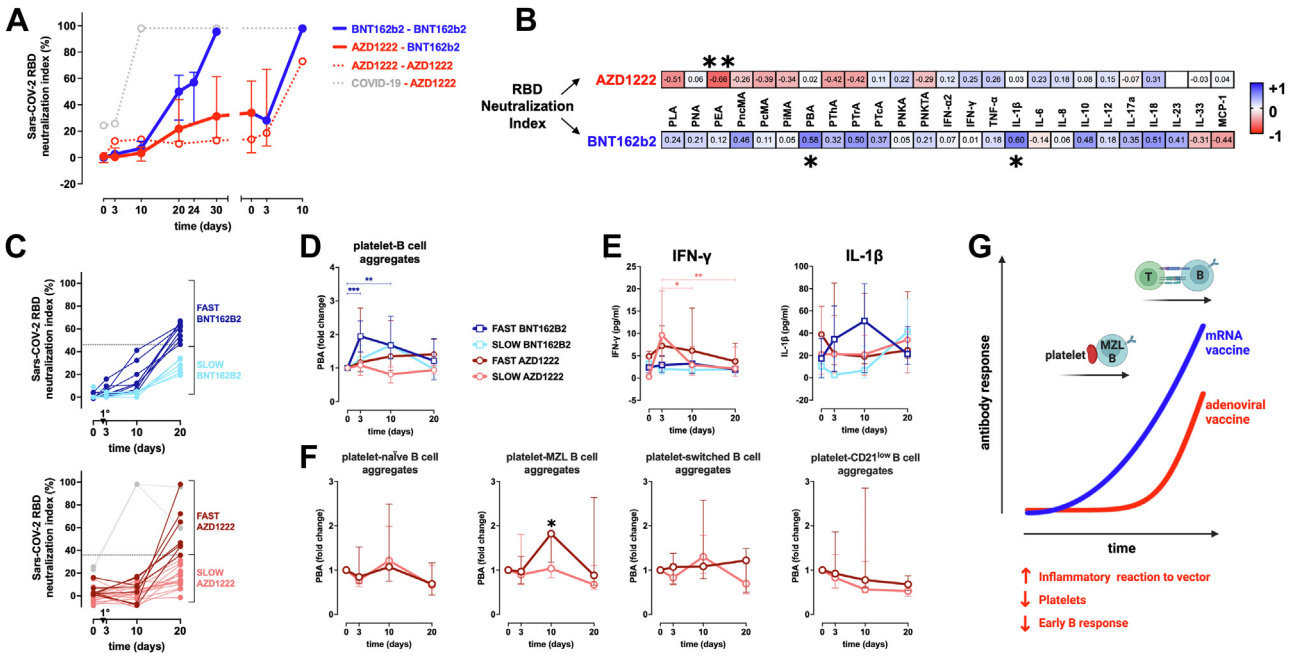

FIGURE 7 Platelet and immune features that correlate with a faster humoral response after the anti-SARS-CoV-2 vaccines. (A) Serum levels of neutralizing antibodies against the receptor binding domain (RBD) of the SARS-Cov2 spike protein of subjects receiving 2 doses of BNT162B2 (blue continuous line), one dose of AZD1222 and one of BNT162B2 (red continuous line), 2 doses of AZD1222 (red dotted line), or one dose of AZD1222 after a previous Sars-COV-2 infection (gray-dotted line) measured by ELISA and expressed as percentage of neutralization index. (B) Spearman rank correlation between the RBD neutralization index 20 days postvaccine and the fold change of platelet-leukocyte aggregates and cytokine levels 3 days after vaccination. In each box is indicated the correlation coefficient r, which ranges from +1 (positive correlation, blue) to −1 (negative correlation, red). (C) Stratification of subjects of the BNT (top) and the AZD cohort (bottom) in FAST (BNT162b2: dark blue, AZD1222: dark red) or SLOW (BNT162b2: light blue, AZD1222: light red) responders based on their ability to reach a Sars-COV-2 RBD neutralization index above the mean of their cohort within 20 days after the first injection. (D) Comparison of the platelet-B cell interactions in AZD1222 or BNT162b2 fast and slow responders. (E) Comparison of the IFN-γ and IL-1β concentration in AZD1222 or BNT162b2 fast and slow responders. (F) Comparison of the interactions between platelets and B-cell subtypes in fast and slow AZD1222 responders. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. Subjects who had a Sars-CoV-2 infection before vaccination (gray lines in panels A and C) were excluded from analysis shown in panels B, D, E, and F. (G) Working hypothesis of the study: both mRNA and adenoviral vaccines induce platelet-marginal zone-like (MZL)-B cells interactions that facilitate a faster antibody response. The antiviral platelet-consuming innate reaction against the adenoviral vaccine AZD1222 dampens the induction of the antibody response. Illustration created with Biorender.com. PLA, platelet-leukocyte aggregates; PNA, platelet-neutrophil aggr.; PEA, platelet-eosinophil aggr.; PncMA, platelet-nonclassical monocyte aggr.; PcMA, platelet-classical monocyte aggr.; PiMA, platelet-intermediate monocyte aggr.; PBA, platelet-B cell aggr.; PThA, platelet-T helper cell aggr.; PTrA, platelet-T regulatory cell aggr.; PTcA, platelet-T cytotoxic cell aggr.; PNKA, platelet-natural killer cell aggr.; PNKTA, platelet-natural killer T cell aggr.; IFN, interferon; IL, interleukin.

providing a further clue that platelets may be implicated in mounting an optimal B-cell response after vaccinations.

4 | DISCUSSION

This study compared the platelet and the immune response of the 2 main first-generation anti-SARS-CoV-2 vaccines administered in Europe in 2021. Our key findings are that (1) a single dose of the adenovirus-based vaccine AZD1222, which is more reactogenic and less immunogenic of the 2, evoked an acute increase of IFN-γ and other inflammatory cytokines that overlapped in time with an increased activation of platelets and granulocytes, increased frequency of circulating platelet-granulocyte aggregates, and a transient decline of the number of platelets and granulocytes in blood; (2) the mRNA-based vaccine BNT162b2, which evokes a faster humoral response compared with AZD1222, associated with a gradual increase of IL-1β, the expansion of regulatory T cells, and an increased interaction of platelets with adaptive immune cells; (3) both types of vaccine induced the formation of platelet-B cell aggregates that associated with a faster antibody response; and (4) aspirin treatment associated with a lower antibody response after BNT162b2 vaccination.

The main features that characterize the short-term response to the adenovirus-based vaccine are typical antiviral innate immune responses [8]. Adenoviral vectors were developed on purpose as vaccine delivery platforms for their ability to induce innate immune signaling that can augment the immunogenicity of the encoded antigen [29,30]. However, AZD1222 achieved a lower and slower antibody response than BNT162b2, which associated with a more gradual and

TABLE 1 Clinical characteristics and antibody responses to the BNT162b2 vaccine of vaccinated health care workers receiving chronic aspirin treatment or not.

	Vaccinated subjects not taking aspirin (n = 56)	Vaccinated subjects taking aspirin (n = 56)	p
Age (y)	59.8±6.5	59.8±5.7	.988
Female sex (%)	18 (32%)	18 (32%)	1.000
BMI (kg/m^2)	26.2±3.1	26.4±4.1	.873
Hypertension (%)	36 (64%)	36 (64%)	1.000
T2DM	9 (16%)	9 (16%)	1.000
Coronary heart disease	9 (16%)	9 (16%)	1.000
History of stroke (%)	3 (5%)	3(5%)	1.000
Anti-Spike IgG (BAU) at T1	**1934 [1287-2083]**	**1139 [571-1701]**	**< .001**
Anti-Spike IgG (BAU) at T2	**359 [222-696]**	**206 [107-420]**	**.004**

Comparison of the antibody response of 112 subjects treated with 100 mg/die aspirin or not, matched for age (mean ± SD), BMI (mean ± SD), sex, and comorbidities, from a cohort of health care workers who received 2 doses of the mRNA-based anti-SARS-Cov-2 vaccine, BNT162b2, between January and March 2021, without having experienced a previous COVID-19 infection. The immunoglobulin G (IgG) levels, expressed as median and [IQR] of the binding antibody units/mL (BAU), were measured by ELISA between 1 month (T1) and 5 months (T2) after the second vaccine dose. The Mann–Whitney analysis between the 2 matched groups was performed with SPSS software. BMI, body mass index

tolerogenic response. Based on our data, we can think of 3 reasons that could explain the lower efficacy and the slower kinetics of the adenovirus-based vaccines.

First, the antiviral response against the vector could be reducing antigen availability. Adenoviruses are not blood-borne viruses, and if they enter the circulation, they are promptly sequestered by macrophages of the liver or the spleen [31,32]. Platelets are the first blood cells to bind adenoviruses, and in the process of shuttling them to macrophages, platelets are cleared as well [33,34]. Intravenous injection of adenoviruses in mice induces platelet activation and thrombocytopenia within 24 hours [35]. Neutrophils are recruited to the spleen and the liver after the platelets and cooperate in eliminating the adenovirus [36] and adenovirus-containing macrophages [37] by phagocytosis. In our study, we detected consistent 20% and 40% reductions of the platelets and granulocyte counts, respectively, and an increased activation of these cell populations 3 days after intramuscular (i.m.) injection of AZD1222, but not after BNT1262b2. We hypothesize that this is an indication that some of the adenoviral vaccine injected in the muscle reaches the blood, and it is sequestered. Thus, less antigen is transduced and is available to evoke the adaptive response. To the best of our knowledge, this is the first study to document a lowering of the platelet count and the second [38] to document an increased platelet activation in healthy subjects vaccinated with AZD1222, possibly because few studies included time points shorter than 7 days [39,40]. In a recent study [27], a similar 20% decline in the platelet count after AZD1222 i.m. injection was shown in mice. The authors speculated that VITT occurs in subjects who accidentally undergo i.v. injection, which drives massive platelet activation and sequestration, but they did not comment the platelet count reduction following i.m. injection because it was moderate and stayed within the normal range. Because AZD1222 contains high levels of EDTA that can increase vascular leakage [41,42], it is plausible to assume that even if the vaccine is properly injected in the muscle, there could be some leakage of the vector in the blood, which is safe as long as it is in small amounts. In fact, the participants of our study did not become thrombocytopenic (platelet count <150 000 platelets/μL) and did not complain of any hemostatic defect post-vaccination. Based on this evidence, it would be advisable to monitor the short-term platelet responses during the development of new and safer vaccine delivery platforms.

A second reason for the uncoupling between the innate and the adaptive responses to the adenovirus-based vaccine could be the wrong timing and intensity of IFN-γ signals. Preclinical [43,44] and clinical [45] studies demonstrated that AZD1222 evokes an increase of IFN-γ that is produced by T helper 1 (Th1) cells and that correlates with an effective stimulation of the humoral response. However, most studies were designed to monitor the immune response starting from 7 to 10 days post-vaccination and did not detect the acute response to AZD1222. Our study provides evidence that AZD1222 evokes an earlier increase in IFN-γ that does not correlate with the production of neutralizing antibodies. The most likely source of IFN-γ 3 days post-AZD1222 are NK cells not Th1 cells because, at this time point, we observed a correlation between the IFN-γ levels and the NK cell count (Figure 2E) and a 2-fold increase in circulating platelet-NK aggregates, suggesting that circulating NK cells were active (Figure 5). Interestingly, the slow AZD1222-responders, ie, the study participants who failed to generate an effective neutralizing antibody response after the first dose, experienced more extreme IFN-γ changes compared with fast AZD1222-responders. Early exposure of peripheral monocytes to high concentrations of IFN-γ has been shown to skew the differentiation of peripheral monocytes into macrophages rather than to functional dendritic cells able to generate antigen-specific T-cell responses [46]. Thus, although a Th1-induced IFN-γ response is desirable to mount an optimal adaptive response to the vaccine, an early and excessive IFN-γ response against the adenoviral vector could be counterproductive.

Another reason why AZD1222 might be less immunogenic is that if platelets and neutrophils are engaged in eliminating the vector, they are not available to perform their costimulatory role in adaptive immunity. Previous studies have demonstrated *in vitro* and in murine models that platelets stimulate B-cell activation and function [15,16]. In a former study, we have identified a correlation between a fast antibody response with the level of circulating platelet-B cell aggregates after mRNA-based vaccination [17]. Here, we provide further evidence that platelets may be modulating B cells in humans because we document a negative effect of the antiplatelet drug aspirin on the

antibody response to the BNT162b2 vaccine. Moreover, we show that platelet-B cell aggregates increase shortly after both types of vaccine, particularly in fast responders. Because circulating B cells express low levels of PSGL1 [47] and P-selectin expression is low after both vaccines, these interactions are likely mediated by other receptor/ligand pairs. Interestingly, we find that platelets bind primarily to MZL-B cells, a B-cell subtype geared to respond rapidly to blood-borne pathogens and bridge the temporal gap between innate and adaptive immunities [48]. Similarly, neutrophils have been shown to stimulate B cells in the marginal zone of the spleen to ensure a rapid defense before the T-dependent humoral response is evoked [49]. However, after AZD1222, the platelet and neutrophil numbers decline, and the platelets bind to innate immune cells rather than to adaptive immune cells. Thus, it is plausible to assume that they are less effective in co-stimulating the adaptive response. These results have significant implications for optimizing the administration of vaccines against SARS-Cov-2 or other viruses such as influenza because a large portion of the population takes antiplatelet drugs for cardiovascular prevention, particularly among the elderly. To exploit our findings for the design of more effective vaccinations, we are currently investigating how different vaccine adjuvants modify the platelet-B cell crosstalk.

Understanding the platelet-B cell crosstalk is also critical to understanding the pathogenesis of VITT and improve vaccine safety. The anti-PF4 antibodies that cause heparin-induced thrombocytopenia are generated by MZL-B cells [50], and after AZD1222 i.v. injection in mice, AZD1222-bound platelets interact with marginal zone B cells in the spleen [27]. However, in our cohort of healthy individuals with no signs of VITT, the formation of platelet-MZL-B cell aggregates was associated with a faster vaccine response. Thus, we hypothesize that in most individuals, the platelet-MZL interaction helps to mount a protective immune response against the antigen and bridge the temporal gap between innate and adaptive immunities. Although in rare cases, probably after accidental i.v. vaccine injection, the adenoviral vector triggers an excessive platelet response and fosters the formation of pathologic antibodies that trigger VITT. Further studies are needed to test this hypothesis.

Being an observational study set at a particular time in history, our study has many limitations. Because we completed the first round of enrollment, most of the Italian population had received the first dose of the anti-SARS-CoV-2 vaccine, and AZD1222 had been suspended. Thus, we could not perform additional experiments to further characterize the platelet-B cell crosstalk, and we lack data on the platelet interaction with B-cell subtypes after the first dose of the BNT162b2 vaccine. Moreover, aspirin has anti-inflammatory effects beyond platelet inhibition [51,52], and we could not perform cell-based assays in vaccinated subjects taking aspirin. Thus, we may not exclude that aspirin dampens vaccine efficacy through mechanisms unrelated to platelets. However, this study provides important insights because it is the first to report the short-term effects of genetic-based vaccines on the platelet-immune crosstalk and provide evidence that antiplatelet drugs for cardiovascular prevention may interfere with vaccine efficacy.

In conclusion, we provide preliminary evidence that platelets are a component of the innate signaling pathways evoked by vaccines and can shape the adaptive response after immunization. We describe how the adenoviral-based vaccine induces an antiviral-like immune response that involves platelet activation and might explain why this vaccine is less immunogenic and why it is associated with a higher risk of VITT. On the other hand, we show that the mRNA-based vaccine is more tolerogenic because it induces a milder innate immune response and increases the number of circulating regulatory T cells. Future studies on the role of platelets in responding to different vaccine platforms and modulating B-cell function may open new avenues to improve the safety, efficacy, and strategic administration of next-generation vaccines.

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AUTHOR CONTRIBUTIONS

L.L. and M.F. equally contributed to the study. L.L. and F.M. performed experiments, analyzed and interpreted the data, and contributed to the final manuscript. R.M. performed the experiments and analyzed the data. A.S., N.S., and D.G. performed experiments: M.M., G.F.R., and B.C. enrolled the study participants. S.P. performed experiments, analyzed the data, and contributed to the final manuscript. A.P. designed the study and critically revised the manuscript. F.P. performed experiments and revised the manuscript. R.C. analyzed the data and contributed to the final manuscript. M.V. analyzed the data and critically revised the manuscript. S.B. designed the study, analyzed the data, and critically revised the manuscript. L.S. designed the study, analyzed and interpreted the data, and wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

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