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Influence of the Immune Checkpoint Inhibitors on the Hemostatic Potential of Blood Plasma

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Keywords
Immune checkpoint inhibitors · Aggregation · Coagulation · Fibrinolysis · Platelet-rich plasma · Platelets · Thrombocytes

Abstract

Introduction: Immune checkpoint inhibitors (ICIs) have revolutionized classical treatment approaches of various cancer entities, but are also associated with a number of side effects. One of these may be life-threatening clotting disorders with the risk of thrombotic or hemorrhagic complications, the mechanisms of which are still poorly understood. In the present study, we analyzed the direct effects of pembrolizumab, nivolumab, and ipilimumab on platelet aggregation as well as plasma coagulation followed by fibrinolysis in an ex vivo model. ***Methods:*** Microplate spectrometry was used to analyze aggregation, coagulation, and fibrinolysis in platelet-free (PFP) and platelet-rich (PRP) healthy donor plasma samples treated with pembrolizumab, nivolumab, ipilimumab, and appropriate isotype controls. Aggregation was induced by TRAP-6. Clotting of PFP and PRP followed by lysis was initiated with a tissue factor in a mixture of phosphatidylserine:phosphatidylcholine and the addition of t-PA. Among other parameters, the area under the curve (AUC) was used to compare the effect of ICIs on aggregation, coagulation, and fibrinolysis. ***Results:*** Upon direct contact with platelets, pembrolizumab stimulated platelet aggregation in PRP, while nivolumab and ipilimumab promoted disaggregation with corresponding changes in the AUC. Pembrolizumab and nivolumab, both PD-1 receptor inhibitors, had no effect on the plasma coagulation cascade. Ipilimumab, a CTLA-4 receptor inhibitor, significantly increased the rate of PRP clotting. When clotting was followed by lysis, all ICIs were found to prolong the growth of the PRP-derived fibrin clot and delay its elimination. This was manifested by an increase in AUC relative to control PRP. ***Conclusion:*** This study characterizes the potential impact of pembrolizumab, nivolumab, and ipilimumab on hemostasis. Nivolumab and ipilimumab are able to reduce aggregation and increase the procoagulant properties of platelets, which can cause side effects associated with hemostatic imbalance leading to thrombosis or bleeding. The observed ICI-specific effects may contribute to our understanding of the mechanisms by which ICI affects platelets and suggest how, in a clinical setting, to reduce coagulation disorders during ICI treatment in the future.

Introduction

Cell surface immune checkpoint inhibitors (ICIs) include a class of checkpoint-blocking antibodies designed to impair tumoral immune-escape mechanisms

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by targeting programmed cell death protein 1 (PD-1), its ligand (PD-L1), or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). ICIs have been used to treat various types of cancers, including melanoma, gastric cancer, lung cancer, bladder cancer, and kidney cancer, serving as first-line therapy drugs today [1].

However, ICIs can also cause side effects, as they can unleash the immune system to attack healthy cells in the body as well. Specifically, ipilimumab, a CTLA-4 blocker, may induce a T-cell-mediated immune attack in a dose-dependent manner in 70–88% of patients causing dermatitis, liver abnormalities, and enterocolitis among other side effects [2, 3]. Pembrolizumab and nivolumab, by blocking PD-1, can disrupt the regulation of self-tolerance mediated by PD-1/PD-L1 interactions [4]. Common side effects include fatigue, rash, hypertension, and inflammation of the organs, such as the lungs or liver. In some cases, these side effects can be severe and even life threatening, in particular, during the development of hematological complications [5]. In recent years, a growing number of studies have documented the increased risk of thromboembolic events associated with the use of ICIs [6–10]. It has been reported that 8.2% of patients with lung cancer treated with nivolumab or pembrolizumab develop cancer-associated thrombosis [8]. In particular, the incidence of venous thromboembolism in patients receiving ICIs ranges from 6% to 18% [9]. A fairly representative number of studies on the risk of thrombosis associated with ICI treatment in cancer patients, including those for specific types of tumors, are presented in the review by Goel et al. [11].

The mechanisms causing these prothrombotic side effects of ICIs are currently poorly understood. ICIs, by disinhibiting immune pathways, enhance inflammatory responses in the vascular endothelium, which can lead to immune-mediated vasculitis causing damage to the endothelium with the formation of a thrombus at the site of injury [11].

ICI immunotherapy has been reported to induce an increase in platelet-derived microparticles (PMPs), exhibiting phosphatidylserine on the surface [12–14]. Phosphatidylserine-containing PMPs are able to concentrate coagulation factors, especially tissue factor (TF), on their surface, which promotes the formation of fibrin and enhances platelet aggregation. Therefore, increased formation of PMPs in plasma can serve as early indicators of prothrombotic side effects of ICIs. Paradoxically, immunotherapy, while increasing the risk of thrombosis, can also cause thrombocytopenia by reducing platelet counts and, depending on the degree of thrombocytopenia, increase the risk of bleeding [15]. Thrombocytopenia as an adverse effect of ICI therapy has been reported in approximately 15–37% of cancer patients [16]. Clinically, it may appear (not always) as petechiae, purpura, ecchymoses, epistaxis, and other hemorrhagic lesions on the skin and, in rare cases, can be associated with severe bleeding events such as intracranial hemorrhage [17]. Although the exact etiology of immune thrombocytopenia is still being debated, the important role of antibodies in promoting platelet elimination is becoming apparent. Many cancer patients which developed thrombocytopenia during ICI therapy have been reported to have high levels of antiplatelet autoantibodies [18].

In a case series [19], it was reported that immune thrombocytopenia occurred in cancer patients treated with pembrolizumab, nivolumab, and ipilimumab. It appears that the frequency of immune thrombocytopenia is low, and is highly dependent on the type of cancer, the nature of the ICI, and the ICI therapy regimen (e.g., mono- or combination immunotherapy). In general, immune thrombocytopenia occurs in 0.5–13% of the total number of cancer patients receiving immunotherapy and can lead to treatment-related death (0.45%–1.2%) [19].

Of note, the use of ICIs in the treatment of cancer is growing rapidly. Thus, in just 2 years, from 2015 to 2017, the number of cases of therapeutic use of PD1/PDL-1 inhibitors in the USA increased by more than 600% [20]. Given this trend, it can be expected that the side effects of immunotherapy will also be more frequent and varied, which will require, in particular, the rapid detection of hematological disorders. The use of conventional aggregometry as well as clotting methods for assessing the coagulability of the plasma of cancer patients seems to be promising. Routine turbidimetry in various versions (coagulometry, microplate spectrometry, aggregometry) can be a useful tool to screen for abnormal hemostasis deviations, including those associated with the action of ICIs. An important advantage of this approach is the possibility of simultaneously obtaining several evaluation parameters that describe various stages of fibrin clot formation, as well as its subsequent degradation. Early detection of hematological adverse events associated with anticancer immunotherapy is critical to recognize and prevent fatal outcomes caused by ICI toxicity.

In our previous in vitro pilot study using platelet concentrates treated with a short 10-min exposure to ICIs [21], we found no effect of ICIs on platelet aggregation, in both nonactivated and TRAP-activated settings. Those results indicate the absence of highly specific binding of the studied ICIs to modify platelet functions. In the present study, by doubling the exposure time and using PRP supplemented with 1 mM Ca^{2+}, we attempted to detect an off-target ICI impact on the functional activity of platelets. Most notably, glycoprotein (GP) IIb–IIIa (integrin $\alpha_{IIb}\beta_3$), which is responsible for platelet aggregation function and its loss during the transition to the procoagulant state [22], can be such a random nonspecific target for ICIs. Accordingly, the aim of this study was

to investigate the direct ex vivo effects of ICIs on platelet aggregation, fibrin clot formation, and fibrinolysis in order to identify ICI-responsive parameters of primary and secondary hemostasis as potential biomarkers of ICI toxicity.

Materials and Methods

Reagents and Kits

Pembrolizumab (Keytruda®) was obtained from Merck Sharp & Dohme (Kenilworth, NJ, USA). Ipilimumab (Yervoy®) and Nivolumab (Opdivo®) were acquired from Bristol Myers Squibb (New York City, NY, USA). Purified human IgG1 Isotype Control and purified human IgG4 Isotype Control were purchased from BioLegend (Dedham, MA, USA). Thrombin Receptor Activator Peptide 6 (TRAP-6) trifluoroacetate salt was obtained from Bachem (Bubendorf, Switzerland). TFs were from PeproTech Germany (Hamburg, Germany), and phosphatidylserine and phosphatidylcholine were purchased from Sigma Aldrich (St. Louis, MO, USA). Rt-PA (Actilyse Cathflo) was obtained from Boehringer Ingelheim Pharma GmbH (Ingelheim am Rhein, Germany).

Blood Collection and Preparation

Whole blood was drawn from 11 healthy blood donors (5 women and 6 men with average age 33.2 years) into 0.106 M trisodium citrate monovettes (S-Monovette, Sarstedt) for anticoagulation. All samples were processed within 1.5 h after donation. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Friedrich-Alexander University Erlangen-Nürnberg (FAU) (#346_18B, #343_18B, #73_19B). Informed consent was obtained from all subjects involved in the study.

Citrated blood was centrifuged at 190 *g* for 15 min without breaks. For aggregation measurements, platelets were counted and adjusted to 300×10^6 cells/mL with autologous platelet-free plasma (PFP). For clotting or clotting/lysis measurements, PRP was concentrated by centrifugation at 360 *g* for 15 min at room temperature. The upper part (1 mL) of this centrifugate was taken and centrifugated at 10,000 *g* for 7 min at room temperature for PFP preparation.

Microtiter Plate-Based Aggregometry of PRP

Aggregation of PRP was measured with a modified microtiter plate assay based on Moran et al. [23]. In detail, aliquots (85 µL) of PRP were dispensed into the appropriate wells of a 96 U-bottomed microtiter plate (Greiner BioOne) containing 10 µL of 0.05 M TBS buffer, pH 7.3 in a control sample or with ICI solution in a test sample with final concentrations of 75 µg/mL, 58.7 µg/mL, and 85 µg/mL for pembrolizumab, nivolumab, and ipilimumab, respectively [21]. Calcium chloride at a final concentration of 1.25 mM was added to the buffer used for the control sample or for ICI dilution. Samples were incubated for 15 min at 37°C and then analyzed for the optical density in duplicates using a BMG Labtech FLUOstar Omega (Ortenberg, Germany). 5 µL of TRAP-6 was added to samples to a final concentration of 15 µM after the 3.5-min reading point. Forty-five measurement cycles were recorded during 15 min at 37°C and 700 rpm of orbital shaking for 3 s prior to each cycle of measurement at the wavelength of 450 nm.

Turbidity Analysis of Plasma Clotting

TF-initiated clotting was evaluated by a one-stage clotting test in accordance to Mackie et al. [24] in which PFP is regarded as single factor-deficient plasma, platelets as lacking factor, and autologous PRP as deficiency-corrected plasma. PFP and autologous PRP clotting were provided using the following scheme: 50 µL of PFP or PRP was placed in a wells of 96-well F-bottom plate, mixed with 50 µL of 0.05 M Tris-HCl buffer with 0.13 M NaCl, pH 7.4 (TBS). The clotting reaction was initiated by the addition of 50 µL of TF/phospholipid (PL) mixture in TBS/Ca^{2+} using a multichannel pipette. TF/PL mixture in TBS/Ca^{2+} contained 30 pM of TF and 15 mM of $CaCl_2$ diluted in 300 µM of PL solution (30:70 of phosphatidylserine to phosphatidylcholine, respectively). Clotting followed by lysis was obtained after the addition of t-PA in TBS (final concentration of 40 IU/mL).

In variants where ICIs were analyzed, 15 µL of ICI solution and 35 µL TBS were added (instead of 50 µL TBS). According to our previous study [21], experimental concentrations of pembrolizumab, nivolumab, and ipilimumab were 75 µg/mL, 58.7 µg/mL, and 85 µg/mL, respectively.

The clot waveform analysis was used to detect the impact of pembrolizumab, nivolumab, and ipilimumab on the coagulation and the fibrinolytic systems. Plasma absorbance during clotting or clotting/lysis was monitored at 405 nm every 5 min employing the BMG Labtech FLUOstar Omega microplate reader (Ortenberg, Germany) until a constant maximum absorbance was achieved in two parallel samples (or 50% reduction in maximum absorbance due to lysis). Because PFP and PRP have different baseline absorbances, all subsequent measured absorbance values were normalized to the baseline.

By plotting the optical density versus time, the OD(t) curve was obtained, and the kinetic parameters were calculated from the waveform of its first derivative [25]. Eventually, the positive values of the first derivative reflected the coagulation velocity and negative values of the first derivative reflected the fibrinolysis velocity. The *initial clotting rate* was estimated as the value of the first derivative in the third minute after exiting the lag period. The *maximum clotting rate* was defined as the maximum positive value of the first derivative. The *initial fibrinolysis rate* was estimated as the first negative value of the first derivative. The *maximum fibrinolysis rate* was defined as the maximum negative value of the first derivative. Maximum absorbance (*ODmax*) represents the maximum value of optical density after reaching a plateau, indicating the completion of clotting. *Time to reach ODmax* was defined as the time when ODmax appears. The *area under the curve* (AUC) was calculated as the integral of the OD(t) function and was used for the assessment of the overall hemostatic balance in plasma.

Statistical Analysis

Data are presented as median with 95% CI. The AUC calculated from the aggregation curves, as well as the clotting parameters, was compared for the ICI groups with those of control plasma or isotype controls using the Wilcoxon rank test for matched pairs or paired *t* test. p values <0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism 9.5.1 (GraphPad Software, San Diego, CA, USA).

Results

ICIs Affect the Aggregation of Platelet-Rich Plasma in Different Ways

The direct effect of ICIs on platelet aggregation was studied in PRP samples adjusted to 300×10^6 cells/mL with autologous PFP. Using a microtiter plate assay, we were able to simultaneously monitor platelet aggregation for several samples, allowing us to obtain greater sensitivity and comparability between control and tested

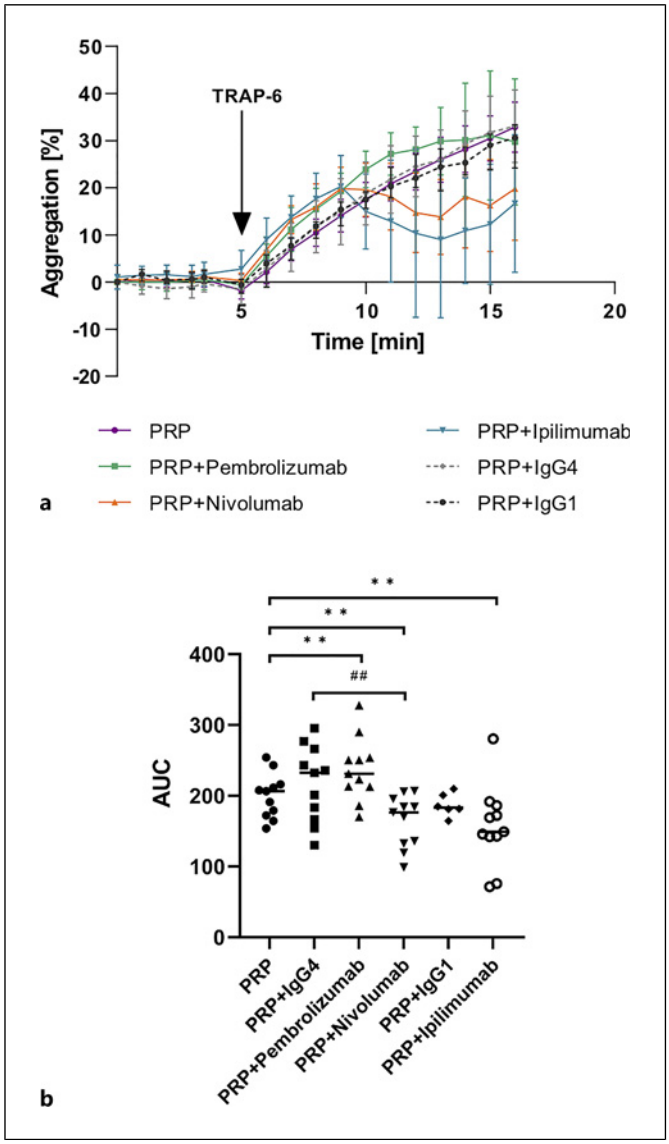

Fig. 1. ICIs directly affect aggregation of platelet-rich plasma. A microtiter plate-based assay was used for the performed aggregometry using a multichannel microplate reader. PRP was adjusted by autologous PFP to 300,000/µL and incubated with pembrolizumab (75 µg/mL, final concentration), nivolumab (58.7 µg/mL, final concentration), and ipilimumab (85 µg/mL, final concentration) as well as appropriate isotype controls or Tris-buffer solution (positive control) for 15 min at RT. After 3 min of measurement under 37°C (negative control), TRAP-6 (15 µM, final concentration) with $CaCl_2$ (1.25 mM, final concentration) was injected and measurements continued. There were 45 measurement cycles in total (20 s each) with 3 s shaking before each cycle. **a** Aggregation curves obtained from median values for each time point (n = 11). **b** Area under the aggregation curve (AUC) is represented as median with 95% CI. Differences were significant for ICI versus PRP at $p < 0.05$ (*) or $p < 0.01$ (**) and for ICI versus Isotype Control at $p < 0.01$ (##) as indicated. RT, room temperature.

samples treated in duplicate with pembrolizumab, nivolumab, and ipilimumab. Within 3.5 min from the start of the measurement, the presence of spontaneous platelet aggregation was checked and, in its absence, TRAP-6 was added to induce PAR-1-dependent aggregation. Tested ICIs (pembrolizumab, nivolumab, and ipilimumab) did not contribute to the induction of spontaneous aggregation; moreover, they presented almost no effect on the initial stage of TRAP-induced aggregation (Fig. 1a). However, starting from the ninth minute on (5–6 min after TRAP-6 injection), significant changes were found in ICI-mediated aggregation patterns. As evidenced by changes in AUC (Fig. 1b) under the influence of ICIs relative to control (PRP), pembrolizumab moderately stimulated aggregation (p = 0.0098, n = 11, Wilcoxon test), while nivolumab and ipilimumab suppressed it (p = 0.0127, n = 11 and p = 0.032, n = 11, respectively, Wilcoxon test). Moreover, the reversal of the aggregation curve for nivolumab and ipilimumab (Fig. 1a) indicates possible platelet disaggregation. Using Wilcoxon test, we also found statistically significant differences between nivolumab-treated samples compared to IgG4 isotype control (p = 0.0068, n = 11) along with no difference for pembrolizumab and ipilimumab compared to their isotype control IgGs (p = 0.123 vs. IgG4, n = 11; p = 0.125 vs. IgG1, n = 6, respectively).

PD-1 and CTLA-4 Blockers Affect Different Targets of the Coagulation System

In order to distinguish if ICIs can affect the coagulation cascade, either through the effect on soluble coagulation factors or through platelets involved in coagulation, TF-induced clotting of PFP and autologous PRP was compared. A direct drug contact with plasma components was provided in an ex vivo model by mixing plasma aliquots with the appropriate inhibitor and inducing coagulation after 15 min of incubation. Clotting dynamics were recorded simultaneously in PFP and PRP samples added to the wells of a microtiter plate. Clotting initiation with the TF/PL mixture was also performed simultaneously for PFP and PRP.

The direct effect of ICIs on PFP clotting was found to depend on their nature: IgG4-like inhibitors, such as pembrolizumab and nivolumab, tended to decrease the initial clotting rate, while ipilimumab and its isotype control protein, IgG1, tended to increase it (Fig. 2a). A marked increase in the initial plasma clotting rate (approximately 20%) under the action of ipilimumab and IgG1, however, was not confirmed by testing for statistical significance due to large fluctuations among donors. Despite these initial differences, ICIs had no effect on the subsequent dynamics of PFP coagulation and showed no procoagulant activity. Moreover, nivolumab, which significantly increased ODmax (p = 0.043, paired t test) (Fig. 2c) and time to reach ODmax (p = 0.0024, Wilcoxon test; Fig. 2d), exhibited rather anticoagulant properties compared with untreated PFP.

When PRP was used instead of PFP, a differential effect of ICIs on clotting parameters was observed.

Namely, pembrolizumab and especially nivolumab in the presence of platelets no longer showed their procoagulant-like properties, while ipilimumab demonstrated them strongly, significantly increasing the maximum clotting rate (p = 0.0186, Wilcoxon test; Fig. 3b) and shortening the time to reach ODmax (p = 0.0245, paired t test; Fig. 3d).

Clearly, the novel method we applied, namely comparing the response of autologous PFP and PRP to drug effects, proved to work. Due to the similar background of humoral coagulation factors in PFP and PRP, as well as the difference due to the presence or absence of platelets, we were able to identify potential targets for the direct influence of ICIs. It appears that pembrolizumab and nivolumab, as PD-1 receptor inhibitors, are able to interact directly with coagulation factors in plasma by slightly stimulating the coagulation cascade. Ipilimumab, an inhibitor of the CTLA-4 receptor, appears to act on the coagulation system indirectly through its effect on platelets in a way that enhances the ability of cells to promote coagulation.

ICIs Presumably Affect Activation Stage of Fibrinolytic Process

We used our comparative approach, which was found to be useful for identifying the effect of ICIs on the coagulation properties of PFP and PRP, to study the responsiveness of the fibrinolysis system to the direct action of ICIs. Namely, we simulated the conditions of the so-called internal (physiological) fibrinolysis, which differs from external (therapeutic) fibrinolysis in that it is induced at the early stages of fibrin formation and develops in close dependence on the accumulation of fibrin during plasma clotting [26]. Therefore, t-PA, a fibrinolysis inducer, was added together with TF/PL, a coagulation inducer.

Unexpectedly, the most significant manifestation of the effect of all ICIs accompanied by t-PA was an increase in the initial rate of PFP clotting. The clearly visible difference of this parameter between the untreated and ICI-treated PFP, however, was not confirmed by statistical tests (Fig. 4a).

Regarding the parameters of fibrinolysis, no significant effect of drugs on the initial and maximum rate of visible fibrinolysis for PFP-formed clots was found. Some exception may be a pembrolizumab-mediated 25% increase in the maximum rate of fibrinolysis (Fig. 4d), but this effect was not statistically significant due to the large scatter of individual values.

In PRP, the influence of ICIs was more obvious, manifesting itself as a change in integrative parameters that reflect the interaction of two systems, coagulation and fibrinolysis. The first parameter is the time to reach ODmax; the second is the AUC. Both are designed to sum up two opposite processes, namely, fibrin production and subsequent degradation of the newly formed fibrin.

In the ICI-treated samples, the time to reach ODmax increased compared to the control (untreated PRP; Fig. 5e). Significant differences were confirmed by a paired t test for inhibitors (p = 0.0004 for pembrolizumab, p = 0.0031 for nivolumab, and p = 0.0084 for ipilimumab) and the respective isotypic control immunoglobulins (p = 0.0088 for IgG4 and p = 0.0024 for IgG1). These data indicate an ICI-induced prolongation of the growth of the fibrin clot and a delay in its elimination. The AUC significantly increased with pembrolizumab (p = 0.0199, paired t test), nivolumab (p = 0.0174, paired t test), and ipilimumab (p = 0.0020, Wilcoxon test; Fig. 5f), indicating a drug-induced shift in the balance between coagulation and fibrinolysis, with the dominance of the former.

Overall, these data suggest that enzymes of the coagulation and fibrinolysis system have different reactivity to various ICIs depending on their state, free or bound to the surface of platelets. It is possible that the direct activating (or destructive) action of ICIs additionally forms a procoagulant or profibrinolytic platelet phenotype.

Discussion

It is generally accepted that the side effects of ICIs used in immunotherapy are most often attributed to their excessive activation of innate immune cells [27, 28]. ICI-induced systemic proinflammatory effects may accelerate coagulation, inhibit fibrinolysis, and affect platelet functions and turnover [29]. The state of platelets, in itself, regardless of the factors of coagulation and fibrinolysis, can contribute to a shift in the hemostatic balance toward thrombosis or hemorrhage.

The possibility of a bimodal platelet response to immunotherapy has been identified, for example, in the treatment with bevacizumab, a humanized immunoglobulin monoclonal antibody (mAb). Side effects of bevacizumab treatment were associated with a possible influence in primary hemostasis and platelet function resulting in an increased rate of both thromboembolism and hemorrhage [30, 31]. Similar to these in vivo studies, our study showed that a direct ex vivo interaction of platelets in PRP with ICIs resulted in pronounced aggregation abnormalities. In particular, we found costimulation of TRAP-induced aggregation by pembrolizumab and aborted aggregation of platelets affected by nivolumab and ipilimumab compared to control PRP. In addition, only nivolumab differed significantly in its action from the IgG4 isotype control, indicating its specific binding to target molecules on the surface of platelets via the variable region of the antibody. Such a significant difference in the effects of nivolumab compared to pembrolizumab was not expected, since both

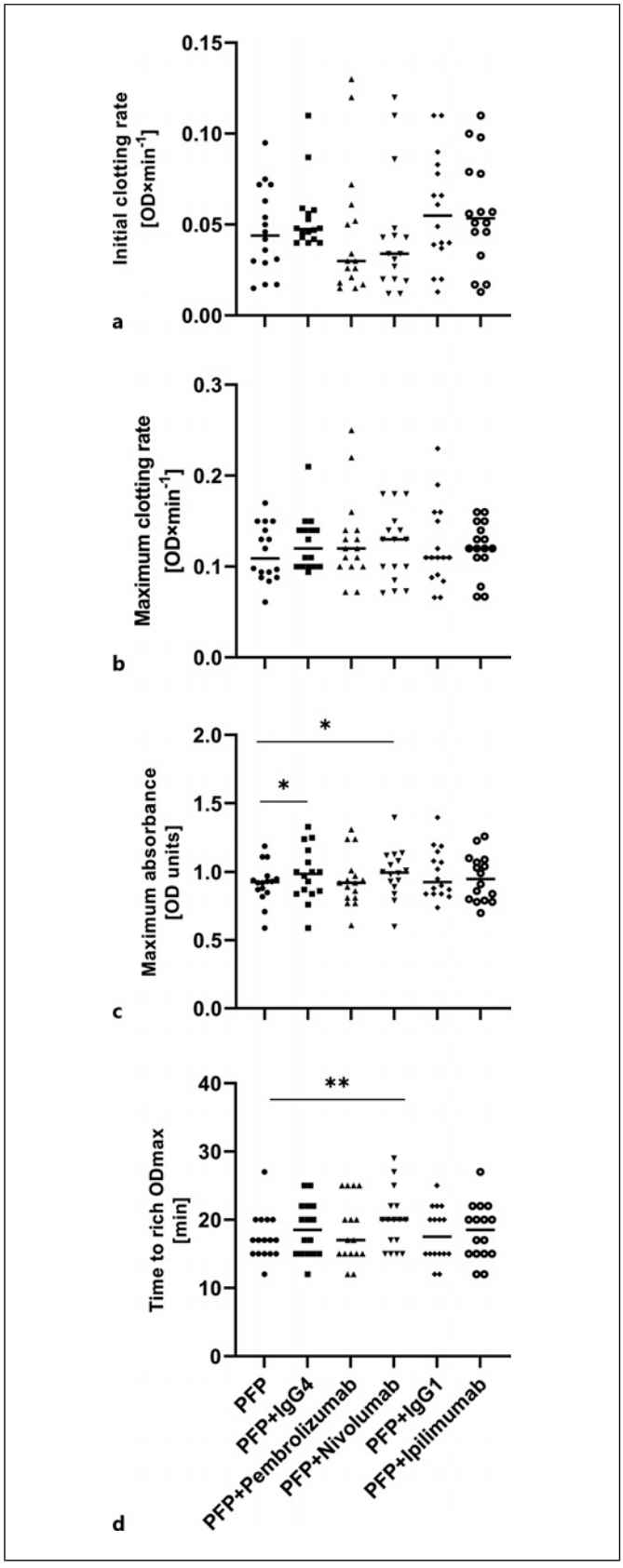

Fig. 2. PFP clotting responds poorly to direct exposure to ICIs. TF-induced clotting was measured turbidimetrically in a microtiter plate-based assay performed with a multichannel microplate reader. Undiluted PFP was incubated with pembrolizumab (75 µg/mL, final concentration), nivolumab (58.7 µg/mL, final concentration), and ipilimumab (85 µg/mL, final concentration) as well as appropriate isotype controls or Tris-buffer solution for 15 min at 37°C. Clotting was initiated by 30 pM TF/PL/$CaCl_2$ mixture. Absorbance curves obtained from median values for each time point (n = 11) were analyzed using WFA. The parameters of the original curve as well as the curve of its first derivative were used for comparative statistics: (**a**) initial clotting rate, (**b**) maximum clotting rate, (**c**) maximum absorbance, and (**d**) the time to rich maximum absorbance. Differences were significant at $p < 0.05$ (*) or $p < 0.01$ (**) as indicated.

antibodies belong to the IgG4 subclass. Moreover, they have a very similar binding mechanism to the targeted PD-1 receptor, interacting with its flexible loops through similar epitopes [32]. Thereby, they cover the surface of the PD-1 to varying degrees and in different spatial positions since pembrolizumab binds to the C′D loop and nivolumab binds to the N-terminal loop on the PD-1 molecule [33]. Perhaps this feature alone or in combination with other structural elements of nivolumab determined its special off-target interaction with aggregating platelets.

Platelets can be considered as immunological targets due to their coating with antigenic membrane components [34–37]. Numerous membrane-associated proteins, including GP receptors (integrins), determine the immunogenicity of platelets. Different platelet GPs can act as alloantigens, autoantigens, and targets for drug-dependent antibodies [35]. For example, antibodies to PI^A (HPA-l, human platelet antigen) epitopes located in subunit III of the receptor GP IIb-IIIa, inhibited clot retraction and platelet aggregation by blocking fibrinogen binding [35].

Heterodimeric receptor GP IIb-IIIa contains many antigenic determinants that significantly enrich the spectrum of immunogenic properties of platelets [38, 39]. But, first of all, GP IIb-IIIa ($\alpha_{IIb}\beta_3$) plays an indispensable role as an activation-dependent receptor responsible for platelet aggregation [40, 41]. After activation, GPIIb-IIIa interacts with bivalent fibrinogen molecules, which form "fibrinogen bridges" between adjacent platelets, involving them in aggregation during the formation of a hemostatic plug or thrombus. The activation of the receptor occurs due to the binding of its two subunits, which is accompanied by a change in their conformation and the appearance of neoepitopes for binding ligands. In addition to fibrinogen, other $\alpha_{IIb}\beta_3$ ligands such as von Willebrand factor, fibronectin, vitronectin, thrombospondin, and CD40 ligand can mediate this subunit cross-linking, thereby modulating platelet aggregation [38]. Accordingly, mAbs can affect the activity of the receptor both by direct interaction and indirectly through modulator ligands.

Specific mAbs against different epitopes on the GPIIb and/or IIIa receptor subunits are able to induce time-

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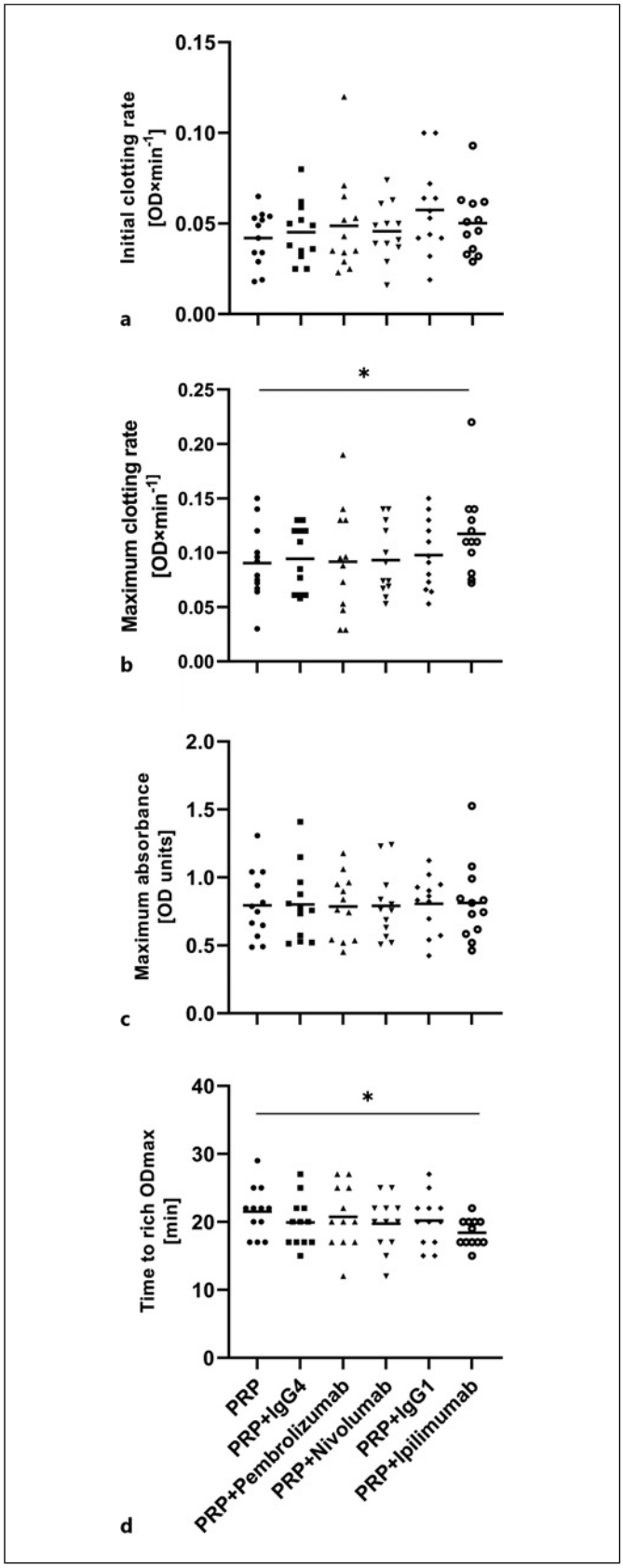

Fig. 3. PRP clotting is accelerated by direct exposure to ICIs. TF-induced clotting was measured turbidimetrically with a microtiter plate-based assay and was analyzed with a multichannel microplate reader. Undiluted PRP was incubated with pembrolizumab (75 μg/mL, final concentration), nivolumab (58.7 μg/mL, final concentration), and ipilimumab (85 μg/mL, final concentration) as well as appropriate isotype controls or Tris-buffer solution for 15 min at 37°C. Final platelet count varied in the range of 230,000–320,000/μL. Clotting was initiated by 30 pM TF/PL/$CaCl_2$ mixture. Absorbance curves obtained from median values for each time point (n = 11) were analyzed using WFA. The parameters of the original curve as well as the curve of its first derivative were used for comparative statistics: (**a**) initial clotting rate, (**b**) maximum clotting rate, (**c**) maximum absorbance, and (**d**) the time to rich maximum absorbance. Differences were significant at $p < 0.05$ (*) as indicated.

dependent clustering and subsequent internalization (after longer antibody exposure) of the receptor-antibody complex in native platelets [42, 43]. Receptor inactivation is a Ca-dependent process, requiring a consistently elevated level of intracellular Ca^{2+} [22]. By arranging prolonged contact of ICIs with platelets (20-min exposure) and maintaining a low millimolar concentration of Ca^{2+} in PRP, we reproduced (to some extent) the conditions necessary for contact of ICIs with the GP IIb-IIIa, which allows us to consider this aggregation suppression mechanism as quite suitable for explaining the effects seen for nivolumab and ipilimumab. Another possible way to inhibit aggregation could be off-target specific (nivolumab) or nonspecific (ipilimumab) occupancy of the GP IIb-IIIa, overlapping fibrinogen binding sites, which causes steric blockade of fibrinogen interaction with the receptor [35]. Since relatively high concentrations of mAb (≈0.5 μM) were used, nonspecific off-target binding of ipilimumab seems quite likely. Apparently, pembrolizumab also had a similar nonspecific effect, which, unlike nivolumab and ipilimumab, acted as a weak proaggregant. The corresponding IgG4 isotype control showed no such effect, but the statistical difference between pembrolizumab and IgG4 was not significant. The apparent discrepancy between these data can be explained by assuming that nivolumab has a higher affinity for $\alpha_{IIb}\beta_3$ receptor than pembrolizumab. By reaching saturating concentrations more easily, nivolumab will counteract aggregation by inhibiting fibrinogen binding, while fewer pembrolizumab molecules can act as a fibrinogen binding stimulator. Similar stimulation/inhibition was found for the anti-PI^{A1} autoantibody and some inhibitory peptides (disintegrins) and has since been known as dose-dependent stimulation versus inhibition of fibrinogen binding [35]. Another possible explanation could be that pembrolizumab has two or more epitope binding sites that may mimic receptor dimerization (clustering) promoting aggregation [32, 44]. A similar activation mechanism has also been described that requires the antibody to first bind to its antigenic determinant on the platelet surface, after which the antigen cross-links to the platelet FcTII receptor via the Fc portion of the antibody [34]. In the systemic circulation,

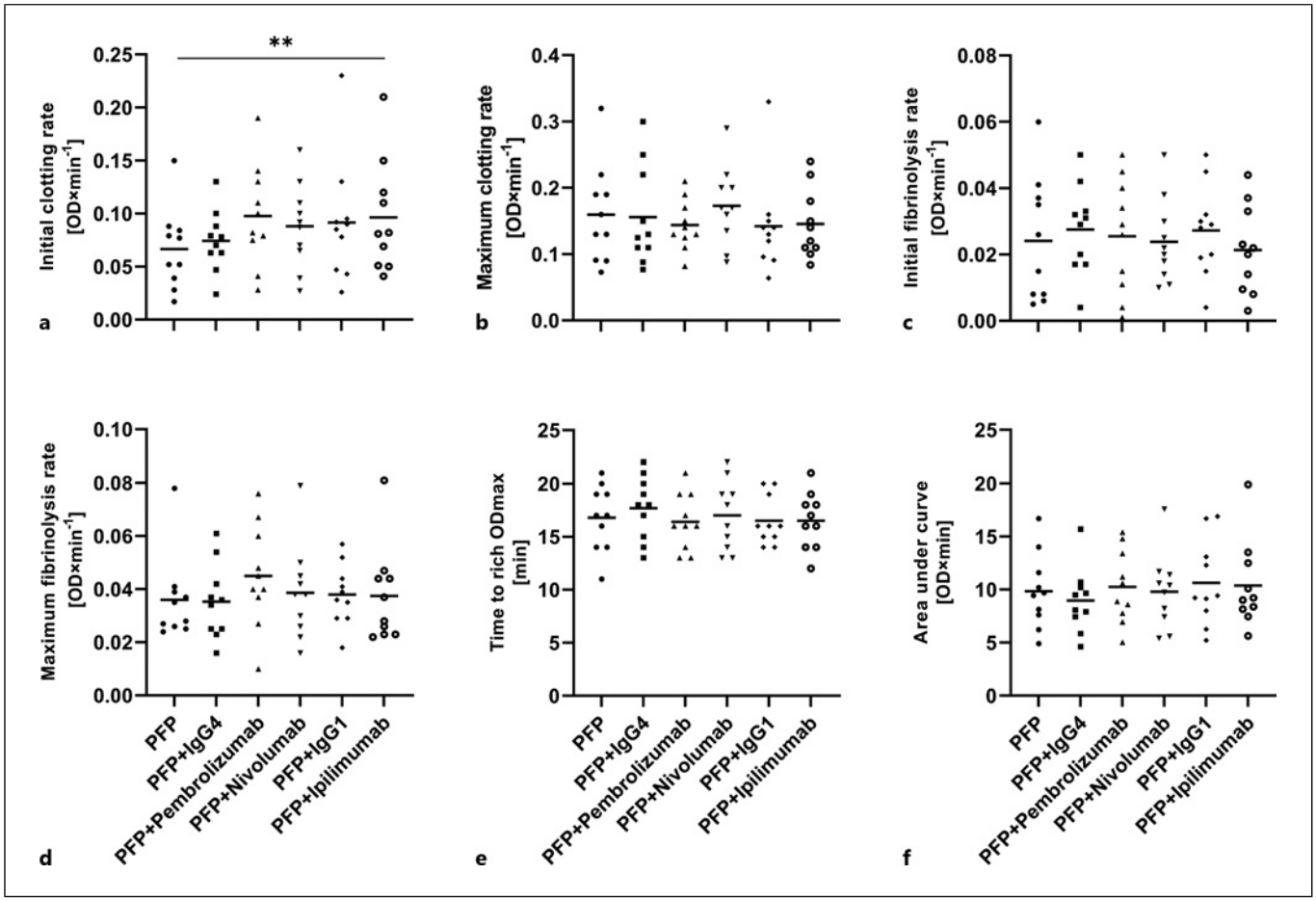

Fig. 4. Direct exposure to ICIs has no proven effect on PFP clotting followed by lysis. TF-induced clotting followed by rtPA-induced lysis was measured turbidimetrically by employing a microtiter plate-based assay performed with a multichannel microplate reader. Undiluted PFP was incubated with pembrolizumab (75 μg/mL, final concentration), nivolumab (58.7 μg/mL, final concentration), ipilimumab (85 μg/mL, final concentration) as well as appropriate isotype controls or Tris-buffer solution for 15 min at 37°C. Clotting/lysis was initiated by adding 30 pM TF/PL/$CaCl_2$ + tPA (40 IU/mL). Absorbance curves obtained from median values for each time point ($n = 10$) were analyzed using WFA. The parameters of the original curve as well as the curve of its first derivative were used for comparative statistics: (**a**) initial clotting rate, (**b**) maximum clotting rate, (**c**) initial fibrinolytic rate, (**d**) maximum fibrinolytic rate, (**e**) the time to reach maximum absorbance, and (**f**) area under the polymerization curve. Differences were significant at $p < 0.01$ (**) as indicated.

the decrease in platelet count, accompanying the blockade of ICIs, may be caused by impaired platelet production or the clearance of circulating antibody-associated platelets provided by the reticuloendothelial system. Several other pathogenic mechanisms are known to be responsible for antibody-mediated thrombocytopenia. It may result from phagocytosis of antibody-bound platelets or their lysis mediated by complement activation [15, 45] or T-cell mediated direct cytotoxicity, causing destruction of platelets [46, 47].

Regardless of the cause, activated and damaged platelets acquire phenotypic features that affect their interaction with enzymes and cofactors of the coagulation and fibrinolysis systems.

According to Sato et al. [48], anti-PD-1/PD-L1 mAb immunotherapy can affect the balance between blood clotting and bleeding and provoke diseases associated with disorders of the blood clotting system and fibrinolysis. Thus, ICI treatment can cause both thromboembolism and bleeding complications in cancer patients. Accordingly, hematological disorders are also usually explained by systemic immune hyperactivation mediated by counterinhibition of immune checkpoints. However, so far we have not been able to find studies of the direct toxic effect of ICIs on the coagulation and fibrinolysis systems in human plasma.

We hypothesize that ICIs-induced hyperactivation and/or destruction of platelets can be a potential pathophysiological mechanism responsible for acquired coagulopathy. To test our hypothesis, we used a comparative approach to identify differences between primary and secondary hemostasis in response to direct exposure to pembrolizumab, nivolumab, and ipilimumab.

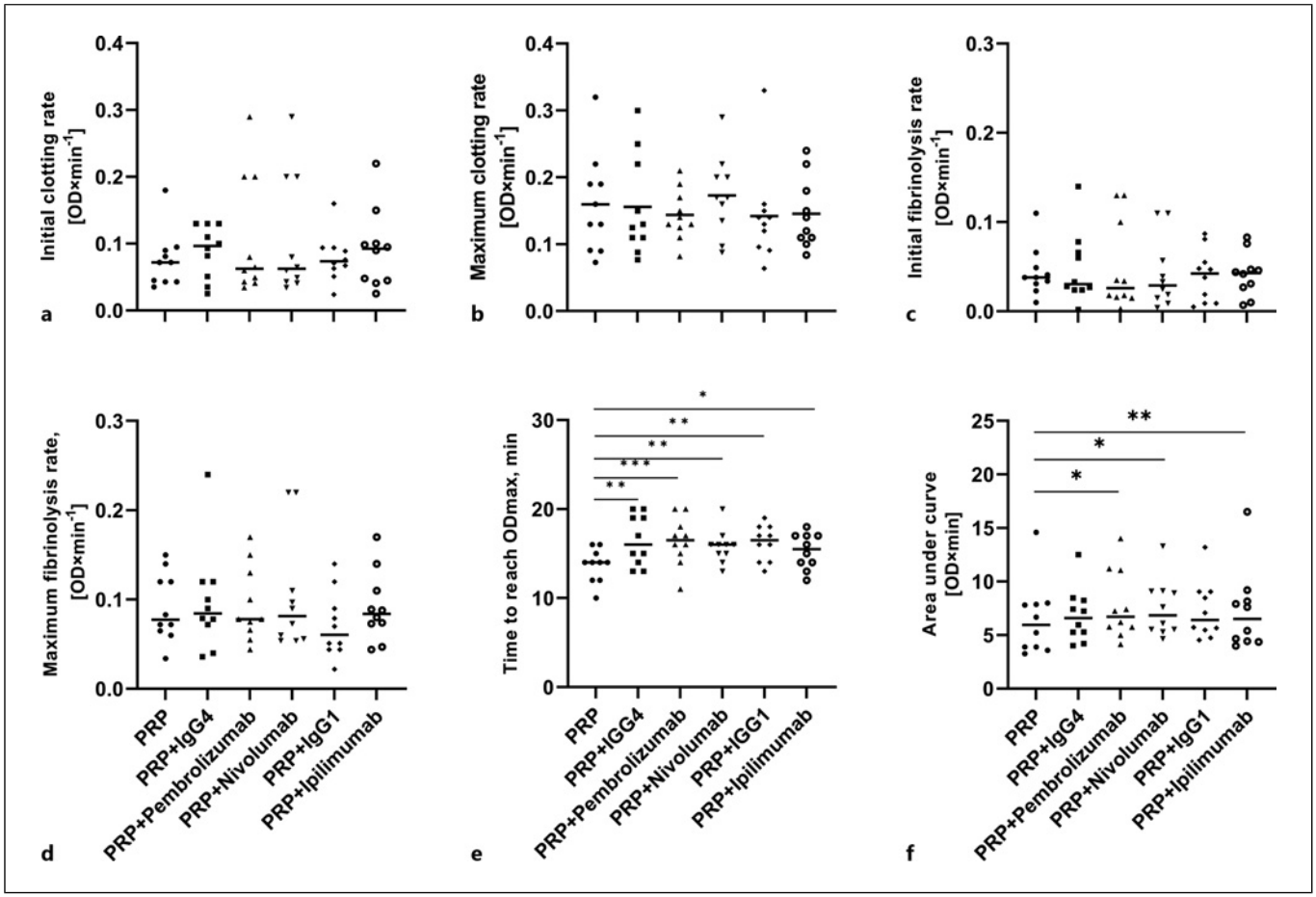

Fig. 5. Direct exposure to ICIs slows down clotting of tPA-added PRP. TF-induced clotting followed by rtPA-induced lysis was measured turbidimetrically using a microtiter plate-based assay performed with a multichannel microplate reader. Undiluted PRP was incubated with pembrolizumab (75 μg/mL, final concentration), nivolumab (58.7 μg/mL, final concentration), and ipilimumab (85 μg/mL, final concentration) as well as appropriate isotype controls or Tris-buffer solution for 15 min at 37°C. Final platelet count varied in the range of 230,000–320,000/μL. Clotting/lysis was initiated by 30 pM TF/PL/$CaCl_2$ + tPA (40 IU/mL) mixture. Absorbance curves obtained from median values for each time point (n = 10) were analyzed using WFA. The parameters of the original curve as well as the curve of its first derivative were used for comparative statistics: (**a**) initial clotting rate, (**b**) maximum clotting rate, (**c**) initial fibrinolytic rate, (**d**) maximum fibrinolytic rate, (**e**) the time to reach maximum absorbance, and (**f**) area under the polymerization curve. Differences were significant at $p < 0.05$ (**), $p < 0.01$ (**), or $p < 0.001$ (***) as indicated.

Comparing the clotting of PRP against autologous PFP, we found that pembrolizumab and nivolumab, which are PD-1 receptor inhibitors, are not able to interact directly with clotting factors in blood plasma and, therefore, do not affect the coagulation cascade. The effect of these drugs on platelet-dependent coagulation pathways was also not confirmed in our experiments. In contrast, ipilimumab, a CTLA-4 receptor inhibitor, significantly accelerated clotting in the presence of platelets by increasing the maximum clotting rate and decreasing the time to rich ODmax. It should be noted that at the molecular level, the initial clotting rate characterizes the formation of fibrin protofibrils, and the maximum clotting rate corresponds to the stage of lateral association of protofibrils and the formation of a three-dimensional fibrin network [49]. The finalization of these events is indicated by the time to reach ODmax, i.e., reaching a plateau. Accordingly, it is expected that ipilimumab, by acting on platelets, can accelerate the later, clot propagation stage, which are amenable to therapeutic correction using direct inhibitors of thrombin and/or coagulation factor XIIIa.

It seems paradoxical, but the addition of t-PA to PRP during simulated internal lysis in our study significantly affected the ability of ICIs to change the dynamics of clot formation, but not the rate of visible lysis of the formed clot. This discrepancy could be due to the initiation of fibrinolysis before the clot reached its maximum amplitude. In this case, the onset of fibrinolysis is masked by the predominant accumulation of fibrin and is indirectly detected by a decrease in the

maximum clotting rate that we observed under the action of ICIs. As it was found out earlier, activated platelets are able to modify both the processes, coagulation and fibrinolysis, affecting the size and lifetime of the fibrin clot [50].

The fibrinolytic process, as is known, takes place in two stages: at the first stage, plasminogen is converted into plasmin by limited t-PA-dependent proteolysis; at the second stage, plasmin-dependent hydrolysis of fibrin occurs [51]. In our study, the beginning of the first stage of fibrinolysis coincides with the stage of lateral association of protofibrils during clotting; therefore, it is masked and manifested itself only after fibrin lysis by generated plasmin began to significantly compete with fibrin formation. By counteracting the accumulation of fibrin, fibrinolysis prolongs the completion of clot formation, which we observed under the action of ICIs. This line of reasoning allows us to predict that the tested ICIs can regulate the first, plasmin-generating stage of fibrinolysis associated with the platelet surface. However, these assumptions require experimental verification.

Conclusions

As this study showed, the effect of ICI on the hemostasis system may be mediated primarily by platelets (primary hemostasis) rather than the activity of the coagulation cascade (secondary hemostasis). ICIs enhance the procoagulant properties of platelets and, presumably, can stimulate the plasmin-generating stage of fibrinolysis, which occurs on the surface of platelets, capable of binding plasminogen and t-PA and facilitating their interaction. Changing the balance between the procoagulant and profibrinolytic effects of ICIs on platelets will result in side effects leading to thrombosis or hemorrhage.

Naturally, our results ex vivo cannot be directly applied to the in vivo situation. Nevertheless, the proposed approach, though it was performed using healthy donor blood, can be used for rapid diagnosis of possible hemostatic abnormalities during immunotherapy, as well as screening other ICIs for their direct hematotoxicity. In addition, this study raises intriguing new questions about the mechanisms of direct toxic effects of ICIs on platelets and outlines possible ways to find adequate answers.

Our study has limitations. First, the study was performed with the blood of healthy donors. An investigation of the impact of ICIs hemostasis of cancer patients would be more clinically relevant. Second, due to the relatively low number of sample tested, the statistical power of the comparative parameter estimation was low. To confirm the proposed hypotheses, it would be necessary to increase the size of the analyzed group. Third, a fairly wide range of individual values of coagulation and fibrinolysis parameters exist, which makes it difficult to conduct a comparative analysis. However, this feature of hemostasis indicators is at the same time an advantage for individualized therapy, since it may reflect a personal combination of etiological and therapeutic factors.

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Statement of Ethics

This study protocol was reviewed and approved by the Ethics Committee of the Friedrich-Alexander University Erlangen-Nürnberg (FAU) (Approval No. #346_18B, #343_18B, #73_19B). Written informed consent was obtained from all subjects involved in the study.

Conflict of Interest Statement

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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Author Contributions

Irina Patalakh: conceptualization, data curation, formal analysis, investigation, methodology, validation, and writing – original draft. Sarah Cunningham: conceptualization, formal analysis, project administration, supervision, validation, and writing – review and editing. Alexandra Wandersee: technical assistance and resources. Julian Schlüter: writing – review and editing. Michael Erdmann: resources and revision of the manuscript. Holger Hackstein: conceptualization, formal analysis, project administration, validation, and writing – review and editing.

Data Availability Statement

Data will be made available on request.

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