which causes mesenteric microbleeding after LPS challenge. How mechanotransduction through GPIIb/IIIa-GPVI is influenced by biomechanical determinants that drive variations in blood hemodynamics across diverse vasculatures in vivo remains a matter of speculation. Differences in endothelial phenotypes and their response to specific infectious or inflammatory cues across varied vascular beds may further add to the complexity of crosstalk between endothelium, platelets, and the immune, coagulation, and fibrinolytic systems. Nevertheless, Kaiser et al convincingly demonstrate the mechanistic mediation of GPIIb/IIIa-GPVI in LPS-induced inflammatory hemostasis in the lungs and postcapillary mesenteric venules. However, the impact of von Willebrand factor and GPIb-mediated mechanosensitivity on the sequential transition of GPIIb/IIIa affinity from a "closed" to an intermediate "extended-closed" state (required to receive mechanosignals and undergo mechanical affinity maturation) to an "extended-open" state[6] remains to be elucidated. Moreover, platelet mechanosensing on collagen matrices increases with substrate stiffness, prompting PS exposure.[7] How and whether this mirrors the optimal trigger for restorative or deleterious inflammatory hemostasis in a hypercoagulatory vascular microenvironment remains undefined. Although the thromboinflammatory CLEC-2–podoplanin axis[9] is suggested to have a minor role in primary hemostasis, it deserves attention, particularly considering the prevalence of podoplanin at sites of vascular breaches, its protective role in attenuating acute lung injury,[9] and the involvement of Syk, shared by GPVI and CLEC-2 signaling[9] in the procoagulant transformation of migratory platelets.

Curiously, platelets help leukocytes accumulate at inflammatory loci, whereas a platelet-assisted hemostatic plug seals endothelial gaps left by transmigrating leukocytes. Inflammatory hemostasis may be substantiated but not entirely reliant on circulatory platelet-leukocyte interactions, unlike the influence thromboinflammatory platelet-leukocyte aggregates have in atheroprogression, in venous thrombosis, or in influencing thrombotic propensity after ischemia reperfusion.[10] Therefore, simultaneous GPIIb/IIIa-GPVI blockade does not reduce platelet-neutrophil aggregates or pulmonary recruitment of neutrophils after LPS challenge, but it does exacerbate alveolar microbleeds that negatively correlate with procoagulant platelet counts. Accordingly, anticoagulants aggravate pulmonary hemorrhage without affecting circulatory platelet-neutrophil aggregates or pulmonary neutrophil infiltration. Nonetheless, neutrophil depletion in LPS-challenged mice affects intravascular fibrin(ogen) deposition in mesenteric vessels, suggesting that neutrophils and platelets may cooperate to reach the inflamed endothelium, but the crucial procoagulant transformation of platelets requires their arrest on collagen and the mechanosensing services of GPIIb/IIIa-GPVI. The therapeutic goal is to sustain hemostatic plugging of the inflamed vessel and prevent pathological thrombosis at the same time. This requires further insights into the intricate details of classical and inflammatory hemostasis. Potential drug targets may emerge from subtle differences between PS-exposing apoptotic vs procoagulant platelets, PAC-1–binding vs PS^+ platelets, and the mechanosensing vs signaling and adhesive involvement of GPIIb/IIIa and GPVI. Therefore, we can expect truly translational findings from continued investigation on the intriguing platelet patrol.

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THROMBOSIS AND HEMOSTASIS

Comment on de la Morena-Barrio et al, page 140

Antithrombin deficiency: no sugar, no diagnosis!

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In this issue of *Blood*, de la Morena-Barrio et al[1] describe 2 novel variants of antithrombin (AT) with altered glycosylation profiles. This study found that although these mutations are undoubtedly associated with thrombophilia, carriers are not identified using routine testing for AT deficiency.

Several studies have reported that, among genetic risk factors for thrombophilia, inherited AT deficiency is underdiagnosed, emphasizing the need for the development of new genetic and functional assays.[2] In their study, de la Morena-Barrio et al add further evidence of this need by identifying 2 novel mutations in the *SERPINC1* gene (encoding for AT) in 4 unrelated thrombophilic families whose proband had normal (or near-normal) AT activity in functional assays routinely used

to diagnose AT deficiency. By a combination of molecular and biochemical analyses, they established that both were missense mutations close to or in an *N*-glycosylation sequon, resulting in an impaired or null *N*-glycosylation of Asn224.

AT is a major endogenous anticoagulant, whose deficiency causes the most severe form of thrombophilia. It is a serine protease inhibitor (serpin) that primarily targets thrombin (factor IIa [FIIa]) and activated factor X (FXa), but also targets other procoagulant enzymes such as FVIIa, FIXa, and FXIa. AT is a glycoprotein with 4 sites of *N*-glycosylation (Asn128, Asn167, Asn187, and Asn224). Most of the AT in the plasma is fully glycosylated (α-AT), although a minor form, lacking a glycosyl sidechain on Asn167 (β-AT), is also present.[3]

This study presents the case of a patient who had an unprovoked portal vein thrombosis at the age of 44 years. His mother and his 2 daughters also had venous thrombosis at the ages of 70, 19, and 18 years, respectively. The screening for inherited risk factors for thrombophilia, including for AT deficiency, led to negative or inconclusive results. Although the patient had slightly decreased AT activity during the acute episode, a second assay a few months later was normal. Based on the severe clinical manifestations in this family, genetic analysis of the proband was undertaken by whole-genome sequencing. It revealed a heterozygous mutation in the *SERPINC1* gene resulting in the Glu227Lys substitution, near the *N*-glycosylation site 224. This mutation, never described before, has also been found in another patient with recurrent thrombotic events starting at an early age.

The biochemical characterization of the variant AT-Glu227Lys, in a recombinant system, showed both reduced secretion and heterogeneous glycosylation, presumably from partial glycosylation in position 224. In addition, the β glycoform of AT-Glu227Lys exhibited impaired anti-FXa and anti-FIIa activities compared with β-AT.

Two other unrelated patients are also described in this study. Both were carriers of another mutation in the same glycosylation sequon, resulting in the Asn224His substitution. These patients, and some of their relatives, also presented with recurrent thrombosis at a young age, with normal or near-normal AT activity. As expected, biochemical analysis of the variant AT-Asn224His, in a recombinant system, confirmed the loss of a glycan chain. Interestingly, although this mutation had no effect on the AT anticoagulant activity in its α-glycoform, it abolished both anti-FXa and anti-FIIa activities of the β glycoform.

This study highlights the difficulty of diagnosing congenital AT deficiency. Routine laboratory tests for AT are primarily functional assays based on the ability of plasma AT to inhibit exogenous FIIa- or FXa-chromogenic activity. Even if these tests are able to detect functional defects, they sometimes lack the sensitivity to detect milder forms. The biochemical analysis of AT variants revealed that mutations had no effect on its anticoagulant activity, at least of its α glycoform, which is predominant in plasma. However, hypoglycosylation of Asn224 affected the anticoagulant activity of the β-AT, which accounts for ∼10% of plasma AT. This may explain why the carriers of this mutation were not diagnosed as having a congenital AT deficiency. The discrepancy between the results of functional assays and the severity of prothrombotic phenotype suggests that β-AT could play a preponderant role in the anticoagulant activity of AT, as previously reported in animal models.[4]

AT anticoagulant activity is closely related to its metastable structure that undergoes a profound conformational change on interaction with its target proteases to form an irreversible covalent complex through a mousetrap mechanism (common to serpins). Thus, a glycosylation defect could alter AT folding and stability, making it more sensitive to external conditions that alter its conformation and thereby its anticoagulant activity.[5] This hypothesis is supported by the higher sensitivity of plasma from patients with Glu227Lys or Asn224His mutation to heat stress. Patient plasma had lower AT activity when heated to 41°C than normal control plasma. It is conceivable that mutations impairing glycosylation could be associated with transient AT deficiency with thrombotic consequences brought on by external factors. This transient AT deficiency state would also make it difficult to diagnose.[6]

The development of more sensitive functional assays could help in the diagnosis of AT deficiency. Indeed, plasma from carriers of the Glu227Lys mutation exhibited a hypercoagulable state in a thrombin generation assay, evidenced by a faster and greater thrombin generation compared with control plasma. In addition, when assessed for anti-FVIIa activity, carriers of the Glu227Lys or Asn224His mutation appeared to have reduced levels of FVIIa-AT complexes compared with healthy controls. Because anti-FXa and anti-FIIa activity of carriers were nearly normal, this result implies that hypoglycosylation of Asn224 could have distinct effects depending on the AT target, and that, in this case, an anti-FVIIa assay could be more sensitive/specific than anti-FIIa or anti-FXa assays. This result is very interesting and requires further investigations to determine the molecular mechanism responsible for this observation.

Finally, even if these results encourage further development of screening methods for inherited AT deficiency; overall, the usefulness of testing for hereditary thrombophilia remains debated.[7] In this context, the development of too sensitive or overly broad-spectrum assays has to be considered carefully as, although it may be useful for the management of thrombophilia, it may also generate many off-target results that could be difficult to interpret.

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

Comment on Atanackovic et al, page 152, and Oh et al, page 157

Buckling up against COVID-19 after CAR T-cell therapy

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In this issue of *Blood*, Atanackovic et al[1] and Oh et al[2] separately report on T-cell immune responses following severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) messenger RNA (mRNA) vaccination in patients with non-Hodgkin lymphoma (NHL) receiving CD19 chimeric antigen receptor (CAR) T-cell therapy. Collectively, their findings suggest a potential seatbelt of immune protection against coronavirus disease 2019 (COVID-19) for patients whose treatment journey requires traveling by CD19 CAR T cells.

COVID-19 is caused by SARS-CoV-2, a single-stranded RNA virus that requires host cells for replication. In this regard, SARS-CoV-2 affixes itself to the host cell using its spike (S) protein comprising S1 and S2 subunits, the former containing the receptor binding protein (RBD) that binds to angiotensin-converting enzyme 2 receptor, enabling the virus to enter the host cell and use its replicative machinery.[3] Effective eradication of SARS-CoV-2 involves coordinated innate and adaptive antiviral responses, while dysregulated host immunity leads to systemic hyperinflammation underlying severe COVID-19.[4]

In December 2020, the Food and Drug Administration (FDA) issued emergency use authorization for SARS-CoV-2 mRNA vaccines, BNT162b2 (age >16 years) and mRNA-1273 (age >18 years), as prevention against severe COVID-19. Since FDA approval of BNT162b2 and mRNA-1273, SARS-CoV-2 mRNA booster vaccination and revaccination have been recommended for patients receiving hematopoietic cell transplantation and CAR T-cell therapy.[5] These effective vaccines induce antiviral innate and adaptive immune responses in immunocompetent persons, most notably antibodies against the S and RDB proteins as well as viral-specific $CD4^+$ and $CD8^+$ T cells.[6,7] Variants of the ancestral SARS-CoV-2 strain, including Delta (B.1.617.2) and Omicron (B.1.1.529), and the variants themselves (ie, Omicron variant, BA.2) are associated with decreased vaccine efficacy attributed to S mutations that attenuate neutralizing antibody effect.[8]

Patients with hematologic malignancies are at significant risk for severe COVID-19, resulting from primary disease and therapy-associated, aberrant or absent immune function.[9] For example, patients receiving CD19 CAR T-cell therapy for relapsed/refractory NHL experience prolonged and profound B-cell aplasia and hypogammaglobulinemia, placing them at higher risk for severe COVID-19 regardless of their receiving supplemental IV immunoglobulin.[10] Although attenuated humoral responses to SARS-CoV-2 mRNA vaccination have been noted in patients receiving CD19 CAR T-cell therapy, vaccine-associated cellular immune responses remain largely undefined.

To this end, Oh and colleagues compare adaptive immune responses following BNT162b2 vaccination between 8 patients with B-cell lymphoma who received CD19 4-1BB-CD3z CAR T-cell therapy and 26 healthy controls. Specifically, the authors measure neutralizing antibody, whole-blood S-peptide-induced interferon-γ (IFN-γ) and interleukin-2, IFN-γ enzyme-linked immune absorbent spot, and activation-induced marker $CD4^+$ and $CD8^+$ T cells before and after vaccination (10, 21, and 90 days following first and second inoculations) as well as vaccine-associated cellular responses against SARS-CoV-2 variants, Delta and Omicron. Notably, second BNT162b vaccination induced levels of functional S-specific T cells in CD19 CAR T-cell recipients, which were greater than those in healthy controls. Furthermore, vaccine-associated T-cell responses were largely preserved against SARS-CoV-2 variants (ie, neutralization of Delta being greater than that of Omicron).

Atanackovic and colleagues compare adaptive immune responses following SARS-CoV-2 mRNA vaccination between 18 patients with NHL receiving mostly CD19 4-1BB-CD3z CAR T-cell therapy (10 received mRNA-1273, 8 received BNT162b2) and 10 healthy controls. Interestingly, despite reduced levels of antibody against SARS-CoV-2 proteins (S1, S2, RBD), even after 3 inoculations and lower levels of peripheral blood B cells and quantitative immunoglobulins, patients receiving CAR T cells had normal levels of immunoglobulin G against recall antigens (influenza A, tetanus toxoid, Epstein-Barr virus, and herpes simplex) and higher numbers of plasma cells ($CD19^-CD38^-$) relative to controls. In contrast, levels of vaccine-induced anti-S $CD4^+$ and $CD8^+$-specific T cells in patients receiving CAR T cells were comparable to or even higher than control levels. In addition, vaccine-induced T-cell reactivity against the Omicron variant was present albeit decreased relative to activity against ancestral SARS-CoV-2 in patients receiving CAR T cells.

What do these studies tell us? First, relative to healthy controls, patients with NHL