

maturational processing, trafficking, membrane localization, signaling interactions, and stability. Several cancer-associated proteins are known to be palmitoylated, a classic example being the RAS family of small GTPases, where palmitoylation dictates trafficking, membrane localization, and signaling properties.<sup>8,9</sup> However, the role of palmitoylation in regulating FLT3-ITD localization and signaling has not been previously shown. Lv et al elegantly demonstrate that S-palmitoylation mediated by ZDHHC6 plays a critical role in determining FLT3-ITD localization and activity (see figure). They show that disruption of palmitoylation promotes trafficking of FLT3-ITD from the ER to the plasma membrane, and leads to activation of AKT and ERK while still maintaining activation of STAT5, and thereby increased FLT3-ITD-mediated leukemic progression. In contrast, palmitoylation did not play a significant role in trafficking of FLT3-WT and TKD mutant proteins to the plasma membrane or their signaling or cellular effects. They further confirmed that FLT3 proteins were palmitoylated, and that ZDHHC6-mediated palmitoylation regulated FLT3-ITD surface expression, signaling and growth in primary human FLT3-ITD<sup>+</sup> AML cells.

It is of note that FLT3-ITD phosphorylation did not affect palmitoylation, and that TKI treatment further increased the surface levels of a palmitoylation-deficient ITD mutant, suggesting that palmitoylation and phosphorylation are separate mechanisms regulating FLT3-ITD intracellular localization. The relationship of palmitoylation to receptor glycosylation and maturation was not evaluated, and requires further study. Palmitoylation-deficient FLT3-ITD mutants retained sensitivity to gilteritinib. Importantly, pharmacological inhibition of FLT3-ITD depalmitoylation using a pan-depalmitoylase inhibitor significantly reduced FLT3-ITD surface expression, inhibited AKT and ERK signaling, and reduced cell growth. The depalmitoylase inhibitor synergized with gilteritinib in inhibiting FLT3-ITD surface localization, AKT and ERK signaling, and abrogating growth of primary FLT3-ITD<sup>+</sup> AML cells. These observations provide new insights into the role of lipid modifications in compartmentalization of FLT3-ITD signaling in AML. Importantly, they indicate that targeting of depalmitoylation could be a potential therapeutic strategy for FLT3-ITD<sup>+</sup> leukemias and support further exploration and development of

clinically applicable inhibitors of depalmitoylation. Because resistance to gilteritinib has been associated with reactivation of RAS/MAPK pathway, it will be of interest to determine whether depalmitoylation inhibitors provide additional benefit in FLT3-ITD<sup>+</sup> AML through inhibition of RAS/MAPK signaling.<sup>3</sup>

The implications of these studies extend beyond FLT3-ITD AML because subcellular localization is a general mechanism that affects activation of RTKs and their downstream pathways.<sup>7</sup> Abnormal maturation and trafficking have also been observed for other oncogenic RTKs, which in addition to being aberrantly active in different cellular compartments, can also generate different signaling outputs depending on localization. The role of palmitoylation in localization, signaling, and transforming activity of other RTKs will doubtless be the subject of future studies.

*Conflict-of-interest disclosure:* The authors declare no competing financial interests. ■

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## PLATELETS AND THROMBOPOIESIS

Comment on Greinacher et al, page 2256

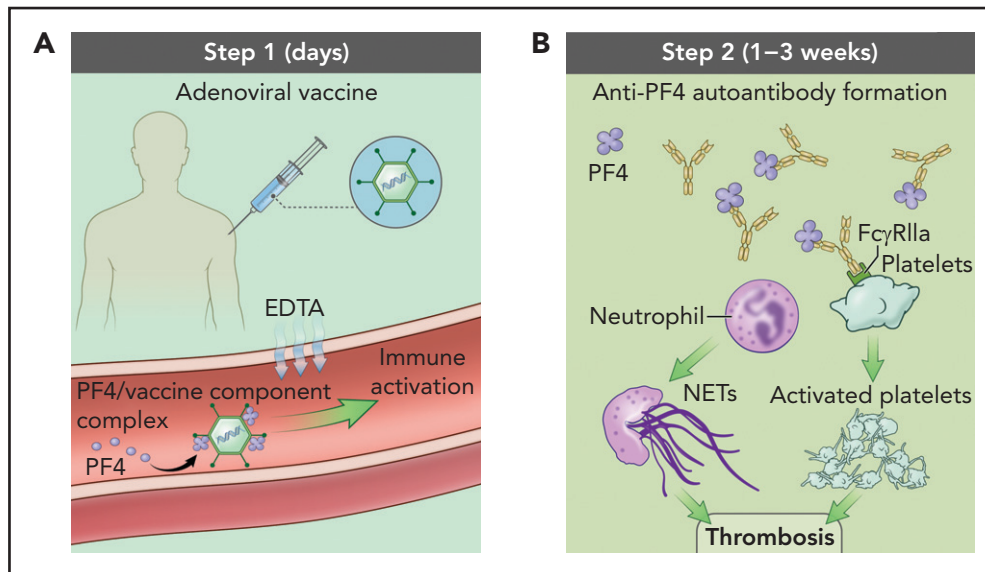
# VITT(al) insights into vaccine-related clots

**Jeffrey R. Strich<sup>1,2</sup> and Yogendra Kanthi<sup>3</sup>** | <sup>1</sup>National Institutes of Health Clinical Center; <sup>2</sup>US Public Health Service Commissioned Corps; <sup>3</sup>National Heart, Lung, and Blood Institute

**In this issue of *Blood*, Greinacher et al<sup>1</sup> propose that the pathogenesis of vaccine-induced immune thrombotic thrombocytopenia (VITT) involves a 2-step mechanism, initiated by binding of PF4 to components of the ChadOX1 nCov19 adenoviral vaccine followed by a prothrombotic antibody response similar to autoimmune heparin-induced thrombocytopenia.**

A number of vaccines targeting the SARS-CoV-2 virus were urgently developed to curtail the infection and the complications of COVID-19. Two of these vaccines, ChAdOx1 nCoV-19 (Oxford-AstraZeneca) and Ad26.COV2.S (Johnson & Johnson/Janssen), use recombinant adenovirus vectors encoding the SARS-CoV-2 spike glycoprotein.<sup>2</sup> These vaccines were extensively

evaluated before regulatory authorization for utilization and did not demonstrate any safety concerns. However, ongoing safety surveillance identified an association between adenovirus-based vaccines and the rare development of thrombocytopenia and thrombosis in atypical locations, including the cerebral venous sinus thrombosis (CVST) and splanchnic veins 1 to 2



Two-step process for VITT pathogenesis. (A) Shortly after vaccine administration, components of the adenovirus vaccine and PF4 generate immune complexes, whereas EDTA sequesters calcium, leading to vascular leak and an inflammatory response serving as a danger signal to provoke antibody generation. (B) One to 3 weeks after vaccine administration, polyanion/PF4/anti-PF4 antibody immune complexes trigger neutrophil extracellular trap formation and platelet aggregation in an FcγRIIA-dependent manner, resulting in thrombosis. Illustration by Alan Hoofring, National Institutes of Health.

weeks after vaccination, termed VITT.<sup>3</sup> Patients with VITT have a high level of circulating immunoglobulins (IgGs) that recognize platelet factor 4 (PF4) and activate platelets in a manner that shares features with autoimmune heparin-induced thrombocytopenia with thrombosis, previously unraveled by elegant work from Greinacher et al<sup>3</sup> and Kelton and Warkentin,<sup>4</sup> among others. In the absence of heparin exposure, the source and nature of the polyanion(s) that associate with PF4 in VITT have been heavily debated. Although VITT is rare among the millions of patients receiving adenoviral-based vaccines, a detailed understanding of the initial steps that result in VITT is needed to inform management at the bedside and the development of future vaccines and therapeutics that may use similar vectors.

To that end, Greinacher et al perform a detailed characterization of a potential mechanism underlying VITT pathogenesis using the ChAdOx1 nCoV-19 vaccine. Using three different imaging techniques, the authors demonstrate that adenovirus and vector components in the vaccine aggregate with PF4 in a charge-driven manner, to which anti-PF4 IgG binds. The authors then investigated vaccine composition and the ability of the ChAdOx1 vaccine to induce inflammation. They determined that approximately half of the proteins in the vaccine were of human origin, likely from the T-Rex HEK293 cells used in vaccine manufacturing. The

investigators also identified EDTA in the vaccine. Used as an excipient, EDTA is a chelating agent that may sequester calcium necessary to maintain local endothelial barrier function. Intradermal injection of either the vaccine or EDTA alone triggered vascular leakage in mice, and reconstitution of calcium in the vaccine mitigated the loss of barrier integrity. The authors also observed that serum from patients with VITT robustly initiated platelet aggregation when presented with PF4. Notably, this effect was fully abrogated by blockade of the FcγRIIA receptor (FcγRIIA). Prothrombotic neutrophil extracellular traps (NETs), known to occur in acute COVID<sup>5</sup> and in autoimmune heparin-induced thrombocytopenia (HIT), also formed when neutrophils were stimulated with VITT serum or affinity purified anti-PF4 IgG in the presence of PF4 and platelets. Greinacher et al and another recent report<sup>6</sup> observed that NETs were more prevalent in CVST tissue from patients with VITT compared with VITT-unrelated CVST.

Taken together, these data support the hypothesis that VITT pathogenesis occurs in a 2-step process (see figure). In the first step, shortly after vaccine inoculation, vaccine components and PF4 form neoantigens, promoting a proinflammatory vascular milieu that amplifies the adaptive immune response including production of anti-PF4 antibodies. In the second step, 1 to 3 weeks after inoculation,

complexes of polyanion/PF4/anti-PF4 antibody activate neutrophils and platelets in an FcγRIIA-dependent manner, leading to thrombosis accretion in atypical vascular beds. Because the number of patients diagnosed with VITT is low, it remains unclear whether VITT has a predilection for thrombosis in unusual sites such as the cerebral venous sinus or whether thromboses in more typical sites such as the peripheral veins do not raise the clinical alarm to trigger diagnostic testing.

These findings provide detailed insight to VITT pathogenesis and the reasons that it may occur after vaccination with adenoviral vector-based vaccines but not mRNA-based vaccines. However, many questions remain. What is the protein(s) in the vaccine that binds to PF4? What is the precise neoantigen generated when PF4 and vaccine components interact? Do human proteins in the vaccine provoke an immune response? Is the prothrombotic antibody repertoire in VITT limited to PF4, the vaccine and its components, or is there overlap with autoantibodies found in acute COVID, autoimmune disease, and other critical illnesses? What is the half-life of immune complexes or the effect of multiple vaccine doses on the autoantibody response? Ultimately, answers to these and other questions will be needed to inform future development of vaccines and therapeutics that use adenovirus- and other virus-based vectors.

As more is understood about the molecular disruptions that occur during VITT, the question remains; what is the best treatment for patients? Proposed therapeutics include IV immunoglobulin (IVIG) because of its success in treating autoimmune HIT, nonheparin anticoagulants, and plasma-pheresis. A small body of evidence from nonrandomized trials and retrospective studies suggests that IVIG may be an effective treatment of VITT, although sometimes incomplete.<sup>7</sup> The results of this study by Greinacher et al bring to light another potential therapeutic, the spleen tyrosine kinase (SYK) inhibitor, fostamatinib that is currently used for the treatment of chronic immune thrombocytopenia. Fostamatinib inhibits activation of Fc receptor by antigen/antibody complexes, and reduced NETosis and platelet activation in ex vivo COVID studies.<sup>8</sup> In hospitalized patients with COVID, where circulating, prothrombotic antibodies that activate neutrophils, platelets, and endothelium have been identified,<sup>9</sup> orally administered fostamatinib reduced adverse events and showed a trend toward clinical benefit.<sup>10</sup> Although large, randomized trials are impractical in VITT given its low incidence, the FcγRIIA-dependent signaling mechanism leading to platelet activation in VITT identified by Greinacher et al provides strong rationale to consider SYK inhibition in the limited therapeutic armamentarium of clinicians treating VITT and perhaps other forms of autoantibody-mediated thrombosis.

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## TRANSPLANTATION

Comment on Cutler et al, page 2278

# ROCKin' cGVHD treatment: has the time come?

Jörg P. Halter | University Hospital Basel

**In this issue of *Blood*, Cutler et al<sup>1</sup> report encouraging results from a randomized, multicenter, phase 2 trial (the ROCKstar Study) of treatment with the ROCK2 inhibitor belumosudil in patients with inadequately controlled chronic graft-versus-host disease (cGVHD) after 2 or more lines of prior therapy.**

When dire diseases are cured by allogeneic hematopoietic cell transplantation, patients still face many obstacles in their struggle to return to normal. Among them, cGVHD is a leading cause of nonrelapse mortality and morbidity. Between 35% and 70% of patients develop cGVHD with 30% to 50% of them having steroid-refractory or steroid-dependent cGVHD. After starting initial systemic therapy for National Institute of Health (NIH)-defined moderate or severe cGVHD, only 1 of 3 patients will be alive and off immunosuppression 5 years later.<sup>2</sup> This significant burden of GVHD in survivors has led to the introduction of a composite end point of cGVHD plus relapse-free survival. Manifestations of cGVHD are heterogenous and affect multiple organs. Among them, keratoconjunctivitis sicca, sclerosis, bronchiolitis obliterans, severe joint/fascia involvement, and esophageal strictures are the most frequently associated with high morbidity<sup>3</sup> and impaired quality of life. Hence, the big question in the field is how to control cGVHD without increasing the risk of serious adverse effects from immunosuppressive treatment.

Cutler et al show that selective inhibition of  $\mu$ 10-associated, coiled-coil-containing protein kinase 2 (ROCK2) with belumosudil (formerly known as KD025) is effective and safe in heavily pretreated patients with persistent cGVHD manifestations after 2 to 5 prior systemic lines of therapy (LOTs). The authors are to be congratulated for this trial in these difficult-to-treat patients with advanced stages of cGVHD. Two-thirds of patients had NIH-defined severe cGVHD. Prior treatment included a median of 3 prior LOTs with 27% having at least 5 LOTs. Many of the patients had received extracorporeal photopheresis, ibrutinib, and/or ruxolitinib. Half of patients had 4 or more organs involved, with a high percentage with skin, joints/fascia, eye, mouth, lung, and/or esophageal involvement. High overall response rates (ORRs) were observed across different organs and prior treatment histories, with partial responses being more frequently recorded in manifestations where fibrosis or permanent organ damage dominated, such as joint/fascia, eyes, skin, or lungs (see figure). Notably, most patients receiving prior ibrutinib or