proteins that interact with the SH3 domain of PSTPIP1 to create a broader picture of the general consequences of the R405C mutation.

Furthermore, the study by Starnes et al provides new insight into the role of PSTPIP1 as a regulator of the transition between podosomes and filopodia on 2D substrates. While there are examples of podosome alterations that correlate with inhibited ECM degradation,[5,8] here, in the absence of podosomes, the delivery of proteases appeared to be transferred to filopodia with an even more efficient matrix-degrading activity in PSTPIP1-R405C cells than in control macrophages. This suggests that filopodia can substitute for podosomes to degrade ECM proteins. Migration in 3D dense matrices such as Matrigel requires ECM degradation for macrophages to create paths.[9] In these environments, macrophages form cell protrusions called 3D podosomes at the tip of which podosome proteins accumulate along with F-actin and matrix proteolytic activity.[9,10] The study by Starnes et al provides a very important observation because it shows that the ability of this patient's cells to migrate in dense matrices is highly efficient despite podosome disruption. It will be interesting to characterize whether filopodia-bearing cells in 2D do form 3D podosome structures in dense matrices.

Mutations in *PSTPIP1* that disrupt the interaction with PTP-PEST result in impaired chemotaxis and 3D migration of macrophages, whereas the R405C mutation triggers an opposite phenotype, which would be expected to lead to different clinical outcomes. Indeed, the patient with the R405C mutation showed aggressive pyoderma gangrenosum but no arthritis, whereas arthritis is usually very prominent in patients carrying A230T or E250Q mutations. Those observations suggest that different mechanisms are involved in the two aspects of this autoinflammatory syndrome. Thus, extension of our knowledge about PAPA syndrome mutations may eventually help to adapt therapies. In line with this, therapies targeting IL-1β lack efficacy in some patients[2] and the study by Starnes et al may help us understand why.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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● ● ● PLATELETS & THROMBOPOIESIS

Comment on Stefanini et al, page 2722

Eliminate dark side from antiplatelet therapy

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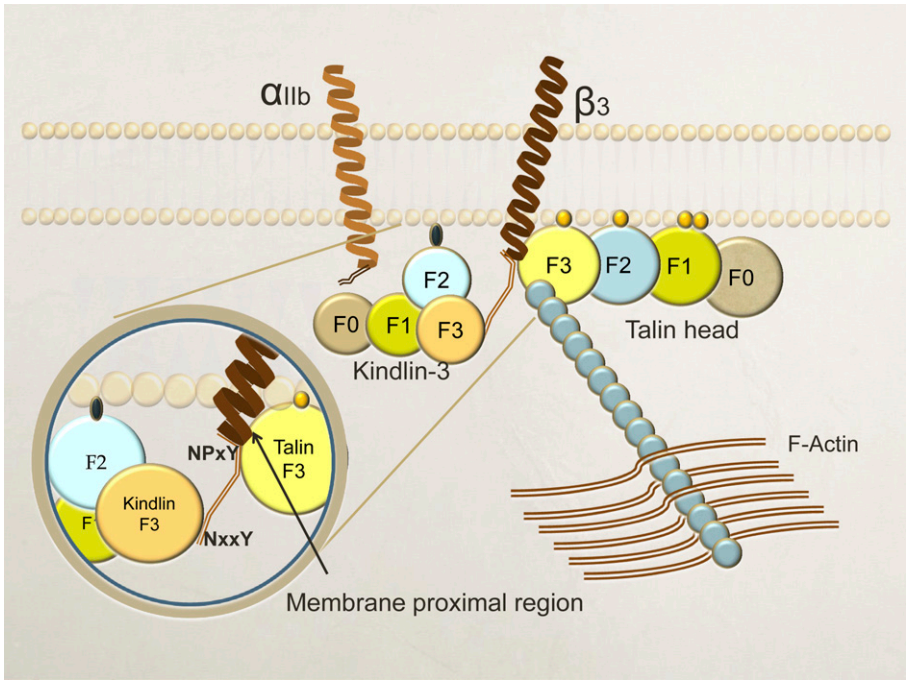
In this issue of *Blood*, Stefanini and colleagues report a new way to regulate platelet function. Mice expressing a talin-1 mutant (W359A) that disrupts its binding to the NPxY motif in β_3 were protected from experimental thrombosis without undermining hemostasis by decelerating αIIbβ3 activation.[1]

In addition to their essential role in hemostasis, platelets play a crucial role in a pathological thrombus formation, particularly within atherosclerotic arteries subjected to high shear stress.[2] As an initial step in thrombogenesis (or hemostasis), platelets adhere to altered vascular surfaces or exposed subendothelial matrices, then become activated and aggregate each other to grow thrombus (or platelet plug) volume. These processes are primarily mediated by platelet surface glycoproteins: GPIb-IX-V, integrin $\alpha_2\beta_1$ (also known as GPIa-IIa), GPVI, and integrin αIIbβ3 (GPIIb-IIIa).[3] Integrins are a family of αβ heterodimeric adhesion receptors that mediate cellular attachment to the extracellular matrix and cell cohesion.[4] Among integrins, platelet αIIbβ3 is a prototypic non-I domain integrin and plays an essential role in platelet aggregation and thrombus growth as a physiological receptor for fibrinogen and von Willebrand factor, as evidenced by the clinical features of congenital bleeding disorder—Glanzmann thrombasthenia.[3] After exposure to subendothelial matrix including collagen and von Willebrand factor and/or several mediators (agonists) including adenosine 5′-diphosphate (ADP), thromboxane A_2, and thrombin, platelets become activated and a series of intracellular signaling events ("inside-out" signaling) that rapidly induce a high-affinity state of αIIbβ3 from its low-affinity state for soluble ligands (αIIbβ3 activation) are generated. In fact, αIIbβ3 activation is indispensable for platelet aggregation, and specific binding of the cytoskeletal proteins, talin and kindlin-3, to the cytoplasmic tail of the β_3 subunit is the final common step in αIIbβ3 activation.[5] Although αIIbβ3 represents a rationale target for antithrombotic therapy, strong inhibition of αIIbβ3 increased bleeding complication. This is also true in the case of $P2Y_{12}$ inhibitors.[6]

Investigators are still searching for the "magic bullet" that selectively targets pathological thrombus formation without



Schema of talin-1 and kindlin-3–dependent αIIbβ3 activation. Talin-1 and kindlin-3 are prerequisite for αIIbβ3 activation via "inside-out" signaling. Talin-1 binds to 2 distinct binding sites in the β_3 cytoplasmic tail: a membrane distal region (a proximal NPxY motif) and a MPR of β_3, whereas kindlin-3 binds to the membrane-distal NxxY motif of the β_3 cytoplasmic tail.

undermining hemostasis.[7] In this issue of *Blood*, Stefanini et al report a new strategy to segregate antithrombotic capacity from bleeding complications by modifying αIIbβ3 activation via disrupting of talin-1 interaction with β_3 cytoplasmic tail. Talin-1 is a large protein (~270 kDa) that consists of a ~50 kDa globular head domain and an elongated flexible 220-kDa rod domain. Talin head domain contains 4 subdomains: F0, F1, F2, and F3, and recent structural and biochemical studies have established that αIIbβ3 activation requires interaction of talin F3 domain with 2 distinct binding sites in the β_3 cytoplasmic tail: a membrane distal region (a proximal NPxY motif) and a membrane proximal region (MPR) of β_3. "Inside-out" signaling first induces the talin F3 domain binding to the NPxY motif, which stabilizes additional interactions between talin F3 and MPR.[8,9] Unlike talin-1, kindlin-3 binds to the membrane-distal NxxY motif of the β_3 cytoplasmic tail[9] (see figure). Both talin L325R and W359A mutants that selectively disrupt talin binding to the membrane proximal region and to the NPxY motif, respectively, have been reported to abolish integrin activation in Chinese hamster ovary cells.[8] In contrast, it has been reported that in a human megakaryocytic cell line, CMK, all talin mutants (L325R, S365D, S379R, and Q381V) selectively disrupting the binding to MPR completely lost their ability to activate αIIbβ3, whereas talin W359A retained some ability to activate αIIbβ3.[10] To further elucidate the functional differences between talin L325R and talin W359A in vivo, the authors generate a series of platelet-specific talin-1 mutant knock-in mice. Because homozygotes for either talin-1 L325R or talin-1 W359A were embryonic lethal, talin-1 (L325R/wt) or talin-1(W359A/wt) mice were crossed with platelet-specific talin-1 knockout (talin-1flox/flox Pf4-Cre$^+$) mice to generate compound heterozygous (talin-1 L325R/flox Pf4-Cre$^+$ and talin-1W359A/flox Pf4-Cre$^+$) and control mice (talin-1wt/flox Pf4-Cre$^+$). Thus, as compared with wild-type mice, these mice express only 50% of talin-1 in platelets, even in control mice (talin-1 wt/flox PF4-Cre$^+$). The phenotype of talin-1(L325R) mice was very similar to that of talin1-deficient mice. These mice were protected from the $FeCl_3$-induced thrombosis model in the carotid artery, whereas tail-bleeding time was markedly increased as expected. Compared with wild-type mice, the 50% reduction of talin-1 expression in platelets in control mice (talin-1wt/flox Pf4-Cre$^+$) used in this report had no effect on occlusion times in the thrombosis experiments. It is especially noteworthy that talin-1 (W359A) mice were protected from the experimental thrombosis without pathological bleeding. Moreover, in vitro experiments revealed that talin-1 (W359A) head domain bound to β_3 tail with 2.9-fold lower affinity compared with wild-type talin-1 head domain, which is likely responsible for slower αIIbβ3 activation, delayed platelet aggregation and markedly reduced ex vivo thrombus formation under high sear rate. However, under low shear rate, talin-1 (W359A) platelets had a capacity to form a small 3-dimensional thrombus.

This report reveals that the interaction of talin-1 with the MPR of β_3 tail plays a more critical role in αIIbβ3 activation than with the NPxY motif in vivo. The disruption of the interaction with the NPxY motif did not abolish, but reduced, the affinity of talin-1 for β_3 tail, thereby decelerating αIIbβ3 activation. It is likely that rapid αIIbβ3 activation on platelets is a prerequisite for pathological thrombus formation, particularly within atherosclerotic arteries subjected to high shear stress. This report newly proposes that deceleration of αIIbβ3 activation could be a smart way to prevent thrombosis without increasing bleeding tendency. Further studies are warranted to find the "magic bullet."

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Comment on Nguyen et al, page 2732

Decrypting C2 inhibitors

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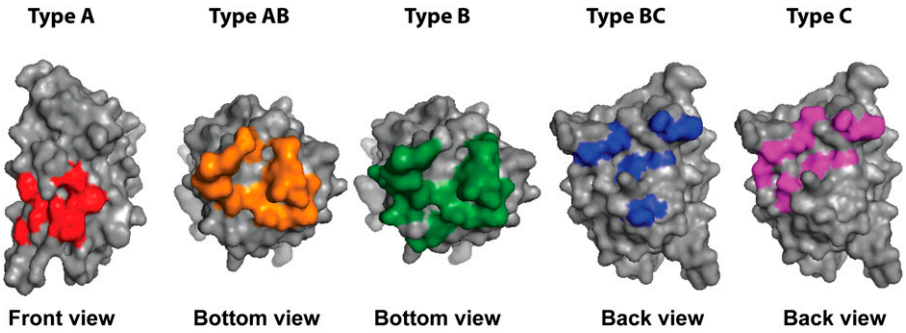
In this issue of *Blood*, Nguyen et al employ high-resolution mapping to precisely define epitopes on the C2 domain of blood coagulation factor VIII (FVIII).[1] Their results nicely complement a recent report in *Blood* describing the structure of a ternary complex of 2 inhibitory antibodies with the C2 domain.[2] Together, these studies reveal fascinating molecular details on the unexpectedly large number of exposed surfaces in the C2 domain that contribute to the binding of inhibitory antibodies. These findings are relevant in the context of neutralizing anti-FVIII antibodies that develop in patients with hemophilia A. Insight into the antigenic properties of the C2 domain is needed to design FVIII variants with decreased antigenicity. Pioneering studies by Dorothea Scandella on the epitope mapping of FVIII inhibitors already pointed toward the C2 domain as a major binding site for FVIII inhibitors.[3] The recent studies by Nguyen and Walter, combined with earlier work by Meeks et al, have provided evidence for 3 major binding sites for inhibitory anti-FVIII antibodies within the C2 domain.[4] The overall dimensions of the C2 domain are small when compared to antibodies. Nevertheless, at least 2 and probably 3 monoclonal antibodies can simultaneously bind to the C2 domain.[2] The mapping studies reported by Nguyen also suggest the presence of 3 distinct clusters of surface-exposed side chains on the FVIII C2 domain (see figure).[1]

The results obtained raise the issue of why so many distinct antigenic sites are present within the C2 domain. The currently available structures of antibodies in complex with the C2 domain reveal that positively charged surfaces contribute to the binding of anti-C2 antibodies.[2,5] These positively charged clusters contribute to the binding of FVIII to negatively charged phospholipids. Conversely, these patches of positively charged amino acids may also direct the immune response toward the C2 domain by promoting the selection of B-cell clones expressing antibody molecules with negatively charged residues in their variable domains.



Inhibitor epitopes on the C2 domain. Binding sites of the 5 different types (A, AB, B, BC, and C) of anti-C2 domain antibodies are displayed. The orientation of the C2 domain is depicted below the image. For type AB and B antibodies, a bottom view of the C2 domain is shown. Image was prepared using the crystal structure of B domain-deleted FVIII (3cdz), using PyMOL.

It should be noted that the majority of monoclonal antibodies analyzed in this study are derived from hemophilia A mice injected with human FVIII. Epstein-Barr virus immortalization and phage display have been employed to isolate human monoclonal anti-C2 antibodies from peripheral blood of inhibitor patients.[6,7] Reactivity of only a single human monoclonal antibody (BO2C11) belonging to the type AB group (see figure) was included in this study. Competition experiments have shown that so-called type BC/C antibodies (see figure) are also present in patients with FVIII inhibitors.[8] Nevertheless, it would be important to extend the innovative studies reported in this article to a panel of human monoclonal antibodies derived from B cells of inhibitor patients.

In their search for antigenic sites on the C2 domain, Nguyen et al focused on antigenic variants that increased the dissociation rate of antibody–C2 domain complexes. This elegant approach has proven to be highly diagnostic for the identification of residues crucial for the high-affinity binding of antibodies to the C2 domain. Modification of the identified C2 domain residues in conjunction with antigenic loops in other antigenic sites within other domains provides an interesting approach for the development of less-antigenic variants of FVIII.

Apart from the proposed modification of B-cell epitopes on FVIII, a number of other approaches are currently being explored to prevent formation or eradicate preexisting inhibitors in hemophilia A patients.[9,10] These efforts, together with the novel half-life-extending bioengineered FVIII molecules and the recent revival of gene therapy approaches, provide exciting new opportunities to further extend the current portfolio of therapeutic options for hemophilia A.

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

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