was associated with significant decreases in AML cells in the bone marrows of 3 of the 6 groups. Examination of marrow sections showed that the mouse cells expressed ASS1, whereas the human AML blasts did not, supporting the conclusion that differential ASS1 expression is the basis for selectivity. In support of this point was the finding that sorted healthy human marrow cell populations with phenotypes of hematopoietic stem cells ($CD34^+CD38^-$) or progenitors ($CD34^+CD38^+$) were enriched in ASS1 mRNA expression relative to lymphocytes or cells with other expression patterns ($CD34^-CD3^-CD19^-$). In particular, ASS1 expression was greater than that of the AML blast samples sensitive to ADI-PEG 20 treatment in vitro.

Administration of subcutaneous cytarabine for 10 days alone, which approximated a low-dose 20-mg twice daily regimen, decreased tumor presence in bone marrow after 4 weeks in 4 of 6 xenograft groups. Combination treatment with ADI-PEG 20 and subcutaneous cytarabine significantly lowered marrow blasts in all the xenografts, some of which failed to respond to either agent alone. When AML blasts were treated with the combination in vitro, synergy in cell killing was demonstrated in 3 of 3 samples.

This report finds that the expression of ASS1 is heterogeneous in AML disease populations. Comparisons of the efficacy of immunologic and reverse transcriptase-polymerase chain reaction assays of ASS1 levels indicated that the latter was the more sensitive candidate for developing a biomarker to identify AML samples that may be sensitive to arginine deprivation. Questions regarding the mechanisms of cell killing by arginine deprivation alone and how it augments active agents such as cytarabine await more definitive answers that likely will vary with disease type and accompanying agents; so too does the understanding of mechanisms by which tumors will become resistant to this approach. More than a dozen clinical trials testing arginine deprivation using ADI-PEG 20 have been initiated, mainly in solid tumors and as a single agent.[9] The information in this paper provides a compelling rationale to initiate clinical trials that will determine whether a cohort of AML is truly addicted to arginine.

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

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● ● ● THROMBOSIS AND HEMOSTASIS

Comment on Lorenz et al, page 4069

CLEC-2: the inside story

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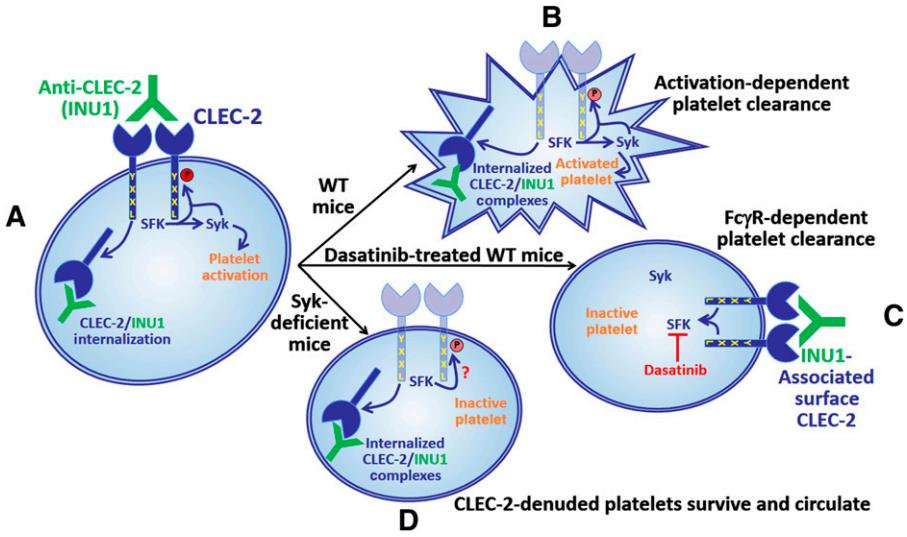
In this issue of *Blood*, Lorenz et al elucidate the mechanisms by which antibody-mediated targeting of platelet C-type lectinlike receptor 2 (CLEC-2) induces receptor downregulation and thrombocytopenia. This information is important because antibody-mediated targeting of CLEC-2 may have therapeutic utility as antithrombotic therapy, especially if thrombocytopenia can be avoided.[1]

Excessive platelet aggregation at sites of atherosclerotic plaque rupture can result in formation of pathological thrombi that reduce or obstruct blood flow to downstream tissues and cause tissue ischemia or infarction.[2] Consequently, antiplatelet drugs have been developed for treatment of acute thrombotic events. Platelet activation and aggregation at sites of vessel damage is, however, also normally required for cessation of bleeding. It is therefore not surprising that antithrombotic therapies that target platelets are often associated with a disconcertingly high risk for bleeding. The search therefore continues for antithrombotic therapies that do not cause bleeding.

The multiple cell surface receptors that are capable of activating platelets fall into 2 main categories depending on whether they signal through activation of heterotrimeric G proteins, such as Gαq or Gαi, or nonreceptor tyrosine kinases (NRTKs) such as Src-family kinases (SFKs) and spleen tyrosine kinase (Syk).[3] The major platelet G-protein–coupled receptors (GPCRs) include the $P2Y_1$ and $P2Y_{12}$ receptors for adenosine 5′-diphosphate (ADP), the protease-activated receptors (PARs) for thrombin (PAR1 and PAR3 or 4), and the thromboxane/prostaglandin endoperoxide receptor for thromboxane A_2. Antiplatelet agents that target the major GPCRs are currently in use, and an elevated risk for bleeding is a well-known side effect associated with each of them.[4] The major NRTK-coupled platelet-activating receptors include the glycoprotein VI (GPVI)/Fc receptor γ-chain (GPVI/FcRγ) collagen receptor complex, the C-type lectinlike receptor for podoplanin, CLEC-2, and (in humans) the low-affinity receptor for the Fc portion of the immunoglobulin γ heavy chain, FcγRIIA.[5] The first 2 of these receptors represent especially interesting targets for antithrombotic therapy because knockout mice whose platelets fail to express either the GPVI/FcRγ complex or CLEC-2 exhibit



A rat monoclonal antibody, INU1, that is specific for mouse CLEC-2 induces activation of SFK and Syk, resulting in platelet activation and internalization of INU1/CLEC-2 complexes (A). In wild-type mice, INU1-bound platelets, which have internalized their INU1/CLEC-2 complexes, are cleared in a manner that depends upon platelet activation (B). In wild-type mice treated with the SFK inhibitor, dasatinib, platelets can neither be activated nor can they internalize CLEC-2/INU1 complexes; consequently, they are cleared in a manner that depends on binding of FcγRs, presumably on phagocytes, to the Fc region of the INU1 IgG heavy chain (C). Interestingly, in Syk-deficient mice, platelets do not become activated but do internalize CLEC-2/INU1 complexes; consequently, they cannot be cleared by either the activation-dependent or FcγR-dependent pathway and therefore continue to circulate (D). These findings show that antibody-mediated CLEC-2 downregulation can be uncoupled from antibody-induced thrombocytopenia, which is a prerequisite for CLEC-2 targeting strategies to be useful as antithrombotic therapies.

impaired thrombus formation in experimental models of arterial injury, but do not bleed more than their wild-type counterparts.[6] Efforts to develop and test antibodies or small-molecule inhibitors of GPVI/FcRγ complexes or CLEC-2 for use as antithrombotic agents are therefore currently under way.

Antibodies that target the GPVI/FcRγ complex or CLEC-2 work to limit thrombosis by inducing loss of the relevant receptor from the surfaces of megakaryocytes and circulating platelets in vivo.[6] Receptor downregulation is, however, preceded by a transient, but profound, thrombocytopenia, which causes bleeding and therefore limits the use of these antibodies as antithrombotic agents. Determining the mechanisms underlying antibody-induced receptor downregulation and platelet clearance is important to enable efforts to uncouple the desired effect of receptor downregulation from the undesired effect of thrombocytopenia. The studies by Lorenz et al[1] demonstrate that, unlike the GPVI/FcRγ chain complex (which is lost from the surfaces of platelets and megakaryocytes primarily as a consequence of antibody-induced, matrix metalloprotease–dependent ectodomain shedding), antibody-induced downregulation of CLEC-2 is due to internalization of antibody/CLEC-2 complexes, which interestingly requires SFK, but not Syk, activity (see figure). The authors additionally show that the CLEC-2–specific monoclonal antibody, INU1, can induce thrombocytopenia in 2 distinct ways. The first mechanism applies to INU1-treated wild-type mice, in which platelets both become activated and internalize the CLEC-2/INU1 complexes that form on their surfaces (see figure, panel B). Because these platelets internalize CLEC-2/INU1 complexes, their clearance does not involve FcγR-dependent recognition. The precise mechanism by which these activated platelets are cleared remains to be determined. The second mechanism applies to INU1-treated wild-type mice that were also treated with the SFK inhibitor, dasatinib. Platelets in dasatinib-treated mice cannot be activated; however, they also cannot internalize CLEC-2/INU1 complexes and therefore end up being cleared in an FcγR-dependent manner (see figure, panel C). Perhaps the most interesting finding of the study is what happens in INU1-treated Syk-deficient mice, in which platelets do not become activated but do internalize CLEC-2/INU1 complexes (see figure, panel D). These platelets cannot be cleared by either the activation-dependent or the FcγR-dependent pathway and therefore continue to circulate. Thus, Syk deficiency uncoupled the undesired effect of thrombocytopenia from the desired effect of CLEC-2 downregulation in INU1-treated mice. These results suggest that combination therapy with a CLEC-2–specific antibody and a Syk inhibitor, one of which is currently in clinical trials for treatment of rheumatoid arthritis,[7] may effectively limit thrombosis without causing significant bleeding.

The finding that INU1-dependent CLEC-2 internalization depends on SFK activity but not Syk was unexpected and has interesting implications for our understanding of events that are most proximal to CLEC-2 engagement. Transduction of activating signals by CLEC-2 requires phosphorylation of a cytoplasmic tyrosine (Y) residue that together with a nearby leucine (L) residue constitutes half (YxxL) of an immunoreceptor tyrosine-based activation motif (hemITAM).[5] Signaling by receptors that contain full ITAMs [consensus sequence: YxxL(x_{6-12}) YxxL], such as the GPVI/FcRγ complex, normally involves SFK-mediated phosphorylation of the 2 ITAM tyrosine residues, which then support recruitment of Syk via its tandem Src homology 2 domains and subsequent SFK-dependent Syk phosphorylation and activation.[5] CLEC-2 is unique in that its hemITAM has been reported to be phosphorylated primarily by Syk, which requires SFK for activation.[8] The finding by Lorenz et al that INU1-induced CLEC-2 internalization requires SFK, but not Syk, activity suggests that either the hemITAM of CLEC-2 can be phosphorylated by SFKs, at least in response to INU1 binding, or that INU1 binding to CLEC-2 induces activation of SFK that contributes in some way other than hemITAM phosphorylation to CLEC-2 internalization (see figure, panel D).

Although CLEC-2 and GPVI are similar to one another in that they both signal through activation of SFKs and Syk, they differ in important ways. Thus, whereas GPVI expression is limited to platelets, CLEC-2 has been reported to be expressed, albeit to a lesser extent, on cells other than platelets, including liver sinusoidal endothelial and Kupffer cells as well as neutrophils and macrophages.[9] In addition, whereas GPVI appears to function only in hemostasis and thrombosis, CLEC-2 plays additional roles in other physiological processes that range from lymphangiogenesis and maintenance of

blood and lymphatic vessel integrity to organ development (eg, brain, kidney, and lung) and tumor metastasis.[9,10] To the extent that efforts to treat thrombotic events by targeting CLEC-2 continue, these additional aspects of CLEC-2 biology must be taken into account.

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

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● ● ● TRANSPLANTATION

Comment on Flynn et al, page 4085

Syk and tired of current chronic GVHD therapies

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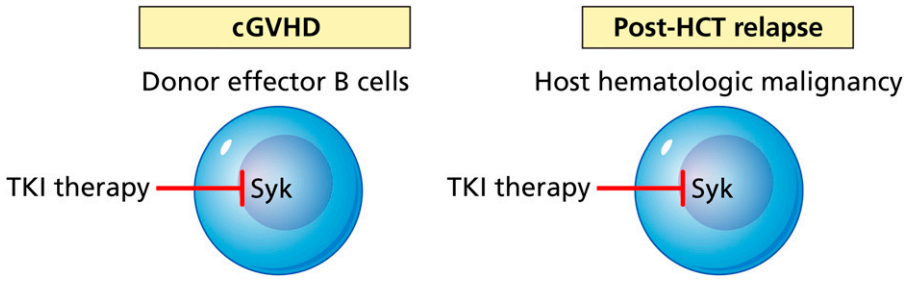
In this issue of *Blood*, Flynn et al[1] provide key data that lend further support to the development of clinical trials of spleen tyrosine kinase (Syk) inhibition for more effective chronic graft-versus-host disease (cGVHD) treatment.

Treatment of cGVHD with medications such as corticosteroids and calcineurin inhibitors is often insufficient or toxic and causes immunosuppression that can increase malignancy relapse. As illustrated by Flynn et al[1] in this issue of *Blood*, an improved understanding of cGVHD biology and the availability of targeted therapies may help solve these obstacles. Flynn and colleagues have identified Syk signaling in murine allogeneic B cells as a mediator of cGVHD, including the devastating complication of bronchiolitis obliterans. Targeting of Syk during established chronic lung GVHD was effective using either conditional genetic ablation or pharmacologic tyrosine kinase inhibitor (TKI) therapy. Syk inhibition was also effective at inducing apoptosis of human cGVHD B cells.



Flynn et al have demonstrated that Syk signaling within allogeneic B cells contributes to murine cGVHD and can be successfully targeted with TKI therapy. The effect was most dramatic in a model of bronchiolitis obliterans, which is a devastating complication of allogeneic hematopoietic cell transplantation (HCT). Because Syk inhibition holds promise for the treatment of multiple hematologic malignancies, targeting this pathway represents an exciting new intervention to improve both the safety and the efficacy of allogeneic HCT. Professional illustration by Patrick Lane, ScEYEnce Studios.

Previously, inhibition of the Syk signaling pathway with fostamatinib prevented experimental murine acute GVHD more effectively than cyclosporine therapy.[2] Importantly, fostamatinib inhibited myeloid-derived antigen-presenting cells (APCs) and T cells (including inhibition of migratory function) yet preserved T-cell cytolytic effects against lymphoma and viral challenge. Fostamatinib was also previously evaluated in a cGVHD model that was somewhat different from the multiple models used by Flynn et al; in this prior study, fostamatinib reduction of skin and lung disease was again primarily attributed to modulation of APC and T-cell function.[3] The experiments by Flynn et al place a new emphasis on the role of B cells in Syk-driven cGVHD as (1) recipients of Syk-deficient donor T cells developed cGVHD, (2) recipients of Syk-deficient marrow had limited B-cell reconstitution and avoidance of cGVHD, and (3) conditional in vivo deletion of Syk-expressing marrow-derived cells (or in vivo therapy with fostamatinib) was effective against established pulmonary cGVHD.

The work of Flynn et al is also instructive because of the multiple experimental models of cGVHD that were evaluated. Although control of cGVHD cutaneous manifestations was incomplete and variable between the models, fostamatinib was relatively consistent in terms of its ability to modulate some of the immunologic end points associated with cGVHD biology. This type of diversity in modeling may help in the design of clinical trials, both with respect to types of cGVHD patients to accrue and immune end points to monitor as potential surrogates for drug activity. The inclusion of well-annotated human samples in this investigation is an advantageous feature that may assist in the more effective screening of promising agents, especially as the number of cGVHD patients and clinical trials are relatively scarce. Indeed, as exemplified in the current work, new efforts in cGVHD drug development