mutations in the target (see figure). The lessons learned about BTK inhibition in CLL may open doorways to new therapeutic approaches for AML, which are urgently needed. Studies such as these are needed for all of the novel agents in development, because the more we understand about the mechanisms underlying the efficacy of these new drugs, the better we will be able to use them to improve the treatment of patients with hematologic malignancies.

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Comment on Klimenkova et al, page 1239

SLPI is essential for granulopoiesis

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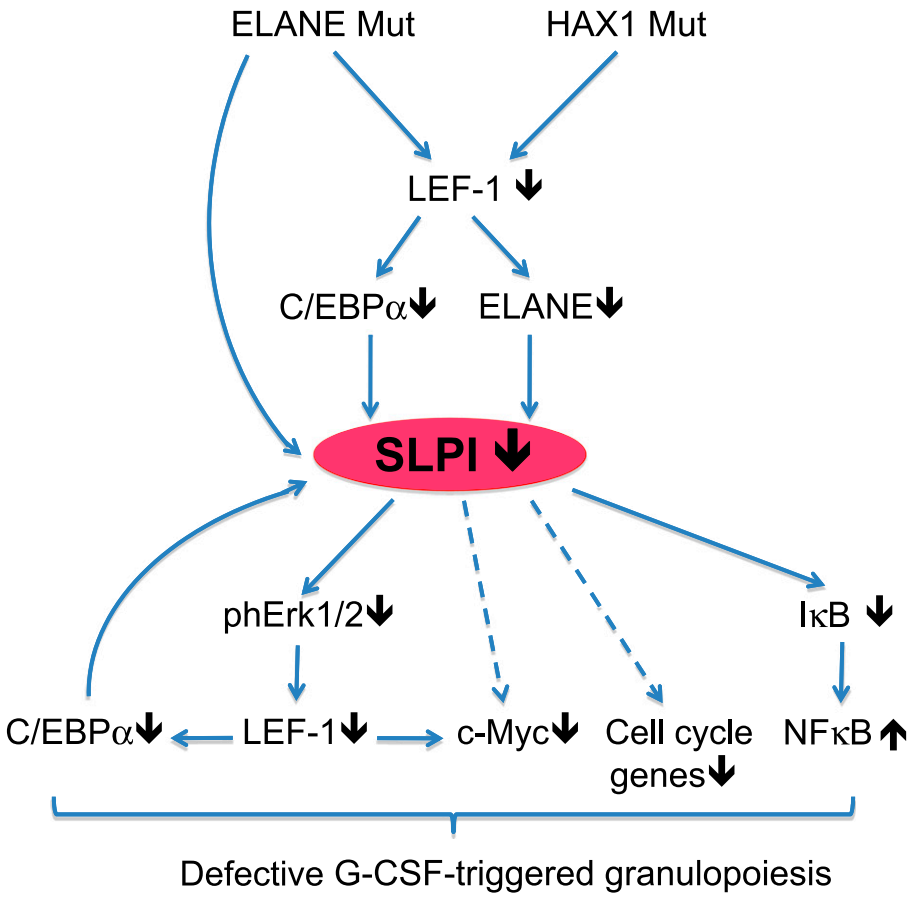
In this issue of *Blood*, Klimenkova et al demonstrate that secretory leukocyte protease inhibitor (SLPI), the natural inhibitor of neutrophil elastase (NE), plays a nonredundant role in human neutrophil differentiation. The authors show that NE itself is both required and sufficient to induce expression of SLPI in myeloid progenitors, which subsequently regulates granulocyte colony-stimulating factor receptor (G-CSFR) signaling and thereby cell proliferation, differentiation, and survival. Patients with severe congenital neutropenia (SCN) were found to have strongly reduced SLPI levels, and this article contributes to unraveling the molecular mechanism(s) underlying the block in neutrophil formation in this disease.[1]

SCN is a primary immunodeficiency syndrome that occurs in approximately 1 in 200 000 individuals and is characterized by a block in the development of neutrophilic granulocytes. The absence of neutrophils makes these patients much more susceptible to



Model for the altered molecular pathways in myeloid progenitor cells of patients suffering from SCN, in which known genetic mutations lead to decreased SLPI expression and subsequently to altered cell signaling and transcription-factor expression, resulting in abrogated G-CSF–induced granulopoiesis. Arrows indicate confirmed (solid) or speculative (dashed) molecular interactions. IκB, inhibitor of NF-κB; Mut, mutated; ph, phosphorylated. See Figure 7 in the article by Klimenkova et al, which begins on page 1239.

invasive bacterial and fungal infections and increases the risk for the development of myelodysplastic syndrome and acute myelogenous leukemia. Mutations in at least 6 different genes can cause SCN, of which *ELANE* (or *ELA2*), the gene encoding NE, is the most frequently affected one.[2] Heterozygous mutations in *ELANE* are sufficient to cause neutropenia, and it has been suggested that the mutated NE protein can accumulate in the cytoplasm and induce apoptosis through activation of the unfolded protein response.[3,4] Yet NE expression in myeloid cells and plasma of SCN patients is actually downregulated, independently of the mutation status of *ELANE* but dependent on the expression of the myeloid transcription factors LEF-1 and c/EBPα.[5] Still, the underlying mechanism by which diminished NE expression impairs neutrophil differentiation remains unclear.

NE is a serine proteinase expressed in neutrophils, monocytes, and mast cells that can intracellularly breakdown phagosome content but, upon secretion, NE can also degrade a variety of extracellular proteins, including receptors for chemokines and cytokines, whereas it can also induce the expression of several inflammatory cytokines.[6] The natural inhibitor of this potent enzyme is SLPI, which is induced by NE and has strong antiprotease activity to NE, but SLPI can also modulate intracellular signal transduction pathways through ERK and nuclear factor κB (NF-κB).[7,8] Therefore, the authors questioned whether reduced levels of NE in SCN could alter intracellular signaling in myeloid progenitors through SLPI and thereby affect the fate of these cells. In their article, Klimenkova et al demonstrate that SLPI expression is strongly reduced in myeloid cells and plasma from SCN patients carrying mutations in *ELANE* but also in patients with mutations in hematopoietic cell-specific Lyn substrate 1–associated protein X-1 (*HAX1*), another gene frequently mutated in SCN.[1] Importantly, treatment of primary myeloid cells with purified human NE was sufficient to induce SLPI messenger RNA (mRNA) and protein expression, as was transduction with wild-type *ELANE*, but not with *ELANE* mutants found in SCN patients. Reciprocally, short hairpin RNA–mediated knockdown experiments revealed that SLPI mRNA and protein expression were dependent on ELANE/NE and HAX1. Further molecular analysis showed that SLPI mRNA expression is regulated by LEF-1–induced activation of c/EBPα and binding of the latter to the *SLPI* promoter (see figure).

These experiments demonstrate that SLPI expression is positively regulated by an intricate interplay between NE and key myeloid transcription factors, but they do not explain how decreased levels of SLPI can impair neutrophil formation. To address this, the authors inhibited SLPI expression in expanded bone marrow progenitors and could demonstrate that SLPI is in fact required for both G-CSF–driven proliferation and survival and for colony formation of neutrophils, but not of monocytes/macrophages or erythroid cells. These functional experiments were corroborated by the finding that knockdown of SLPI blocked G-CSF–induced phosphorylation of STAT5, ERK1/2, and LEF-1 and led to diminished levels of the LEF-1 target genes *c-Myc*, *survivin*, and *cyclin D1*, which are key drivers of proliferation and survival (see figure). Gene expression analysis by microarrays also showed that c-Myc–triggered signaling and cell-cycle signaling are impaired on SLPI downregulation. Interestingly, the same pathways were also downregulated in myeloid progenitors from SCN patients compared with healthy controls or patients with idiopathic neutropenia. Overall, this article demonstrates that SLPI is an important mediator of neutrophil differentiation, because it positively regulates signal transduction downstream of the G-CSFR in myeloid progenitors and thereby enhances their proliferation and survival. Since both NE and HAX1 control SLPI expression, it becomes more evident why mutations in these genes strongly contribute to the development of SCN. Although the authors did not experimentally address this, it would be of interest to determine whether the block in neutrophil differentiation in SCN patients can be overcome by exogenous treatment with NE or overexpression of SLPI.

The elegant work by Klimenkova et al[1] adds another dimension to the anti-inflammatory function that SLPI fulfils in the immune system. In macrophages, SLPI is induced by lipopolysaccharide (LPS) and can suppress LPS-induced activation of NF-κB and thereby the production of nitric oxide and tumor necrosis factor α.[7] SLPI is also expressed in dendritic cells, particularly in mucosa-draining lymph nodes, in which it regulates tolerance of T cells to mucosally administered antigens.[9] The novel finding that SLPI can also regulate neutrophil differentiation in the bone marrow raises the question of whether this mode of regulation is also important during inflammation. Bone marrow output changes upon infection, and we have shown that interferon γ (IFN-γ) produced on viral infection enhances monocyte formation but strongly inhibits neutrophil formation through the induction of suppressor of cytokine signaling 3 in myeloid progenitors, thereby inhibiting G-CSF–induced neutrophil development.[10] Yet since IFN-γ can suppress SLPI expression,[7] it would be of interest to determine whether suppression of the newly identified SLPI/G-CSFR/LEF-1/c-Myc pathway[1] (see figure) also plays a role in the inflammatory feedback to the bone marrow on viral infection. Conversely, given the important protective function of neutrophils in the defense against bacterial or fungal pathogens, it can be envisaged that this pathway is instead turned on during these infections, but this awaits further investigation.

In conclusion, SLPI is a versatile molecule, not only inside and outside the cell, but also in both developing and fully mature myeloid cells. The discovery that SLPI is an essential regulator in neutrophil differentiation that is downregulated in SCN will open new avenues for further research into this disease, hopefully leading to novel treatment options.

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

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● ● ● PLATELETS & THROMBOPOIESIS

Comment on Tyagi et al, page 1250

Inhibit the calpain to climb the mountain

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In this issue of *Blood,* Tyagi et al shed some light on the mechanism of thrombosis induced by a high altitude, hypoxic environment.[1] Using proteomic analysis of platelets and in vivo models of thrombosis, the authors elegantly demonstrated that enhanced calpain activity, regulated by CAPNS1, significantly contributes to platelet reactivity and thrombosis under hypoxic conditions. The observations from animal models were further supported by human data showing increased calpain activity and elevation in markers of platelet activation in the plasma of patients who developed deep vein thrombosis at high altitude.

Who does not know about the increased risk of developing deep vein thrombosis during long air travels? Or heard about pulmonary or cerebral thrombosis causing death of people trekking Himalaya? Have you ever thought that calpains may have something to do with that? Calpains are calcium-dependent, nonlysosomal cysteine proteases expressed ubiquitously in mammals. There are 2 major forms of calpains, the μ-calpain (also called calpain-1) and the m-calpain (calpain-2), which require micro- or millimolar calcium concentration for full activation, respectively. The calpain proteolytic system includes the calpain proteases, the small regulatory subunit CAPNS1, and the endogenous calpain-specific inhibitor, calpastatin. A previous study with recombinant calpastatin provided the first demonstration of the role of calpain in platelet secretion, aggregation, and spreading.[2] Subsequently, another group used μ-calpain–knockout mice to demonstrate attenuated thrombin- and adenosine 5′-diphosphate–induced platelet aggregation and clot retraction.[3] Because current calpain inhibitors are unable to differentiate between μ- and m-calpain, it is unknown which calpain plays the dominant role in hypoxia-mediated platelet activation and thrombosis. Data from a recent study examining the effect of calpain on the platelet proteome and reactivity in diabetes mellitus suggest that m-calpain may be primarily involved in platelet spreading, whereas μ-calpain primarily contributes to platelet adhesion.[4]

Platelet activation and aggregation are critical for clot formation, and attenuation of these functions certainly contributed to reduction of thrombus size in hypoxic rats treated with calpain inhibitors.[1] However, additional mechanisms could also influence the outcome of this study. Recently, much attention was focused on procoagulant microparticles released from many cell types, including platelets, and their contribution to activation of coagulation and thrombosis. Interestingly, the calpain-dependent release of procoagulant microparticles from platelets has been previously described.[5] In addition, tissue factor (TF) expressed by monocytes has been shown to promote thrombosis in a mouse model of oxygen deprivation.[6] Of note, calpain inhibition can attenuate the activation of the nuclear factor κB pathway that plays a critical role in the regulation of TF gene expression.[7] Unfortunately, the authors did not analyze monocyte TF expression in their study.

Another interesting aspect of the paper by Tyagi and colleagues is the demonstration of TF in rat platelets.[1] The authors observed the splicing of TF pre–messenger RNA (pre-mRNA) and increased levels of TF protein in platelets isolated from hypoxic rats. The splicing of TF pre-mRNA was previously shown in activated human platelets[8]; however, the ability of platelets to synthesize TF protein is still debated. For example, it has been proposed that the presence of TF on platelets isolated from patients with sepsis and acute coronary syndromes most likely results from the docking of monocyte-derived TF-bearing microparticles rather than de novo synthesis.[9] Because hypoxia upregulates TF expression on monocytes,[6] the same mechanism could contribute to the increased levels of TF protein in platelets isolated from hypoxic rats.

This study raises a number of interesting questions regarding the role of calpain and platelets in hypoxia-induced thrombosis. What is the relative contribution of the 2 major forms of calpain to hypoxia-induced thrombosis? The tail-bleeding time was not affected in μ-calpain–knockout mice.[3] Would that suggest that specific targeting of this calpain can effectively reduce thrombosis without interfering with hemostasis? In addition, follow-up studies should provide more mechanistic insight into how calpains regulate platelet responses during hypoxic conditions. Finally, in contrast to rat platelets, pre-mRNA for TF was not observed in mouse platelets, precluding the use of mouse models to study the role of platelet TF.[10] Because genetically engineered rat strains are now being routinely produced, it would be of interest to generate rats with conditional, platelet-specific deletion of the TF gene to address the controversy surrounding the role of platelet TF in thrombosis.

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

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