Moreover, super β-sheet (ie, intermolecular β-sheet) formation is widely recognized as one of the “strongest” linking mechanisms between monomers. Additionally, numerous favorable side-chain interactions and the burial of proportionately large surface area (close to one-third of the entire monomer surface) all contribute to the ultrastability of the CTCK dimer (see figure). This dimer structure is truly a “beauty.”

From a technical perspective, the structure determination of CTCK was no small feat because of complications such as glycosylation and proper disulfide formation. Successful protein expression was achieved using mammalian cells, a complex task in itself. Although protein production in HEK293 cells is not amenable to routine selenomethionine labeling for standard crystallographic phasing, fortunately, the successful crystallization condition contained zinc metal which served as a heavy atom for solving the phase problem. Furthermore, the unusually high solvent content (88%) greatly helped improve the map quality which would otherwise have been challenging for such low-resolution data.

As with most determined structures, the structure determined by Zhou and Springer provides immediate insight into the structural mechanisms involved with VWF. Not only does it provide direct knowledge of the VWF dimer binding mechanisms, but it also offers clear structural rationale for CTCK point mutations implicated in von Willebrand disease: they all weaken this dimer interaction. Moreover, it is also anticipated that this structure will help facilitate the characterization of related homologs involved in numerous cellular processes. However, like many other structures, the CTCK structure also raises some interesting questions. For example, the CTCK dimer structure seems incompatible with the fact that shear stress can provoke a rearrangement of disulfide bridges in the C-terminal part of the protein, a process that contributes to the incorporation of new VWF molecules into the assembled VWF multimers at the vascular surface. Based on the crystal structure, it is difficult to comprehend how some sort of disulfide bridge swapping could be accomplished without greatly compromising the triple reinforcement. Clearly, more studies are needed to gain a better understanding of the complex and multifaceted VWF molecule. From a clinical perspective, although the CTCK structure is a big step forward in our basic understanding of the dimer formation, developing any potential therapeutic benefits from this structure is likely a far way off. The challenge here will be to “outsmart” Mother Nature and design either (1) an even stronger dimer that is less compromised by disease–causing mutations (though the challenges of delivering a genetic fix are still formidable) or (2) finding novel compounds that can enhance CTCK dimer formation for those with deleterious point mutations.

This structure serves as a poignant example of the capacity for evolution to engineer exquisite solutions to highly specific problems, in this case marshalling an array of structural components that make the CTCK assembly as stable/destruction resistant as possible. In essence, CTCK acts like protein superglue that holds VWF together. Indeed, this structure is akin to highly–reinforced concrete blocks strengthened not only by standard steel bars but also by polymers and/or alternate composite material.

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**Lymphocytosis and ibrutinib treatment of CLL**

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**Comment on Woyach et al, page 1810**

In this issue of Blood, Woyach et al clarify that prolonged lymphocytosis is composed of biologically inert leukemic cells and does not anticipate poor outcome or relapse. Prolonged lymphocytosis may be perceived as a failure of chronic lymphocytic leukemia (CLL) treatment with ibrutinib.

Besides the accumulation of genetic lesions, active signaling through the B-cell receptor (BCR) plays a central role in CLL pathogenesis and progression. The restricted and frequently stereotyped repertoire of the BCR in CLL has been interpreted as a result of BCR-driven selection initiated by specific antigens or autoantigens that may promote the expansion of the CLL clone and has been taken as the major proof of the addiction of CLL to BCR signaling. The Bruton’s tyrosine kinase (BTK) is at the crossroad of the BCR pathway (see figure). On BCR activation by the microenvironment, BTK transduces the signal to the phosphatidylinositol 3-kinase (PI3K)/murine thymoma viral oncogene homolog (AKT), mitogen-activated protein kinase (MAPK), and nuclear factor (NF)-κB pathways, resulting in prevention of apoptosis, as well as in promotion of cell adhesion, cell migration, and other cellular processes that can help survival and proliferation of leukemic cells.

This strong biological rationale makes BTK an ideal target for therapy of CLL and other B-cell malignancies. Ibrutinib is a selective tyrosine kinase inhibitor that covalently and irreversibly binds BTK and consequently blocks survival, proliferation, and migration of CLL cells in in vitro models of the tumor microenvironment. Recently,
ibritinib has been shown to exert impressive clinical activity in CLL. In relapsed and refractory patients, ibritinib monotherapy induces an estimated 26-month progression-free survival (PFS) in 75% of cases. In treatment-naïve patients, the drug leads to a projected 24-month PFS in 95% of patients. Beside antiproliferative activity, ibritinib induces the redistribution of tissue-resident CLL cells into the blood with rapid shrinkage of the lymph nodes. In this context, cellular redistribution across anatomic compartments causes an increase of the absolute lymphocyte count in the peripheral blood. Such ibritinib-induced lymphocytosis is transient in most patients, resolving within 8 months, but may be more prolonged in a fraction of CLL patients, lasting >12 months. The biological characteristics of the CLL cells egressed from the lymph nodes and sustaining ibritinib-induced lymphocytosis, as well as the clinical impact of this phenomenon, have not been investigated in detail and are a matter of current debate.

By taking advantage of cases enrolled on the PCYC-1102-CA phase 1b/2 trial of single agent ibritinib in patients with relapsed or refractory CLL, Woyach et al identify 20% of CLL cases that responded to ibritinib but showed a prolonged lymphocytosis, defined as an elevation of the tumor lymphocyte count that had not resolved to normal or <50% of baseline within 12 months. Patients with prolonged lymphocytosis were more likely to carry mutated immunoglobulin genes, which is a favorable prognostic marker in CLL, and deletion of 13q. The genetics and immunogenetics profile of the leukemic cells composing the prolonged lymphocytosis did not vary significantly over time, indicating the lack of emergence of clonal diversity and selection in this clinical context. In patients with prolonged lymphocytosis, the delay in the clearance of the peripheral blood from leukemic cells was not due to a suboptimal inhibition of BTK activity by ibritinib or to the selection of a resistant clone, because biochemical assays proved the effectiveness of ibritinib in blocking phosphorylation of BTK targets. In contrast to the biochemical inhibition of BTK, downstream pathways, namely AKT and extracellular signal-regulated kinase (ERK), were still activated at enhanced levels in the lymphocytes of patients with prolonged lymphocytosis, conceivably because CLL cells still remain responsive to stimulation outside of BTK or the BCR. As suggested by Woyach et al, such BTK-independent activation of AKT and ERK might provide a biological explanation for the survival of lymphocytes in the peripheral blood of patients displaying prolonged lymphocytosis. Despite these clues of signaling downstream of BTK, lymphocytes composing the prolonged lymphocytosis observed during ibritinib treatment appear to be transcriptionally inert, as revealed by the absence of variations in the BCR signalosome and BCR target gene expression program. Consistent with the biological features described above, CLL cells of prolonged lymphocytosis patients had a low mitotic index and were not actively proliferating, thus simulating a state of anergy and quiescence. The minimal change in gene expression profile combined to the absence of proliferation provide a biological rationale to the clinical observation that persistent lymphocytosis has no impact on ibritinib treatment outcome and does not represent a risk factor for progression.

Published guidelines define the outcome of CLL therapy based on surrogate markers of tumor burden reduction, including the decrease of the peripheral blood lymphocyte count, which enable the physician to assess the clinical benefit of a given CLL therapy. Under ibritinib treatment, the emergence of lymphocytosis and its persistence for a long time, even in the presence of a clear clinical benefit for the patient, may confound response classification according to the current criteria and can generate the impression of resistance to treatment. The advent of new guidelines for ibritinib-treated CLL is therefore desirable and important.

As inhibitors of the BCR signaling become more widely available and enter the standard of care of CLL patients, it is important that physicians understand that lymphocytosis, even when persistent for many months, is not a sign of treatment resistance or disease aggressiveness, to prevent unnecessary drug discontinuation. The study by Woyach et al provides the proof of principle that
Comment on Desmond et al, page 1818

Eltrombopag: a stem cell cookie?

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In this issue of Blood, Desmond et al present an extension of their earlier phase 2 study of the thrombopoietin receptor (TPO-R) agonist eltrombopag to treat 43 patients with refractory severe aplastic anemia (SAA). Hematologic responses, including trilineage response, were maintained despite later discontinuation of the drug. They propose that eltrombopag directly stimulates residual hematopoietic stem cells (HSCs) in SAA. This represents a novel approach to the treatment of SAA.1 2

The immune basis of acquired SAA for most patients is now well established in vitro, characterized by clonal expansion of CD8+ T cells, Th1 cells, reduced regulatory T cells (Tregs) that are also dysfunctional in their ability to suppress T effectors, and increased Th2 and Th17 cells.3 4 From clinical observations, response to immunosuppressive therapy (IST) with antithymocyte globulin and ciclosporin occurs in approximately two-thirds of patients. The reasons for nonresponse to IST in the remainder have remained an enigma. An alternative diagnosis of constitutional aplastic anemia accounts for only 5% to 10% of patients. 5 Lessons learned from using hematopoietic growth factors (HGFs), 4 such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor, stem cell factor, and thrombopoietin, in an (erroneous) attempt to treat the underlying disorder, showed that they were ineffective, but it was also assumed that there were too few HSC remaining in SAA following the initial insult to the bone marrow (BM), despite supraphysiological doses of circulating HGF. However, the results of this study appear to negate this latter assumption.

This study first confirms the earlier observations from the phase 2 study that eltrombopag can not only stimulate the platelet count in SAA but also induce bi- or trilineage hematologic responses and that overall responses occur in 40% of patients. Second—and this is an even more exciting observation—out of 14 responders who continued the drug, 5 patients later discontinued eltrombopag and maintained sustained hematologic response. What is the possible explanation for these observations? Direct stimulation of HSC is likely because the TPO-R, c-mpl, is present not only on megakaryocytes but also on HSC and progenitor cells and HSCs are deficient in c-mpl knockout mice.6 7 This study suggests that the remaining few HSCs in SAA can be stimulated by high eltrombopag levels and/or by moving them from a quiescent state. However, future studies using long-term culture initiating cells before and after eltrombopag may provide more evidence for this. The authors propose that a critical number of HSCs is required for IST response as a possible explanation for lack of response to IST. But could there be other explanations, such as an off-target effect of eltrombopag? Could eltrombopag have an immune-modulatory role, analogous to improvement in Treg function, demonstrated by the suppression of autologous T effectors seen in chronic immune thrombocytopenic purpura patients responding to eltrombopag? 8

Alongside these important observations, however, is the concern regarding clonal evolution to myelodysplasia. Out of 43 patients, 8 developed clonal cytogenetic abnormalities, most frequently monosomy 7, known to be associated with a high risk of transformation to myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML) in SAA. Furthermore, abnormal clones were detected very early after starting eltrombopag, most frequently by 3 months. This is much earlier than the onset of clonal evolution following antithymocyte globulin (ATG) treatment. The normocellularity of BM trephines seen in responding patients is also different from typical marrow appearances after ATG, where some degree of residual hypocellularity is more frequent. The changes observed with eltrombopag may reflect a greater recovery of stem cells, but careful morphologic and molecular characterization is required to exclude changes due to MDS. Although only 2 patients showed dyserythropoiesis, follow up of patients is too short, and it is too small a series because 5 of the 8 patients were subsequently transplanted. In SAA, the remaining HSCs are under constant pressure to support adequate hematopoiesis and peripheral blood counts. Combined with shortened telomeres, this

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