that total BTK declines over time during continuous BTK inhibitor therapy (see figure panel A).[9]

In addition to these analyses, Sun et al measured BTK occupancy in paired PBMC and lymph node or bone marrow biopsy specimens, during a planned 36- to 48-hour dosing interruption from day 3 to 5. This allowed them to determine trough BTK occupancy in different tissue compartments and to directly calculate the rate of BTK resynthesis. BTK occupancy was higher at drug trough in lymph nodes within the 100-mg twice-daily than the 200-mg daily cohort (95.8% vs 90.1%). Resynthesis rates for BTK were similar in PBMC and in lymph nodes (14.5% vs 11.2% per day), with a tight correlation seen between BTK occupancy in PBMCs and bone marrow or lymph nodes at all time points (see figure panel B). This is critical information for any future studies of BTK occupancy during acalabrutinib therapy, as it demonstrates that testing of BTK occupancy in PBMC samples, which are far more readily obtained, can be reasonably used to infer BTK occupancy in lymph nodes.

Importantly, this study did not just perform BTK occupancy analysis. Transcriptomic analysis using RNA sequencing from purified circulating tumor cells and from whole lymph nodes revealed suppression of gene signatures related to BCR, NF-κB, cytokine signaling, and cellular metabolism. These pathways were more profoundly impacted by twice-daily than daily dosing, and these differences became more pronounced over time, indicating the biological importance of different levels of BTK occupancy and supporting twice-daily dosing.

Taken together, these data are supportive of the current 100-mg twice-daily dosing of acalabrutinib in CLL. However, several questions remain. First, although it appears from the correlative data that the 100-mg twice-daily dosing provides optimal target coverage and biological effect, the study was not powered to detect differences in clinical outcome between the 100-mg twice-daily group and the 200-mg daily group, so the clinical importance of these findings remains uncertain. Second, although 100-mg twice-daily dosing appeared to provide optimal target coverage, it is not clear whether the 100-mg dose is necessary to achieve this, or whether twice-daily dosing using lower doses of acalabrutinib could provide similar target coverage. Third, the study demonstrated that BTK occupancy at drug trough increased over time; consequently, could lower doses be used at later time points? A pilot study demonstrated that sequentially reducing ibrutinib dose from 420 mg/d to 140 mg/d over 3 months achieved >95% BTK occupancy in PBMCs at all dose levels.[10] A confirmatory randomized study is planned. Exploration of reduced doses of BTK inhibitors is attractive, as lower doses could attenuate costs, and potentially, toxicity. However, until clinical data are available demonstrating equivalent efficacy of lower doses, BTK inhibitors should be dosed at FDA-approved doses, unless toxicity mandates dose reduction.

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MYELOID NEOPLASIA

Comment on Shide et al, page 106

Mutant *CALR* functions: gains and losses

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In this issue of *Blood*, Shide et al separate the roles of loss of a normal *CALR* allele and gain of a mutant *CALR* allele in *CALR*-driven essential thrombocythemia (ET).[13]

Approximately 1 in 4 patients with ET, a blood cancer characterized by overproduction of platelets, has a frameshift mutation in the gene encoding calreticulin (*CALR*).[2,3] Research to date has demonstrated a gain-of-function role for the frameshifted CALR protein in binding to the thrombopoietin receptor (TpoR), thus

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acting as a rogue ligand and triggering proliferation and megakaryopoiesis. However, there has been less investigation of the effects of the loss of a normal *CALR* allele: *Calr*$^{-/-}$ mice are embryonic lethal because of a malformation of the heart, which prevents studying hematopoiesis in these mice.[4]

Shide et al generated a mouse with hematopoietic-specific deletion of the *Calr* allele to allow separation of gain- and loss-of-function effects of the *Calr* mutation. Loss of 1 or both *Calr* alleles in hematopoietic cells had little effect on bone marrow hematopoiesis and did not cause ET, although loss of both alleles increased extramedullary hematopoiesis. In transplantation experiments, loss of 1 or both alleles increased bone marrow repopulation in primary recipients, but only loss of a single allele maintained this advantage in secondary transplants, indicating that *Calr* haploinsufficiency yields a competitive advantage. The authors further demonstrate that a *CALR*del52 transgene can drive a myeloproliferative neoplasm (MPN) phenotype (thrombocytosis) in transplants only when wild-type (WT) *Calr* is haploinsufficient. Finally, they used transcriptomic data to investigate potential mechanisms that underlie the advantage of *Calr*$^{+/-}$ cells. Compared with WT stem/progenitor cells, they find that *Calr*$^{+/-}$ cells have increased expression of E2F targets, suggestive of higher cell cycling. When comparing WT and *Calr*$^{+/-}$ cells that also express transgenic *CALR*del52, the authors again show upregulation of E2F target genes, in addition to an increase in stem cell self-renewal pathways and decreases in pathways responsive to the pro-inflammatory cytokines tumor necrosis factor α and interferon γ.

These results advance the field in several ways: one of these is by demonstrating a previously unknown role for normal CALR in hematopoiesis. In addition, they shed light on important questions about differences between mouse models and human patients: human patients typically show clonal disease, but mouse models to date have shown either no[5,6] or a slow-rising[7] competitive advantage. The results of Shide et al suggest that an approach wherein 2 WT *Calr* alleles are maintained[6] may show no competitive advantage, because this requires loss of a normal *Calr* allele. Therefore, knockin approaches may be more informative, because they have 1 WT and 1 mutant allele, thus more closely resembling patients. One mouse model with knockin of a humanized *Calr*del52 allele gave rise to a strong ET phenotype but no advantage within transplants.[5] A separate CRISPR-Cas9–based mouse model of the *Calr*del52 mutation within the mouse *Calr* gene exhibited a lower platelet phenotype and a slow-rising competitive advantage in primary transplants, consistent with the requirement for *Calr* haploinsufficiency to yield a stem cell advantage.[7] The differences between the models remain unresolved but may reflect differences in how murine and human frameshifted CALR protein binds to TpoR[7] or other differences in the generation of the models. Furthermore, the results of Shide et al emphasize that the level of *Calr*del52 expression is crucial: progression to myelofibrosis in mouse models is seen only when *CALR*del52 is highly expressed via retroviral expression[6] or homozygosity of the knocked-in *Calr*del52 allele.[5] Together, these results underscore the importance of ensuring that model systems resemble the situation in human patients, who have 1 mutant and 1 WT allele. Similarly, although results from transplant experiments can give insight into stem cell function, it is important to remember that these are artificial settings that do not resemble steady-state hematopoiesis in patients.

Going forward, more research will be necessary to understand why *Calr* haploinsufficiency is so critical for bestowing a competitive advantage on stem cells: although there are indications of increased cell cycling and self-renewal, no direct mechanism has been proposed. It remains to be seen whether this mechanism will rely on one of the canonical functions of CALR, such as protein chaperoning or calcium signaling,[8] or whether the mechanism will be as surprising as the discovery of TpoR activation by frameshifted CALR protein. Because the transcriptomic data were obtained from stem/progenitor cells from transplant recipients, they may be confounded by the stresses of the transplantation protocol on both donor cells and the recipient bone marrow niche. Overall, this study is an important advance in our understanding of mutant *CALR*-driven ET: in addition to the well-studied role of mutant CALR as a rogue ligand for TpoR, Shide et al show a role for the loss of a WT *Calr* allele that warrants further investigation.

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