cofactor for Tet2, augmented Tet2 activity, increased conversion of 5-mC to 5-hmC, and increased HSC differentiation.

The results of the Aljoufi et al study raise several interesting questions: first, Does physioxia sustain HSC numbers and HSC self-renewal capacity solely by inhibition of Tet2-mediated oxidation of 5-mC to 5-hmC? Or does physioxia also suppress other important Tet2 epigenetic functions (eg, histone modification via regulation of O-linked β-N-acetylglucosamine transferase)?[9] Furthermore, How does the function of Tet2 in regulating the HSC response to oxygen shock/stress relate to the function of cyclophilin D, which Mantel et al[3] previously demonstrated to have an integral role in promoting MPTP induction, ROS generation, and HSC loss in ambient air?

The application of physioxia conditions could improve HSC transplantation or HSC gene editing, but it raises practical challenges. The results of the Aljoufi et al study suggest that targeting the molecular mechanisms underpinning physioxia could, in principle, sustain HSC numbers and function ex vivo. Interestingly, Guan et al[10] recently described a Tet-specific inhibitor that produced synthetic lethality for *Tet2*-mutant myeloid neoplastic cells while displaying no pro-proliferative effects on normal hematopoietic progenitor cells. Although even temporary inhibition of the Tet2 tumor suppressor in HSCs would require caution, an alternative strategy could utilize supplementation with 2-hydroxyglutarate, which antagonizes α-KG as a co-factor for Tet2 dioxygenase,[6] thereby inhibiting Tet2 enzymatic activity and mimicking physioxia. The article by Aljoufi et al provides an important step toward understanding the molecular mechanisms that govern the HSC response to extraphysiologic stress and how such mechanisms might be targeted.

Conflict-of-interest disclosure: J.C.P. declares no competing financial interests. ■

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MYELOID NEOPLASIA

Comment on Jutzi et al, page 1291

Mutant *CALR*'s "sweet tooth"

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In this issue of *Blood*, Jutzi et al[1] present compelling evidence of glycosylation as a potential therapeutic vulnerability in calreticulin (*CALR*)-mutated myeloproliferative neoplasms (MPNs).

Classic MPNs, which include polycythemia vera, essential thrombocytosis, and primary myelofibrosis, result from aberrant signal transduction, which induces derangements in hematopoietic stem cell function.[2] This is evidenced by the frequency of activating mutations in the thrombopoietin receptor *MPL*, the downstream kinase *JAK2*, or the endoplasmic reticulum chaperone *CALR*. Mutant CALR confers constitutively active MPL signaling and cytokine independence on hematopoietic cells. *CALR* mutations are found in 20% to 25% of all MPNs, and no precision oncology approaches are available at this time to treat patients with MPNs who harbor this specific mutation.[3-5]

A key mechanism of oncogenesis by the CALR mutation is through direct binding of the CALR-mutant isoform to MPL within the endoplasmic reticulum, which then facilitates its cell surface expression and activation in a ligand-independent fashion[6,7] (see figure). Activation of MPL by mutant CALR then induces cytokine-independent cellular proliferation. Importantly, the interaction between CALR and MPL, as well as proper MPL cell surface expression, are dependent on the addition of carbohydrates, specifically N-linked glycans, to MPL. N-linked glycosylation is a complex, multistep process by which proteins have carbohydrates added to asparagine residues, which then influence protein stability, folding, subcellular localization, and function. Although the role of N-glycosylation is well known to regulate the mutant CALR-MPL interaction to promote abnormal signaling,[8] whether this is potentially targetable is unknown.

To identify potential therapeutic targets in *CALR*-mutant MPNs, Jutzi et al used a genome-wide CRISPR dropout screen and discovered N-linked glycosylation as a key requirement for cytokine-independent proliferation.[7] Importantly, multiple potential enzymes critical to N-linked glycosylation, including DPM2, DPY19L1, MPDU1, PMM2, MOGS, and others were identified through the

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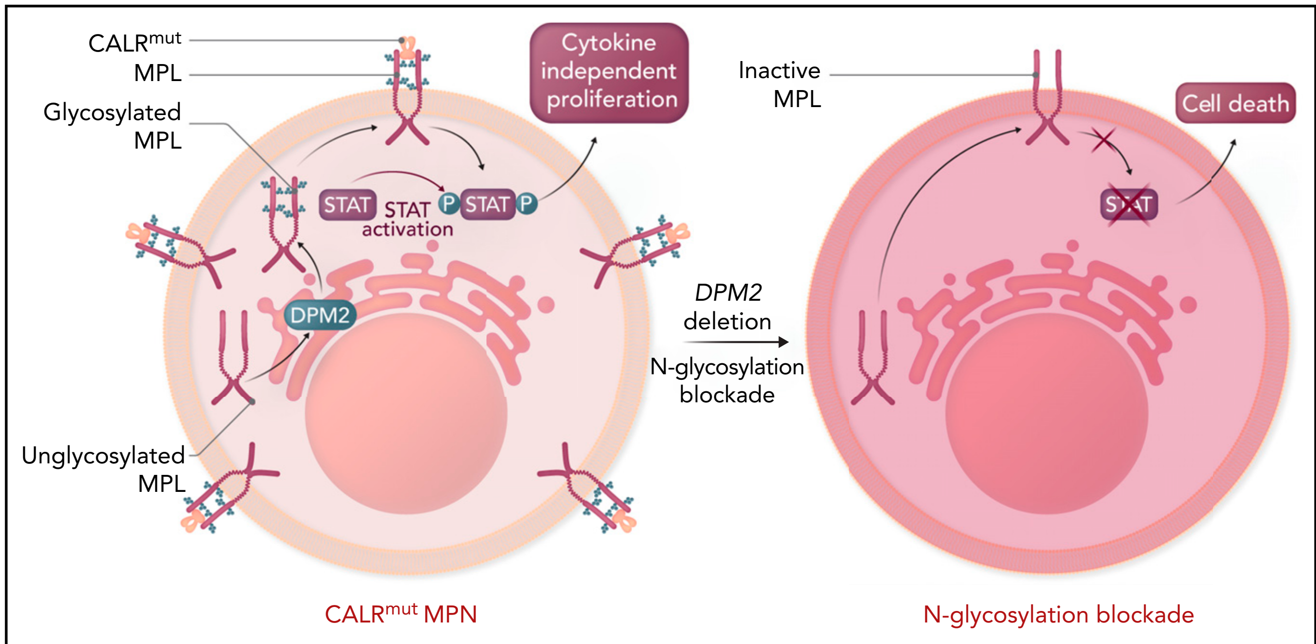

The thrombopoietin receptor (MPL, maroon) requires N-linked glycosylation (blue) via a multistep enzymatic process in the endoplasmic reticulum and association with calreticulin (orange) to be translocated to the cell surface. CALR binding to glycosylated MPL in the cytoplasm facilitates transport to the cell surface. Mutant CALR (red) induces constitutive MPL signaling to confer cytokine-independent proliferation to hematopoietic cells, the ultimate cause of MPNs. Although the mutant CALR was known to require N-glycosylation, dependent on the enzyme DPM2 for its association with MPL, whether this was potentially targetable was unknown. Genetic loss of *DPM2* or inhibition of N-glycosylation with small molecules, including 2-DG, reduced cell surface expression of MPL and decreased MPL activity as measured by STAT activation. Collectively, this indicates that targeting N-glycosylation is a potential therapeutic vulnerability in MPNs. Please note that CALR binds to MPL likely in both the cytoplasm and extracellular space. Professional illustration by Somersault18:24.

screen, indicating that disruption of any stage of the complex enzymatic process had an impact on mutant CALR function. The requirement for DPM2, a critical enzyme central to N-glycosylation, was confirmed for the cytokine-independent growth induced by constitutive MPL activation, a hallmark of MPNs. Given that N-glycosylation is a multistep enzymatic process, the authors then examined >60 different small molecules known to block various enzymes within the process. Half of these small molecules blocked cytokine-independent growth induced by *CALR* mutations or an activating mutation in *JAK2*^{V617F}, suggesting that N-glycosylation was critical to the oncogenic effects of both mutations. Collectively, this suggests N-glycosylation is a potential therapeutic target in *CALR*-mutant MPNs.

The central tenet of precision oncology is that targeted approaches should be more effective against cells that harbor the mutation than against normal cells to increase efficacy and reduce toxicity. The widespread role of N-glycosylation in a multitude of biological processes raised the potential that targeted inhibition may be nonspecific to *CALR*-mutant cells and may be toxic to normal cells. To address this concern, Jutzi et al focused subsequent studies on the N-glycosylation inhibitor 2-deoxy-glucose (2-DG), which has been used in clinical trials for refractory solid tumors on the basis of their differential requirements for glucose as an energy source.[9] By using a combination of genetic rodent models in vivo and primary human patient samples ex vivo, it was possible to examine the effects of 2-DG on both *CALR*-mutant and *CALR* wild-type (wt) cells. In vivo treatment with 2-DG selectively targeted *CALR*-mutant cells by increasing apoptotic pathway activity, consistent with a loss of cytokine-independent proliferation (see figure). Importantly, treatment with 2-DG ameliorated the preferential growth advantage of *CALR*-mutant long-term hematopoietic stem cells when mixed with wt cells, implying that selectivity of N-glycosylation was blocked on mutant cells. Primary patient samples confirmed these findings, implying that the requirement for N-glycosylation is conserved across species. Although *JAK2*^{V617F} cells were not studied as extensively as *CALR* mutations, they showed a response similar to that with 2-DG treatment, which indicates that targeting N-glycosylation may be more broadly applicable across different MPN mutations.

The findings presented in Jutzi et al represent a preclinical step critical to identifying N-glycosylation as a potential therapeutic vulnerability in MPNs. Importantly, additional hits from the CRISPR screen identified protein secretion and unfolded protein response as additional potential targets. Whether these pathways are independent or dependent on N-glycosylation needs to be explored, but they have the potential to identify additional novel targets. Given the central requirement for hyperactive MPL signaling across different genetic mutations in MPNs, it is not surprising that inhibiting N-glycosylation may be broadly applicable, but it also represents a potentially novel target. This may point to therapies for MPNs that lack *JAK2*^{V617F}, a collection of diseases that would benefit from an expanded precision oncology approach.[10] In conclusion, by identifying N-glycosylation as a potential therapeutic vulnerability in MPNs,

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Jutzi et al have identified an entirely new pathway for precision oncology approaches.

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

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TRANSPLANTATION

Comment on Gournay et al, page 1305

AML relapse after a TIGIT race

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In this issue of *Blood*, Gournay et al[1] dissect donor immune reconstitution after allogeneic hematopoietic stem cell transplantation (HSCT) with mass cytometry and identify T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and CD161-expressing CD4$^+$ T cells as early immune correlates of subsequent acute myelogenous leukemia (AML) relapse after HSCT.

The high relapse rate of AML after stem cell transplant has remained one of the most tenacious problems in malignant hematology. With the graft-versus-leukemia (GVL) effect at the core of successful long-term disease control, the early posttransplant course can be thought of as a delicate race between nascent donor immune reconstitution and recovering malignant cell populations that seek to escape GVL. AML relapse after HSCT thus often associates with donor immune cell dysfunction through upregulated immune checkpoint molecules or reduced antigen presentation, which leads to leukemic relapse through impaired recognition and killing of malignant cells.[2]

Clinical studies with immune checkpoint blockade have demonstrated that relapsed AML after transplant can be successfully treated by closing loopholes in donor immunity, for example, using CTLA-4 blockade in cases of extramedullary AML, in which it induced long-term disease control through local CD8$^+$ T-cell infiltration.[3] However, such durable remissions following immune checkpoint blockade are the exception rather than the norm, and a deeper understanding is still lacking regarding the composition and interactions among donor immune cell populations that are needed to drive such effective GVL responses.

Mass cytometry is a systems immunology approach for the unbiased discovery of immune cell subpopulations and their dynamics at single-cell resolution that builds on the capability to capture dozens of surface markers. This permits extension of classical flow cytometry approaches to a much higher resolution and can identify rare, previously unknown cell subsets. The wealth of information provided per cell by mass cytometry requires dimensionality reduction techniques and unsupervised clustering for data analysis, which overcome the limitations of classical manual gating strategies to achieve the analytical depth and speed required for analysis of such data.

Gournay et al studied peripheral blood samples prospectively collected from 2 large cohorts of patients following allogeneic stem cell transplantation, which they analyzed using mass cytometry with a 45-antibody panel (see figure). The first cohort consisted of peripheral blood collected longitudinally at 3, 6, and 12 months after transplant from 37 patients with AML/MDS in remission, providing the opportunity to define immune cell kinetics during the crucial first year after transplant, when most cases of AML relapse occur. As comparator, the authors profiled peripheral blood from 20 healthy donors. As expected, based on longstanding characterizations in the field, immune reconstitution early after transplant (3 months) was dominated by innate cell types such as natural killer (NK) cells and monocytes, whereas adaptive immunity and professional antigen-presenting cells gradually recovered at later timepoints. Notably, however, they demonstrated that the circulating immune compartment after transplant, despite ongoing reconstitution, remained profoundly altered even after 12 months. Globally increased expression of immune checkpoint molecules, such as PD-1, LAG3, and TIGIT, were detected across T and NK cells. Moreover, higher TIGIT expression on several T-cell subsets was associated with subsequent AML relapse, suggesting that the immune checkpoint molecule is of high relevance for posttransplant relapse.

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