were not present at diagnosis. As FL cells are characterized by ongoing SHM in the antigen-binding sites, the authors then examine the clonal diversity of the 9-bp region encoding N-gly motifs of each subclone. Despite ongoing SHM, N-gly sites are never lost but replaced by new variants through synonymous and non-synonymous mutations. Thus, although the evolutionary processes are clearly at work within the tumor cells as evidenced by substantial subclonal BCR and N-gly diversification, there is a selective pressure to keep this unique BCR modification during progression. The authors then looked at the outcome of rare N-gly^{neg} subclones emerging from random SHM and showed that they represent a minor component of the tumor bulk (<1%), and when present, they are deleted from subsequent disease events leading the authors to suggest that N-gly^{neg} cells are outcompeted by the clonal expansion of N-gly^{pos} clones. Although we cannot exclude that the genetic landscape of N-gly^{neg} subclones might be different to explain their deletion, this study supports the fact that N-gly^{neg} cells might not receive a similar type of BCR signal induction and survival input from microenvironmental lectins than is received by the N-gly^{pos} one.

Collectively, this and previous reports[7,8] support a model whereby N-gly motifs are early events during lymphomagenesis that must occur by SHM in the first GC transits of t(14;18)^{pos} cells, which may even precede most aberrations acquired by the CPC. Glycan residues might allow the retention/reactivation of t(14;18)^{pos} subclones within the mutagenic GC context leading to a growth/survival advantage and further (epi)genomic modifications. The hierarchy of founding CPC mutations remains to be established by directly purifying those cells, but glycosylated motifs have been found in in situ follicular neoplasia, 1 established premalignant FL stage, corroborating the present conclusions.

Besides BCR N-gly motifs, recent studies have highlighted the link between individual (epi)genetic alterations and the capacity of malignant B cells to interact with and subvert their GC-like microenvironment in both patients and genetically engineered mouse models.[2,4,9] An important perspective of this study would be to identify how the triad of individual mutations, BCL2 translocations, BCR glycans, and epimutations, work together to trigger CPC genesis and impact the crosstalk between tumor cells and their supportive niche and how those cross-dependencies can be exploited therapeutically. Mouse models that mimic the introduction of altered glycans into the BCR are needed.

Regarding clinical applications, precision medicine approaches disrupting glycan-lectin interactions and blocking downstream BCR kinase cascades might represent good candidates to inhibit the repopulating potential of this reservoir of relapse-initiating cells.[7] Whether the addiction for glycan-lectin interactions is preserved in vivo in all relapse settings remains unknown. In vitro functional genomic CRISPR screens in GC-derived lymphomas recently reported a dependency of the tumor cells for a mode of oncogenic BCR signaling that promotes survival in a BCR-PI3K–dependent but microenvironment-independent fashion,[10] suggesting some advanced FL have lost this requirement and necessitate distinct therapeutic approaches.

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MYELOID NEOPLASIA

Comment on Zhang et al, page 845

Cooperating mutations: joint forces, novel vulnerabilities

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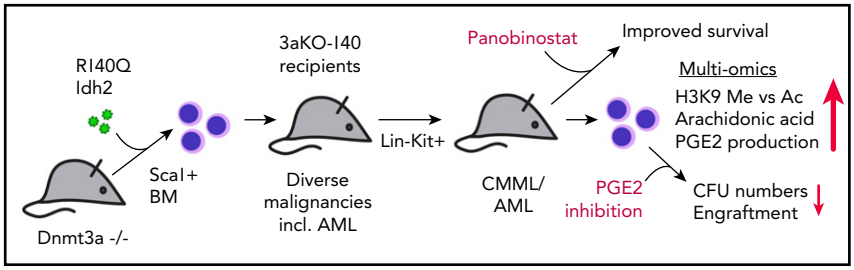
In this issue of *Blood*, Zhang et al report on their investigation to elucidate the mechanisms by which loss of function of the DNA methyltransferase 3A (DNMT3A) cooperatively induces hematologic malignancies when cooccurring with IDH1/2 mutations.[1]

Acute myeloid leukemia (AML) is a complex disease that regularly involves mutations in several oncogenes and tumor suppressors. Many mutations have been investigated on the single-gene level. However, cooccurring mutations are likely to alter phenotypes, leukemogenic mechanisms, and potentially even drug responses. To model the scenario of combined mutations in DNMT3A and either IDH1/2 as observed in 5% to 8% of patients with myelodysplastic syndrome/AML,[2,3]



Sca1$^+$ bone marrow (BM) cells from 3aKO mice were retrovirally transduced with R140Q mutated Idh2 (140) and subsequently transplanted in recipient mice in comparison with their single- and nonmutated WT counterpart cells. Recipients of double-mutated (3aKO-140) cells develop severe hematologic malignancies, including AML, earlier than mice receiving 3aKO cells alone. Lin$^-$Kit$^+$ cells harvested from 3aKO-140 injected mice induce CMML or AML in all secondary recipients. Multi-omics approaches reveal an imbalance of H3K9 methylation vs acetylation and overproduction of AA and PGE2. Treatment of mice with the HDACi restores normal levels of H3K9 methylation and acetylation and improves survival of 3aKO-140 injected mice. Ex vivo treatment with celecoxib of 3aKO-140, but not single- or nonmutated progenitors, reduces colony-forming units (CFU) and leukemic engraftment upon retransplantation. incl., including.

Zhang et al overexpressed the neomorphic Idh2 R140Q mutated protein in hematopoietic progenitors derived from Dnmt3a knockout mice (hereafter called 3aKO-140 cells) and observed development of different hematologic disorders, including overt AML in recipient mice. Using multi-omics approaches, they identified imbalanced H3K9 methylation vs acetylation levels and overproduction of prostaglandin E2 (PGE2) as hallmarks of 3aKO-140 cells. In line with these findings, they found that double-mutant cells from diseased mice were specifically sensitive to histone deacetylase (HDAC) and PGE2 inhibition. The data presented by Zhang et al underline the importance of understanding synergistic mutational events in the development of leukemia. They suggest that the combination of HDAC and prostaglandin synthesis inhibition might represent a novel therapeutic approach for patients with AML harboring both mutations, in particular, for those who develop resistance against available IDH2 inhibitors (see figure).

Patients with AML are currently risk-stratified mostly based on single mutations[4] (except for NPM1 and FLT3-ITD). Small molecules targeting mutated proteins, such as FLT3[5,6] or IDH1/2 inhibitors (reviewed in Golub et al[7]), have shown promising efficacy in patients with AML. So far, most clinical trials assessing the efficacy of compounds targeting mutated proteins usually require only the presence of the respective mutation regardless of comutations. Consequently, the impact of comutations on therapy response is analyzed only retrospectively. Moreover, a substantial fraction of patients will develop resistance against these drugs via diverse mechanisms, including additional mutations in the targeted protein, and will then require alternative therapeutic approaches.

One example of concurrent mutations frequently found in hematologic malignancies involves the genes DNMT3A and IDH1 or IDH2.[2,3] Interestingly, DNMT3A and IDH1/2 are antagonizing epigenetic modifiers, as DNMT3A methylates DNA, while IDH1/2 enzymes cause DNA and histone hypomethylation through generation of α-ketoglutarate, an important cofactor of histone and DNA demethylases, such as TET proteins (summarized in Im et al8). Consequently, cooccurrence of mutations in DNMT3A and IDH1/2 partially reverses the effects observed when only one of them is mutated.[9] This raises the question as to why mutations in both modifiers cooccur in hematologic malignancies and how they cooperate to induce the disease. To address these questions, Zhang et al overexpressed R140Q-mutant Idh2 in hematopoietic progenitor cells harvested from Dnmt3a homozygous KO (3aKO) mice and their wild-type (WT) counterparts. Although a complete loss of the DNMT3A protein is not identical with a heterozygous DNMT3A mutation as observed in the majority of patients, the authors observed that their 3aKO mouse model recapitulates the phenotype of heterozygously mutated Dnmt3a models, such as expansion of hematopoietic stem and progenitor cells (HSPCs).[10] Primary recipients of 3aKO-140 cells developed severe hematologic disorders, including overt AML in some recipients with a shorter median survival compared with 3aKO-Idh2 WT mice. Recipients of only Idh2 R140Q engineered HSPCs did not die of hematologic diseases during the observation period. These results clearly demonstrated synergy between the 2 events in inducing hematologic malignancy. To corroborate their findings, they retransplanted the cells in secondary recipients and observed chronic myelomonocytic leukemia (CMML) or AML in all mice injected with Lin-Kit$^+$ cells from primary 3aKO-140 mice.

To gain insight into the mechanism, they performed reduced representation bisulfite sequencing and H3K4me3 and H3K27ac ChIP-seq. These experiments revealed not only increased H3K9 methylation in 3aKO-140 cells but also reduced H3K9 acetylation, which they suggested to be the consequence of competition between enzymes involved in H3K9 methyl- and acetylation. In line with their hypothesis, histone deacetylase inhibitors (HDACi) reversed the observed imbalance and improved survival of mice injected with 3aKO-140 cells. To confirm that they had identified a specific vulnerability of 3aKO-140 cells, they treated single- and double-mutant as well as WT cells with HDACi and inhibitors of H3K9 methylation (G9a inhibitor) and observed specifically high sensitivity of 3aKO-140 cells against these molecules.

To obtain more hints about the cooperative mechanisms, the authors performed a metabolomics approach, which revealed higher abundance of arachidonic acid (AA) in 3aKO-140 cells. Because AA is the precursor of prostaglandin synthesis, they also studied more components of the pathway and found higher levels of PGE2 and overexpression of FADS2 messenger RNA, a key enzyme for AA synthesis, in 3aKO-140 cells, which they suggest to be due in part to loss of enhancer DNA methylation through the loss of Dnmt3a. Given these results, the authors suggested that PGE2 synthesis inhibition should represent another vulnerability of double-mutant cells. To test this, they again treated WT, single, and double 3aKO-140 cells with the PG synthesis inhibitor celecoxib and PTGER antagonists and found specific sensitivity of double-mutant cells toward PGE2 synthesis inhibition.

Together, the paper highlights the need for understanding cooperative events in leukemia development to identify more efficient synergistic treatment options.

Their results suggest that patients with AML harboring mutations in both DNMT3A and IDH2 might benefit from combination treatment with HDAC and prostaglandin synthesis inhibitors when IDH2 inhibitors are not applicable. The findings are highly relevant, because HDAC inhibitors as well as prostaglandin synthesis inhibitors are already available in the clinic.

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THROMBOSIS AND HEMOSTASIS

Comment on Dhanesha et al, page 857

Integrin α9β1: a new target to fight thrombosis

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In this issue of *Blood*, Dhanesha et al explore the role of myeloid cell–specific integrin α9β1 in arterial thrombosis.[1] They demonstrate that genetic ablation or pharmacologic inhibition of the integrin reduces thrombosis and, importantly, this effect is not accompanied by impairment of normal hemostasis.

Integrin α9β1 is expressed on resting neutrophils at a level similar to that of α5 integrin. After activation, its expression increases two- to threefold, and it becomes the most abundant β1 integrin on the neutrophil surface. The role of α9β1 has been demonstrated in such processes as migration on tenascin substrates and vascular cell adhesion molecule 1 (VCAM-1), but no information about its function in thrombosis has been reported so far.

This study demonstrates a novel role of α9β1 on the neutrophil membrane and raises several important questions for further investigation. For example, mechanisms of neutrophil recruitment to the site of thrombus development remain elusive. Fewer neutrophils get recruited to the thrombus in $\alpha 9^{fl/fl}$LysMCre^{+} mice, which suggests that α9β1 binds a ligand on certain cells in the thrombus or in the vessel wall. This integrin binds VCAM-1 on the activated endothelium[2]; however, it is generally assumed that ferric chloride denudes endothelium and therefore VCAM-1 may not be a good candidate for neutrophil recruitment in this model. A potentially dispensable role of endothelial receptors is further confirmed by the clear antithrombotic phenotype in $\alpha 9^{fl/fl}$LysMCre^{+} mice in the laser injury model in which a thrombus grows on a relatively small area of contact with the endothelium.

It is known that α9β1 engagement activates inducible nitric oxide synthase (iNOS), and the resulting nitric oxide (NO) can mediate neutrophil adhesion and recruitment.[3] However, NO is a potent inhibitor of platelets and therefore, if this mechanism was involved, a prothrombotic phenotype in $\alpha 9^{fl/fl}$LysMCre^{+} mice could be expected. Another potential mechanism through which α9β1 could potentiate cell migration is modulation of potassium channel permeability.[4]

Osteopontin is an extracellular matrix protein involved in the pathogenesis of various inflammatory diseases. Osteopontin is one of the ligands for α9β1 that is capable of inducing neutrophil chemotaxis in an α9β1-dependent fashion.[5] It is currently unknown whether osteopontin is directly involved in thrombosis, but it was shown to be a potential biomarker for atherothrombotic ischemic stroke and deep vein thrombosis (DVT).[6,7] Thus, osteopontin could be implicated in neutrophil recruitment and the process of thrombosis, which should be explored in future studies.

Another question to be addressed is the role of α9β1 in venous thrombosis. Both neutrophils and neutrophil extracellular traps (NETs) are critical for DVT.[8] In addition to retaining red blood cells in the thrombus, components of NETs exert a procoagulant effect (nucleosomes) and stimulate platelet aggregation (histones). Consequently, exploring the role of α9β1 in DVT may be a promising line of research.

Dhanesha et al demonstrate that platelets could be activated by cathepsin G released from neutrophils in an α9β1-dependent manner. However, it has also been reported that pharmacologic inhibition or genetic ablation of cathepsin G leads to prolonged tail bleeding time in vivo,[9] whereas findings by Dhanesha et al demonstrate that tail bleeding time remained unchanged. Consequently, another molecule or molecules released by neutrophils might be involved.

Another interesting line of inquiry is the mechanism through which engagement of α9β1 promotes formation of NETs. One of the potential mechanisms could