Diffuse large B-cell lymphoma (DLBCL) remains incurable in nearly 30% of patients, who do not achieve durable remission with current first-line immunochemotherapeutic approaches. It is now clear that a major barrier to improved outcome is the remarkable heterogeneity of this disease, which makes the generic nontargeted R-CHOP regimen (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone) only partially effective. Of the two molecular subtypes recognized in the updated World Health Organization classification, the activated B-cell–like (ABC)-DLBCL is characterized by a less favorable prognosis (1). Further heterogeneity was revealed by the analysis of the DLBCL coding genome, which led to the discovery of distinct genetic clusters defined by the cooccurrence of specific genetic alterations underlying the involvement of distinct oncogenic survival pathways (1, 2). In this context, ABC-DLBCL is highly enriched in three subtypes—including MCD (or the partially overlapping C5 cluster), defined by recurrent MYD88L265P and CD79B gene mutations; BN2 (or cluster 1), defined by BCL6 translocations and NOTCH2 mutations; and the rare N1 (NOTCH1) group— with MCD cases displaying inferior clinical outcome (1, 2). Precision medicine approaches tailored to subtype-specific tumor addictions are being developed to further our ability to cure these patients.

A hallmark of ABC-DLBCL, and particularly of the MCD subtype, is the presence of mutations targeting the B-cell receptor (BCR)–driven NF-κB signaling pathway, including the genes encoding for the BCR subunits CD79α/β, the CARD11 component of the signalosome complex, the negative regulator TNFAIP3, and the MYD88 scaffolding protein, which relays signals from TLR9 to the NF-κB transcription complex (Fig. 1). These mutations maintain a “chronically active” form of BCR signaling that culminates in the constitutive activation of NF-κB, thereby promoting cell proliferation and survival (3). The critical importance of BCR signaling in the pathogenesis and viability of ABC-DLBCL cells was revealed by functional genomic screens and by the selective toxicity of drugs targeting this pathway to ABC-but not germinal center B-cell–like (GCB)-DLBCL models (3).

Following on these insights, the first phase I/II clinical trial for relapse/refractory (r/r) DLBCL treated with the small-molecule irreversible inhibitor of Bruton tyrosine kinase (BTK) ibrutinib has shown significant clinical activity in patients with ABC-DLBCL, including exceptional responses in tumors that belong to the MCD subtype (4). High response rates were also seen in primary central nervous system lymphoma, which shares a similar genetic profile (3). However, median progression-free survival was short, and most patients experienced rapid disease progression. Signal transduction through the BCR also plays a critical role in other B-cell malignancies such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), where its activity is sustained by a variety of tyrosine kinases in addition to BTK. Consistently, treatments such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), where its activity is sustained by a variety of tyrosine kinases in addition to BTK. Consistently, treatments that selectively target the BCR have revolutionized the clinical approach to CLL, where ibrutinib is highly effective, and are among the preferred standards of care for patients with r/r MCL. Nonetheless, ibrutinib as a single agent does not eradicate the malignant B-cell clone due to emerging drug resistance and/or recurrence upon drug discontinuation (5). To reduce side effects and overcome resistance, a new generation of irreversible or reversible BTK inhibitors (BTKi) is being developed, and promising results were recently reported in DLBCL with acalabrutinib monotherapy, a second-generation covalent BTKi with enhanced kinase selectivity and possibly lower toxicity (6).

The best-characterized mechanism of ibrutinib resistance (IR), particularly in the context of CLL progression after initial response, is represented by genetic mutations affecting components of the BCR pathway. These comprise amino acid substitutions of the cysteine 481 in BTK, which prevent the covalent binding of ibrutinib, or gain-of-function mutations in its downstream substrate PLCγ2 (5). However, a
simple genetic mechanism causing resistance is questioned by several observations suggesting that the mutations may not be the initial drivers, including their timing and the subclonal heterogeneity of resistant disease (7). Notably, a persistent or progressively expanding CLL clone that lacks detectable BTK/PLCG2 mutations or carries them at less than 10% variant allele frequency could be detected in nearly as many as 50% of patients in stable remission on ibrutinib (8). In DLBCL, data are limited, but monitoring of circulating tumor DNA in three patients receiving ibrutinib monotherapy revealed the appearance of likely independent subclonal heterogeneity of resistant disease (7). Notably, a double oval is used to illustrate the downstream activation of the NF-κB complex. The My−T−BCR supercomplex (MYD88–TLR9–BCR) is also indicated. In sensitive cells, BTK inhibition by ibrutinib blocks proliferation and survival (left). The mechanism of epigenetic ibrutinib resistance described by Shaffer and colleagues is illustrated in the middle, where increased amounts of RAC2 and enhanced RAC2–PLCγ2 interaction substitute for BTK in rewiring BCR signaling to downstream NF-κB. This phenotypic shift is driven by TCF4-dependent enhancer activation of critical target genes (only RAC2 and TLR9 shown). On the right, genetic mutations blocking the covalent binding of ibrutinib to BTK or activating PLCγ2 (red thunderbolts) lead to irreversible ibrutinib resistance. BTK, Bruton tyrosine kinase; RAC2, RAC2 inhibitor.

**Figure 1.** Modes of ibrutinib resistance in ABC/MCD-DLBCL. Simplified schematic of the chronic active BCR signaling pathway leading to constitutive NF-κB activation in ABC-DLBCL of the MCD genetic subgroup. Positive and negative pathway regulators commonly targeted by genetic mutations are color-coded in orange (CD79α/β) and magenta (CARD11, TNFAIP3, and MYD88). A double oval is used to illustrate the downstream activation of the NF-κB complex. The My−T−BCR supercomplex (MYD88–TLR9–BCR) is also indicated. In sensitive cells, BTK inhibition by ibrutinib blocks proliferation and survival (left). The mechanism of epigenetic ibrutinib resistance described by Shaffer and colleagues is illustrated in the middle, where increased amounts of RAC2 and enhanced RAC2–PLCγ2 interaction substitute for BTK in rewiring BCR signaling to downstream NF-κB. This phenotypic shift is driven by TCF4-dependent enhancer activation of critical target genes (only RAC2 and TLR9 shown). On the right, genetic mutations blocking the covalent binding of ibrutinib to BTK or activating PLCγ2 (red thunderbolts) lead to irreversible ibrutinib resistance. BTK, Bruton tyrosine kinase; RAC2, RAC2 inhibitor.

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role for nonmutational retuning of BCR signaling in the escape of ABC-DLBCL cells under the selective pressure of ibrutinib treatment. This mode of resistance could sustain the persistence of a polyclonal population while providing a reservoir for the acquisition of later genetic events, such as BTK and PLCγ2 mutations. In further support of this model, a sophisticated genetic barcode experiment that allowed monitoring of the dynamics of expansion of single cells from the starting population showed a pattern incompatible with the outgrowth of a rare genetic alteration for the vast majority of cells surviving treatment for up to 5 weeks. Together, these data suggest that epigenetic changes may be the initial mechanism by which ABC-DLBCL cells adapt to ibrutinib treatment (“persistent cells”) before the occurrence of genetic events irreversibly locks the resistant phenotype and allows its clonal expansion.

The transcriptional signature shared by persistent and resistant cells was significantly enriched in “BCR signaling” genes, including the small GTPase RAC2. In normal B cells, RAC2 contributes to PLCγ2-mediated calcium mobilization downstream of BCR engagement. Shaffer and colleagues observed that, in IR cells, the increased abundance of RAC2 leads to its enhanced biochemical interaction with multiple proteins that participate in proximal BCR signaling, including, among others, PLCγ2 and IgM, thus essentially bypassing BTK in the activation of NF-κB (Fig. 1, middle). RAC2 upregulation resulted in part from an increased accessibility of its enhancer domain to TCF4, and, indeed, the program modulated by TCF4 was among those significantly upregulated in both persistent and resistant cell populations, in line with the preferential enrichment for TCR4-binding motifs in regions of open chromatin that were specific to epigenetic IR cells. Most importantly, knockdown of TCF4 or RAC2 was consistently more deleterious in IR pools as compared with parental cells. In accordance with this observation, a small-molecule inhibitor of RAC2 was among the most differentially toxic drugs in a high-throughput screen for selective vulnerabilities of IR cells.

This wealth of new data opens the way to a number of important questions and opportunities for future investigations: Does in vitro generated resistance reflect the one that develops in vivo, where signals from the microenvironment could potentially bypass the BCR? And will the proposed epigenetic resistance mechanism similarly arise in other B-cell malignancies that depend on active BCR signaling? Also, will it apply to new generation BTKi? Unfortunately, primary DLBCL samples could not be interrogated in this study due to the lack of available material from ongoing studies of ibrutinib monotherapy. Nonetheless, an increased association of RAC2 with PLCγ2 could be detected in post-ibrutinib treatment CLL samples from patients with persistent or progressive disease as compared with pretreatment samples. Although these patients carried subclonal PLCγ2 or BTK mutations, the very low fraction of variant alleles (<15%) was unlikely to explain this protein interaction, which was detected in virtually all tumor cells. Together, these findings reinforce the clinical significance of the TCF4–RAC2–PLCγ2 axis, and suggest its validity as a general mechanism of IR that could also be implicated in CLL and possibly MCL.

An important question that remains is whether targeting epigenetic resistance will prevent the development of genetic resistance. While the answer to this question awaits further studies, the authors observed that cells with genetic IR mutations and, to a lesser extent, parental ABC-DLBCL cells also displayed some response to EHT1864 in culture. Moreover, EHT1864 decreased PLCγ2 activity in one clone with a PLCγ2 mutation, indicating that RAC2 regulates PLCγ2 in genetic IR as well. In line with this model, treatment with EHT1864, while ineffective alone, augmented the ability of ibrutinib to control the growth of sensitive parental ABC-DLBCL xenografts.

Finally, the study by Shaffer and colleagues has several implications for both the development of new predictive biomarkers of epigenetic IR and rationally designed clinical trials that maximize the targeting of BCR signaling using new dosing strategies and drug combinations.

The linear quantitative relationship between RAC2–PLCγ2 interactions and IR, revealed by proximity ligation assays in both DLBCL models with nongenetic resistance and IR CLL patient samples, could have important clinical applications and should be exploited as a potential biomarker for patient stratification and treatment decisions.

From a therapeutic perspective, the identification of RAC2 and other altered dependencies in IR ABC-DLBCL (e.g., BCL2, BRD4, or AKT) as promising new targets provides the foundation for the development of more effective regimens combining ibrutinib with drugs modulating the activities of these proteins, either sequentially or in combination. In this regard, one interesting observation of the present work is that, analogous to cell pools with genetic resistance, the epigenetic IR phenotype was remarkably stable when cells were rechallenged with ibrutinib after short-term withdrawal. However, this was not the case in cultures that were exposed to ibrutinib for only 1 week. Thus, one may speculate that future clinical trials involving ibrutinib may benefit from the use of intermittent dosing schedules in which BTK inhibition could be combined with standard R-CHOP chemotherapy or with drugs targeting the acquired addictions of RAC2–IR resistant cells. Such an approach may also contribute to reducing the toxicity associated with continuous ibrutinib treatment.

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