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Supplement Article

II. Therapy of DLBCL based on genomics

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The activated B-cell-like (ABC) and germinal centre B-cell-like (GCB) subtypes of diffuse large B-cell lymphoma (DLBCL) were defined by their apparent derivation from different stages of B-cell differentiation and their differential response to chemotherapy [1]. With current chemotherapy supplemented with Rituximab, the ABC DLBCL subtype remains less curable [2,3], necessitating new approaches to its treatment.

The view that the ABC and GCB subtypes represent molecularly distinct diseases has been underscored by studies of translocations, genomic copy number changes and somatic mutations (reviewed in ref. [4]). Translocation of BCL2 is restricted to GCB DLBCL, whereas translocation of BCL6 is three times more common in ABC DLBCL. ABC DLBCL is characterized by recurrent deletions of the *INK4a/Arf*, *PRDM1* and *TNFAIP3* loci and gain/amplification of the *SPIB* and *BCL2* loci. GCB DLBCL, on the other hand, is characterized by recurrent amplification of the mIR-17-19 microRNA cluster and deletion of PTEN, both of which activate the PI[3] kinase pathway. Somatic mutations in CD79B and MYD88 are frequent ABC DLBCL, whereas GCB DLBCL sustains mutations in EZH2, TNFRSF14, GNA13, SGK1 and BCL2 that are not found in ABC DLBCL. Thus, the derivation of these DLBCL subtypes from different stages of B-cell differentiation [1] appears to dictate different stereotyped genetic pathways to malignancy.

Treatment strategies have emerged from a regulatory pathway-centric view of ABC DLBCL. Activation of the anti-apoptotic NF-κB pathway appears to be a universal feature of ABC DLBCL [5]. Besides potently blocking apoptosis, NF-κB induces expression of IRF4, which acts together with SPIB to promote plasmacytic differentiation. Another obligate genetic event in ABC DLBCL appears to be inactivation of Blimp-1, a key regulator of tumour plasmacytic differentiation. The requirement for Blimp-1 inactivation may be to cause an accumulation of plasmablastic cells that could be the precursors to ABC DLBCL.

The constitutive activation of NF-κB in ABC DLBCL appears to stem from two prominent upstream signalling pathways that emanate from the B-cell receptor (BCR) and from the signalling adapter MyD88. Combined evidence from RNA interference genetic screens and cancer gene resequencing led to the appreciation that some ABC DLBCL tumours have a 'chronic active' form of constitutive BCR signalling [6]. This pathway is activated proximally by mutations in the BCR subunits CD79B and CD79A in 21% of cases [6] and distally by mutations in CARD11 in 10% of cases [7]. CD79A/B mutations serve at least two purposes. First, BCRs with these mutations are preferentially retained on the cell surface because they fail to be endocytosed [6]. In addition, the CD79B mutations blunt the activation of LYN kinase, which is a negative regulator of BCR signalling. It is important to emphasize, however, that the CD79B/A mutations cannot initiate BCR signalling on their own but rather amplify pre-existing BCR signalling in ABC DLBCLs. The fact that CD79B and CD79A mutations are recurrent in ABC DLBCL but are absent in GCB DLBCL highlights the pathogenetic importance of BCR signalling in ABC DLBCL.

Because constitutive BCR signalling plays a role in the pathogenesis of several lymphoma subtypes, it is important to define precisely the molecular nature of BCR signalling in each instance. BCR signalling can be usefully dichotomized into 'active' and 'tonic' forms (reviewed in ref. [8]). Active BCR signalling, such as occurs when a normal B-cell encounters antigen, is characterized by immobile clusters of the BCR on the cell surface and the engagement of several downstream pathways that promote proliferation and survival, including the NF-κB, PI[3] kinase and MAP kinase pathways. Tonic BCR signalling, on the other hand, is necessary for the survival of all mature B-cells in the mouse and is likely to be antigen-independent. In this mode of BCR signalling, the BCRs are not organized into large clusters in the cell membrane and are freely mobile. Tonic BCR signalling activates the

PI[3] kinase pathway but notably does not activate NF-κB. Within this framework, ABC DLBCLs utilize active BCR signalling [6], whereas Burkitt lymphomas utilize tonic signalling for survival [9].

The second major pathway that engages NF-κB in ABC DLBCL centres on the signalling adapter MYD88, which is somatically mutated in 39% of ABC DLBCLs [10]. One *MYD88* mutation, L265P, occurs in 29% of ABC DLBCL cases but is rare or absent in GCB DLBCL. This mutation affects an amino acid at the hydrophobic core of the MyD88 TIR domain, presumably altering its three-dimensional structure in a way that facilitates signalling. Among the many recurrent MYD88 mutations, MYD88 L265P occurs most frequently in ABC DLBCL, presumably because it is the most efficient mutant in activating downstream signalling pathways such as NF-κB. Subsequently, MYD88 L265P has been reported to be highly prevalent in several other subtypes of lymphoid malignancies, making it one of the most common gain-of-function mutations in these cancers.

MYD88 L265P coordinates a signalling complex involving the kinases IRAK4 and IRAK1, leading to IRAK4-dependent phosphorylation of IRAK1, which is a convenient biochemical assay for the activity of this pathway [10]. In addition, MYD88 L265P activates p38 MAP kinase, which together with NF-κB promotes the secretion of IL-6 and IL-10, which also provides survival signals in an autocrine fashion. In addition, MYD88 L265P promotes the secretion of type I interferon, which is actually toxic to ABC DLBCL cells. This turns out to be an 'Achilles heel' that can be exploited therapeutically using the drug lenalidomide (see succeeding text) [11].

Faced with the observation that cell line models of ABC DLBCL die upon inhibition of NF-κB signalling [5], we initiated a programme at the National Cancer Institute to conduct clinical trials of drugs targeting this pathway in DLBCL. When we first appreciated the importance of this pathway, the only clinically available drug was the proteasome inhibitor bortezomib, which inhibits NF-κB by blocking the proteasomal degradation of IkB We initiated a phase I/II clinical trial in relapsed refractory DLBCL combining bortezomib with dose-adjusted EPOCH chemotherapy because it is well known that NF-κB blocks the pro-apoptotic effects of chemotherapy. Overall survival was significantly prolonged in ABC DLBCL relative to GCB DLBCL, which was associated with a higher rate of complete and partial remissions in ABC DLBCL [12]. The combination of bortezomib plus multi-agent chemotherapy is now being tested in several randomized phase III trials in the USA and in Europe. However, given that bortezomib targets the proteasome and thus inhibits many intracellular pathways, it is will be important to develop more precise methods to target NF-κB in ABC DLBCL.

A more surgical way to inhibit NF-κB in ABC DLBCL emerged with the development of the Bruton agammaglobulinemia tyrosine kinase (BTK) kinase inhibitor ibrutinib. In the active form of BCR signalling, the kinase activity of BTK is essential for NF-κB activation, whereas this kinase is not required for tonic BCR signalling. Accordingly, knockdown of BTK kills ABC DLBCL cell lines with chronic active BCR signalling. Ibrutinib is an irreversible small molecule inhibitor of BTK kinase that is a potent killer of ABC DLBCL cell lines *in vitro* and in xenografts [6,11]. A pilot clinical trial was opened at the National Cancer Institute examining ibrutinib in patients with relapsed/refractory ABC DLBCL, as determined by molecular profiling. Both complete and partial responses were observed, including a complete response in one patient that has been sustained for 2.5 years by daily oral ibrutinib alone [13]. Importantly, ibrutinib has been very well tolerated with only infrequent grade three or four toxicities. On the basis of these encouraging results, a multicentre, phase 2 trial of ibrutinib in relapsed/refractory DLBCL was launched. Interim analysis shows a significant response rate in ABC DLBCL, with very little activity in GCB DLBCL, as hypothesized [13]. Importantly, complete and partial responses have been observed in patients with primary refractory disease who had never had a response to conventional chemotherapy. Future clinical trials will aim to combine ibrutinib with chemotherapy and with other more targeted agents based on a mechanistic understanding of pathological signalling in ABC DLBCL.

One such drug combination that appears promising in ABC DLBCL is the pairing of ibrutinib with lenalidomide, which exploits the concept of 'synthetic lethality'. In genetic studies of model organisms, synthetic lethality refers to the phenomenon that mutation of one gene can make cells vulnerable to inactivation of a second gene, even though neither gene alone is essential for survival. When applied to cancer therapy, synthetic lethality refers to the fact that an oncogenic lesion affecting one protein can render a cancer cell vulnerable to an inhibitor targeting a second protein or pathway. The value of this concept is that normal cells, lacking this oncogenic lesion, will not be affected by the inhibitor, thus providing a therapeutic window.

In ABC DLBCL, the MYD88 L265P mutation promotes secretion of type I interferon [10,11]. This propensity to make type I interferon is a liability to ABC DLBCL cells, because this cytokine is itself toxic for these cancer cells. The first hint was that the case came from investigations of lenalidomide, a drug approved for use in myeloma and myelodysplastic syndrome [11]. A phase II trial of lenalidomide in relapsed/refractory DLBCL showed that the drug induces complete and partial remissions, with most responses occurring in non-GCB DLBCL cases (including ABC DLBCL) [14]. Investigation of lenalidomide *in vitro* unexpectedly revealed that the drug induces a brisk production of interferon β and a consequent type I interferon response in ABC DLBCL cell lines [11]. This interferon

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production contributes to the toxicity of lenalidomide for ABC DLBCL lines because antibody blockade of the type I interferon receptor or knockdown of its subunits reduced this toxicity.

The importance of the type I interferon pathway in ABC DLBCL was also revealed by the analysis of the transcription factor IRF4, which is highly expressed in ABC DLBCL as a consequence of constitutive NF-κB pathway activation. RNA interference-mediated knockdown of IRF4 is toxic for ABC DLBCL cells and is associated with a type I interferon response. The mechanism behind this interferon response was revealed by ChIP-seq analysis of genomic IRF4 binding sites. IRF4 binds to many genomic loci in ABC DLBCL cells together with the ETS-family transcription factor SPIB, which is overexpressed in ABC DLBCL by translocation and chromosomal amplification [15]. IRF4 and SPIB directly repress the transcription of *IRF7*, which encodes the key transcription factor responsible for induction of type I interferon. In normal immune cells, IRF7 is recruited to the MYD88 signalling complex, where TRAF6 ubiquitination is required to make it transcriptionally active, and presumably, the mutant MYD88 isoforms in ABC DLBCLs would act similarly. In addition to downregulating type I interferon signalling, IRF4/SPIB promote NF-κB engagement by the BCR pathway by directly transactivating the *CARD11* gene. Thus, IRF4 promotes the survival of ABC DLBCL cells in two ways, by preventing the toxicity of interferon and by augmenting the anti-apoptotic NF-κB pathway.

The actions of lenalidomide and IRF4 on the interferon pathway are one and the same phenomenon, because lenalidomide treatment of ABC DLBCL cells downregulates both IRF4 mRNA and protein [11]. Indeed, ectopic expression of IRF4 prevents the killing of ABC DLBCL cells by lenalidomide. The action of lenalidomide on IRF4 depends on its ability to inhibit a ubiquitin ligase containing the protein cereblon. Accordingly, cereblon depletion by RNA interference decreases IRF4 mRNA and protein levels in ABC DLBCL.

Because IRF4 is a direct NF-κB target gene, inhibition of NF-κB activation by the BCR pathway should synergize with lenalidomide, which blocks IRF4 expression by a different mechanism. As predicted, ibrutinib and lenalidomide display synergistic toxicity for ABC DLBCL lines, both *in vitro* and *in vivo* in xenografts [11]. On the basis of these pre-clinical results, a trial of ibrutinib plus lenalidomide is rational in relapsed/refractory ABC DLBCL.

Given the promising early clinical trial results of ibrutinib in ABC DLBCL and its outstanding safety profile, it is likely that ibrutinib will serve as a platform for the development of combination therapies for this lymphoma subtype. The key to rapid progress towards the most effective combinations will be a deep mechanistic understanding of the interconnections between the variety of signalling pathways that sustain proliferation and survival of ABC DLBCL cells.

Conflicts of interest

The author has no competing interest.

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