REVIEW ARTICLE

Evolving insights into the genomic complexity and immune landscape of diffuse large B-cell lymphoma: opportunities for novel biomarkers

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Abstract

Recently, comprehensive genomic analyses have allowed a better molecular characterization of diffuse large B-cell lymphoma (DLBCL), offering novel opportunities in patient risk stratification and management. In the era of precision medicine, this has allowed us to move closer toward a more promising therapeutic outcome in the setting of DLBCL. In this review, we highlight the newly reported heterogeneous mutational landscapes of DLBCL (from two whole-exome sequencing studies, and from a more recent work targeting a 293-gene of a hematologic malignancy-designed panel. Altogether, these studies provide further evidence of the clinical applicability of genomic tests. We also briefly review established biomarkers in DLBCL (e.g., MYC and TP53), and our understanding of the germinal center cell reaction, including its epigenetic regulation, emphasizing some of the key epigenetic modifiers that play a role in lymphomagenesis, with available therapeutic targets. In addition, we present current data regarding the role of immune landscapes in DLBCL (inflamed versus non-inflamed), how the recently defined molecular DLBCL subtypes may affect the cellular composition of the tumor microenvironment and the function of the immune cells, and how this new knowledge may result in promising therapeutic approaches in the near future.

Standard of care and classic prognostic factors

Despite the availability of several prognostic tools to predict response to treatment, efforts to tailor therapeutic interventions for specific subtypes of diffuse large B-cell lymphoma (DLBCL) have yielded limited success, and rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) is still considered the frontline therapy across most DLBCL subtypes, with relatively few exceptions [1–5]. Over the past three decades, outcome prediction methods in DLBCL have utilized a diverse array of data sources, including clinical and laboratory factors (i.e.,: cell-of-origin (COO) and genetic subgroups). The two

prognostic factors classically applied in DLBCL include the international prognostic index (IPI) and the COO. MYC as a prognostic biomarker, will be discussed later in the manuscript.

The international prognostic index (IPI)

Developed more than 25 years ago using stepwise regression analysis, it provides risk assessment based on five clinical characteristics (age, stage, lactic dehydrogenase (LDH) level, performance status, and number of extra-nodal sites) [6, 7]. Although the IPI constitutes an accessible clinical tool to effectively predict survival, one of its limitations is its inability to identify targetable vulnerabilities to guide the use of individualized therapy [8].

Cell-of-origin (COO)

In an effort to understand the heterogeneity of DLBCL, gene expression profile (GEP) studies, using an early genomic technology "the DNA microarray", were performed almost 20 years ago on a large cohort of DLBCL patient samples [9]. Clustering of these GEP data revealed two dominant molecular subgroups, germinal center B-cell

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(GCB)-like and activated B-cell (ABC) type, with roughly equal frequencies. The GCB-like subgroup is marked by expression of genes commonly found in GC B-cells, such as amplification of the *c-REL* gene on chromosome 2p [10], in addition to recurrent t(14;18) translocations; and lack of expression of early post-GC markers [9, 11, 12]. In contrast, the ABC-like subgroup expresses genes characteristic of circulating B-cells that have been acutely stimulated through CD40, notably including many NF-κB target genes [9, 12]. The importance of this study resides in predicting the outcome of patients following R-CHOP regimen, as it appears to be less favorable in ABC than GCB-DLBCL (~40% versus ~75% 3-year progression-free survival (PFS), respectively) [13]. However, the prognostic value of the COO classification has not been uniformly reproducible [14]. This suggested that residual diversity within these two subsets exists, and carries valuable prognostic information [14, 15]. In addition, data from two prospective DLBCL trials found no significant differences in survival between ABC and GCB DLBCL, but confirmed the predictive value of MYC/BCL-2 expression [16]. Furthermore, the MYC/BCL-2 status, but not the COO classifier, was recently associated with an increased risk of CNS relapse in de novo DLBCL treated with R-CHOP [17]. Nevertheless, COO types have been officially incorporated as molecular subtypes of DLBCL-NOS in the most recent revision (2017) of the WHO classification of lymphoid neoplasms [18]. Immunohistochemical (IHC) algorithms that function as surrogates for GEP are frequently used in clinical settings, the most popular one has been proposed by Hans et al. [19], and uses the expression of CD10, MUM/IRF4, and BCL-6. Recent advances in technology have improved the use of formalin-fixed paraffin-embedded (FFPE) tissue to apply GEP approaches, such NanoString, for a more reliable COO assignment [20–22]. In light of the recently described novel mutational subtypes of DLBCL (see below), we believe that the clinical use of the COO classification will be limited in the near future.

The germinal center reaction

Somatic hypermutation (SHM) of the variable (V) regions of the Ig heavy and light chain genes of B cells occur in the GC, and result in BCR diversity with varying antigenic affinities. Based on the affinity of BCR for these antigens, B-cells are primed to generate plasma cells and memory B-cells. Class switch recombination (CSR) is a process by which the heavy chain class of an antibody produced by a GC B-cell clone changes from IgM to IgG, IgA, or IgE. CSR has been shown to be triggered prior to differentiation into GC B-cells or plasmablasts, and seems to be greatly diminished in GCs [23]. Both SHM and CSR are mediated by activation-induced cytidine deaminase (AID) [24, 25]. AID can also target other regions in the genome, triggering mutations or chromosome translocations, with major implications on oncogenic transformation.

GCs are polarized into two distinct microenvironments, the dark and light zones. The dark zone is densely packed with centroblasts, dividing rapidly and undergoing SHM. In the light zone, B-cells undergo a process of selection where GC B-cells with increased antigen affinity are selected over those with lower affinity. The selection process includes reentry into the dark zone for further rounds of mutation and selection.

There is evidence indicating that processes involved in GC biology are deregulated in DLBCLs, resulting in failure to activate GC exit programs. Some of the main regulators of the GC reaction, which are key players in the pathobiology of DLBCL, are:

BCL-6, a master regulator of the GC reaction acts as a transcriptional repressor [26] and is strongly upregulated by GC B-cells (and GC T-cells). BCL-6 coordinates a gene expression program that blocks B-cell activation and plasmacytic differentiation (silencing *PRDM1*), and establishes the hyperproliferative status of GC B-cells in the dark zone, while allowing them to tolerate DNA breaks associated with SHM, without eliciting cell-cycle arrest and apoptotic responses. It cooperates with the histone methyltransferase enhancer of zeste homolog 2 (EZH2) to maintain many gene regulatory elements in a bivalent chromatin state, thereby enforcing the GC phenotype while allowing signal-induced activation of certain genes [27]. *BCL6* translocations occur in both ABC and GCB DLBCLs, although more often in the ABC subtype (24%) than in the GC subtype (10%) [28].

MYC is characterized by a bimodal pattern of expression in the setting of the GC reaction: upon contact of antigen-exposed B-cells with T-helper cells in the light zone, MYC is expressed for a short period, allowing B-cell recirculation back to the dark zone for further affinity-based positive selection [29–31]. In the dark zone, activity of AID ensures an additional round of SHMs, however BCL-6 upregulation directly inhibits MYC expression [32]. The balancing of MYC levels in normal GC seems to be essential to reduce the number of B-cell divisions, proper migration between light and dark zone of GC, and affinity maturation [33, 34]. B cells which do not express MYC in the light zone, continue their differentiation pathway to either memory cells or plasmablasts. Plasmablasts' conversion to plasma cells is mediated by the powerful MYC repressor, BLIMP1 [35]. Conversely, B-cells with MYC overexpression undergo multiple cycles of dark zone reentry, leading to cellular proliferation imbalance (Fig. 1).

NF-kB signaling constitutive activation is the hallmark of ABC-DLBCL [36]. CD40:CD40L interaction in the light zone results in the activation of NF-kB and upregulation of
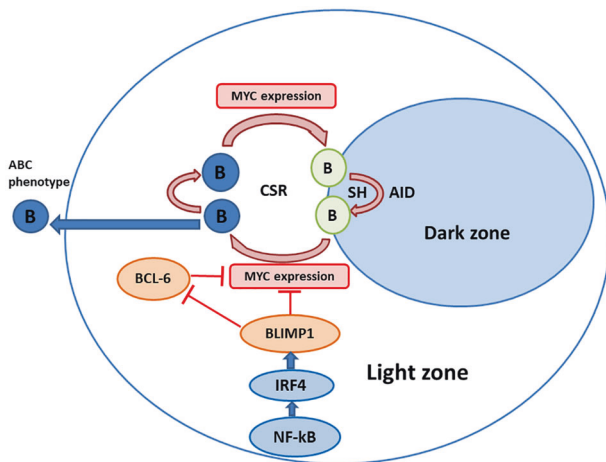
Fig. 1 The role of MYC expression in terminal B-cell differentiation. BCL-6 upregulation directly inhibits MYC expression, which reduces the number of B-cell divisions, through controlled migration between light and dark zone of GC, and affinity maturation. Activation of NF-kB results in upregulation of IRF4, which is critical for the induction of BLIMP1 and termination of the GC cycle, through the suppression of MYC expression and BCL-6. AID activation-induced cytidine deaminase, SH somatic hypermutation, CSR class switch recombination, GC germinal center, ABC activated B-cell.

IRF4, which is critical for terminal B-cell differentiation [37]. Expression of IRF4 is essential for the survival of ABC-DLBCL cells, and is responsible of the plasmacytic phenotype seen in this DLBCL subset [12]. IRF4 can repress *BCL6* through the induction of BLIMP1, encoded by *PRDM1* gene [38] (Fig. 2). Consequently, the phenotype of ABC-DLBCL is likely to mirror that of plasmablasts. However, several genetic events in ABC-DLBCL block full plasmacytic differentiation by reducing expression of BLIMP1. These include genetic events that inactivate PRDM1 itself [39], as well as translocations or amplifications targeting *BCL6* and *SPIB*, both of which repress *PRDM1* transcription [40].

Epigenetic regulation of the GC through EP300, CREBBP, and EZH2 are able to quickly accommodate and coordinate responses to microenvironmental signals. This reprograming process requires the activity of histone/chromatin modifying enzymes, catalyzing the deposition of specific histone marks associated with open or closed chromatin. Bivalent chromatin markers play a key role in establishing the GC B-cell phenotype, and preventing their premature differentiation to plasma cells [41]. Alteration of bivalency at poised promoters affects normal GC B-cell plasma cell differentiation, and results in potential malignant transformation [41]. The most frequently somatically mutated epigenetic modifiers in DLBCL include EP300, CREBBP, and EZH2, among others.

EP300 and CREBBP encode wide-ranging expressed enzymes, that in turn act as global transcriptional co-activators by interacting with more than 400 transcription factors, and by catalyzing the modification of lysines on both histone and nonhistone proteins [42, 43]. In the GC B-cells, there are two critical nonhistone substrates of CREBBP and EP300-mediated acetylation: the tumor-suppressor gene *TP53*, which requires acetylation for its transcriptional activity [44] and the proto-oncogene *BCL6*, a potent transcriptional repressor that is impaired by CREBBP and EP300-mediated acetylation [45]. By catalyzing H3K18 and H3K27 acetylation at promoter and enhancer regions, CREBBP modulates the expression of a selected number of genes that are implicated in GC exit, including signaling pathways triggered by engagement of the BCR and CD40 receptor, the plasma cell regulator IRF4, and antigen processing and presentation through the major histocompatibility complex class II (MHC-II) complex [46]. The GC-specific CREBBP transcriptional network encompasses almost all BCL-6 direct target genes, suggesting a critical role for this acetyltransferase in opposing the oncogenic activity of BCL-6, while ensuring the rapid activation of programs that sustain terminal differentiation in the GC light zone [47]. *EP300* and *CREBBP* mutations result in either loss of function or dominant negative effects, that in turn lead to failure to induce acetylation of BCL-6 target enhancers, preventing termination of the *BCL6* transcriptional program, and favoring the constitutive activity of the *BCL6* oncogene at the expense of the *TP53* tumor-suppressor gene [43] (Fig. 2).

EZH2 and BCL-6, form part of the epigenetic switches that control the GC reaction. EZH2 creates bivalent promoters that control the transcription of genes involved in the negative regulation of cell cycle (CDKN1A and B) and in terminal B-cell differentiation (IRF4, PRDM1). It transiently places the B-cell differentiation program into a poised bivalent state, allowing B-cells to proliferate and undergo SHM. The termination of EZH2 activity is required for B-cells to exit the GC reaction and undergo terminal differentiation. This occurs through two players, CD40 and BCR signaling which strongly induce activation of differentiation, thus terminating the EZH2 poising effect, and presumably switching bivalents promoters to an active state [48]. BCL-6 forms a complex with SMRT at active enhancers (marked with H3K4me1) causing their H3K27 deacetylation through HDAC3 and placing them in a repressed/poised configuration. Both EZH2 and BCL-6 switches result in a transcriptional repression of genes that define the centroblast phenotype. Missense mutations of *EZH2* occur in 25% of patients with follicular lymphoma [49, 50], 22% of patients with GC-DLBCL, and are absent in ABC-DLBCL [51]. These mutations are always heterozygous and the vast majority introduce changes at the tyrosine residue 641 [52]. They result in an enzymatic gain of function that increases H3K27me3 at target gene promoters,

Fig. 2 NF-Kb and CREBBP effects on terminal B-cell differentiation and GC exit. Activation of NF-kB leads to upregulation of IRF4, which is critical for terminal B-cell differentiation. IRF4 can repress BCL-6 through the induction of *BLIMP1*, which is required to initiate the plasma cell program. In a physiologic state, BCL-6 function is impaired by CREBBP-mediated acetylation. *CREBBP* mutations result in either loss of function or dominant negative effects, that in turn lead to failure to induce acetylation of BCL-6 target enhancers, preventing the termination of *BCL6* transcriptional program. GC germinal center.
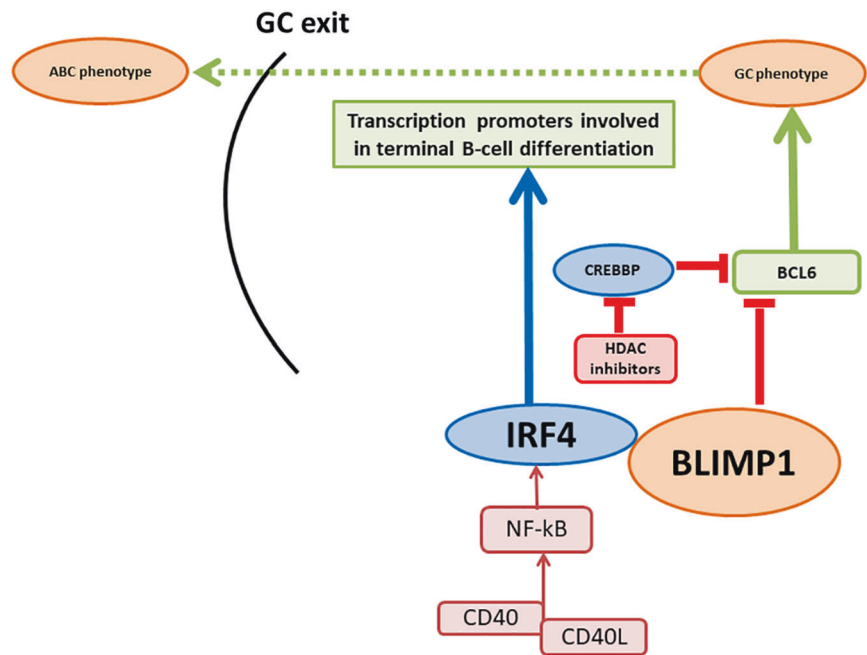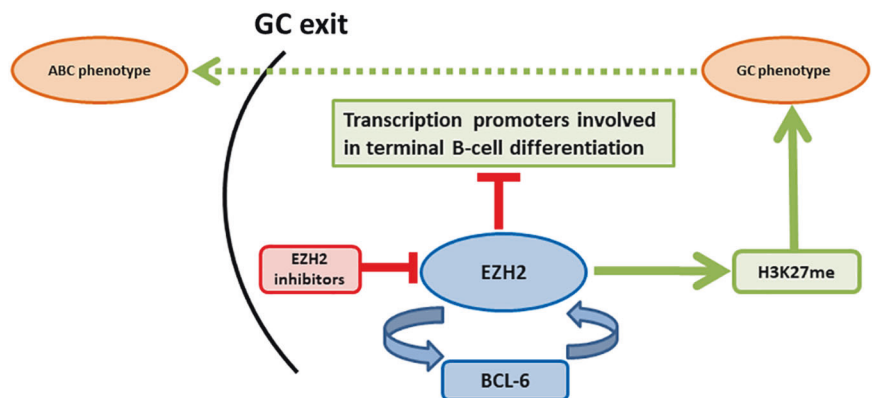

Fig. 3 The role of EZH2 and BCL-6 interaction in terminal B-cell differentiation suppression. BCL-6 cooperates with the histone methyltransferase EZH2 to maintain many gene regulatory elements in a bivalent chromatin state, thereby enforcing the germinal center (GC) phenotype while allowing signal-induced activation of certain genes. GC germinal center.

causing their transcriptional repression, and imposing a centroblast transcriptional program (Fig. 3).

Established prognostic biomarkers in DLBCL

MYC deregulation in DLBCL contributes to the maintenance and disease progression, and is often the result of gross genetic abnormalities, including copy number alterations, chromosomal translocations, increased enhancer activity through aberrant signal transduction leading to increased MYC transcription, or increased MYC mRNA, and protein stability.

The incidence of *MYC* gene rearrangement (GR) in DLBCL is ~12% [53–63]. *MYC* translocation, termed "single hit", lymphoma has been associated with inferior outcomes [55]. In addition, increased *MYC* copy numbers or

MYC gene amplification has been shown to be an independent poor prognostic factor in DLBCL [64, 65], although a recent large retrospective study (9715 cases) has shown that *MYC* amplification did not bear prognostic significance [66].

In contrast to *BCL2* translocations that seem to occur mostly in GC lymphomas as an early event [67, 68], *MYC* translocations appear to be a secondary event, and can be associated (~8% of the cases) with translocations involving *BCL2* and/or *BCL6* genes [69, 70]. These lymphomas, with concurrent rearrangements involving *MYC* and *BCL2* and/ or *BCL6*, previously referred to as double or triple hit lymphomas (DHL/THL) [71], have been shown to portend an aggressive clinical course [62, 70] and were incorporated in the 2017 revised edition of the WHO Classification under the name "high-grade B-cell lymphomas with rearrangements involving *MYC* and *BCL2* and/or *BCL6*" [18].

Approximately 65% of patients categorized as DHL harbor translocations in *MYC* and *BCL2*, 14% demonstrate translocations involving *MYC* and *BCL6*, and the remaining 21% of patients have all three rearrangements [72]. DHL and THL seem to overwhelmingly affect the GC subtype (in ~80–90% of cases) [60, 62, 73]. Lymphomas with both *MYC* and *BCL6* translocations (*MYC/BCL6*) are likely different biologically from those with *MYC* and *BCL2* rearrangements (*MYC/BCL2*) [71], as they occur in the ABC subtype, are more likely to exhibit immunoblastic morphology, and have less frequent *TP53* mutations and less cytogenetic complexity [71, 74]. *MYC/BCL6* lymphomas are also clinically distinct from their *BCL2* rearranged counterparts [74]. Some authors suggested that cases with *MYC/BCL6* rearrangements seem to have a significantly worse overall survival (OS) in comparison with *MYC/BCL2* cases, after the exclusion THL cases [72, 75], although this finding has not been uniformly reproducible [73].

Recent studies have identified a DH gene expression signature (DHITsig) in a subset of aggressive DLBCL that do not harbor *MYC* and *BCL2* GRs, by analyzing RNA sequencing data from 157 de novo GCB-DLBCLs, including 25 with HGBL-DH/TH-BCL2 [76]. This DHITsig was shared with the majority of B-cell lymphomas with high-grade morphology tested [76]. A NanoString assay ("DLBCL90") that recapitulated the GEP signature of HGBL-DH/TH-*BCL2* has since been developed allowing further identification of this aggressive subset of DLBCL on routinely available FFPE biopsy specimens [76].

The impact of *MYC* rearrangement on prognosis is influenced by *MYC* partner gene (i.e.,: immunoglobulin "*IG*" or a *non-IG* gene) [77]. An early study demonstrated *MYC* GRs in 51/574 patients, where the *MYC* translocation partner was an *IG* gene in 24 cases (*MYC-IG*) and a *non-IG* gene (*MYC-non-IG*) in 26 of 50 evaluable cases [63]. This study showed that *MYC-IG* patients had shorter OS compared with *MYC*-negative patients, whereas no survival difference was observed between *MYC-non-IG* and *MYC*-negative patients [63]. Based on these results, identifying whether *MYC* partner gene is one of the *IG* genes was recommended [63]. The impact of *MYC* partner gene on prognosis has been recently reemphasized by Rosenwald et al., who evaluated a large cohort of patients through the Lunenburg Lymphoma Biomarker Consortium [77]. The authors found that patients with DHL or THL in which *MYC* is translocated to an IG partner have a worse prognosis, and suggested that diagnostic strategies should be adopted to identify this high-risk cohort [77].

Several studies have found that an increase in MYC and BCL-2 protein expression in DLBCL also represented an unfavorable subgroup with inferior outcomes after standard frontline therapy and autologous stem-cell transplantation [16, 62, 78]. Nevertheless, recent observations from the GOYA trial have shown no association between MYC/BCL-2 dual expression and disease relapse [79, 80]. Cases with MYC/BCL-2 dual expression have been termed double expressor lymphoma (DEL). The IHC threshold of ≥40% for MYC and >50% for BCL-2 is used to define DEL [18]. Using cutoff values of >70% for MYC and >50% for BCL-2, Ziepert et al. identified an isolated group of patients with a significantly inferior clinical course [81]. In addition, this threshold of >70% MYC expression was also better in predicting the presence of MYC GRs (88% of cases), and generally more reproducible among hematopathologists [81]. Nevertheless, DEL status is not considered a surrogate to DHL (or vice versa), as DEL and DH disease are not identical or even strongly overlapping groups (at least with the current WHO criteria) [8]. In fact, unlike the case for DHL/THL, DEL seems to occur more often in non-GC DLBCL (~63%) compared with GCB DLBCL (~37%) [62]. In the absence of chromosomal translocations, MYC and BCL-2 overexpression is likely attributable to gene amplification and post-translational processes [59, 82, 83]. On the other hand, up to 20% of patients with DHL do not demonstrate overexpression of MYC and BCL-2 at a protein level, and show improved outcomes when compared with those with DHL with concurrent dual protein expression [58, 78].

TP53 mutations are independent poor prognostic markers in the setting of DLBCL [84]. In addition, they are identified in 20–30% of patients with DHL, and are thought to be negative prognostic markers [85]. The occurrence of *TP53* mutations seems to be more common in *MYC/BCL2* DHL, in comparison with DHL with *MYC/BCL6* rearrangements and classic DLBCL [85]. In addition, *TP53* mutations may be synergistic with *BCL2* translocations through inhibition of apoptosis, conferring a competitive advantage in clonal evolution [85]. DLBCL cases with *MYC* rearrangements and *TP53* mutations demonstrate worse survival than patients with *MYC/BCL2* DHL, suggesting the need to evaluate *TP53* mutations in cases of *MYC*-rearranged DLBCL [86]. TP53 overexpression, defined as ≥50% positive cells, was seen in 33% of evaluated cases in one study, and seems to be associated with a negative prognosis, especially in DLBCL with *MYC* rearrangements, MYC overexpression, and DEL [87].

New genetically defined DLBCL subsets/clusters

The pronounced genomic heterogeneity in DLBCL has been recently scrutinized using comprehensive multiplatform genomic analyses.

A study by Schmitz et al. integrating whole-exome and transcriptome sequencing, array-based copy number

analysis, structural variants, and targeted amplicon re-sequencing has identified four genomic subtypes in DLBCL characterized by [1] *CD79B/MYD88L265P* double mutations (MCD subtype), [2] *NOTCH2* mutations or *BCL6* fusions in ABC or unclassified DLBCL (BN2 subtype), [3] *NOTCH1* mutations (N1 subtype), and [4] *EZH2* mutations or *BCL2* translocations (EZB subtype) [14]. These subtypes had prognostic relevance even after accounting for COO assignment, with inferior responses found in patients with *CD79B/MYD88L265P* double mutations (MCD subtype) and *NOTCH1* mutations (N1 subtype) [14]. It is worth mentioning that these genomic subtypes represented less than half of the investigated cases, suggesting the presence of a distinct subset with genomic diversity in the remaining patients [14]. An additional comprehensive genomic analysis of a large cohort of untreated DLBCL patients by Chapuy et al. [15] identified specific genomic subsets of DLBCL as follows [1]: high-risk ABC DLBCLs with near-uniform *BCL2* copy gain, frequent activating *MYD88L265P, CD79B* mutations, and extra-nodal tropism (cluster 5) [2]; low-risk ABC DLBCLs with genetic features of an extra-follicular, possibly marginal zone, origin (cluster 1) [3]; high-risk GC DLBCLs with BCL2 structural variants, inactivating mutations and/or copy loss of *PTEN* and alterations of epigenetic enzymes (cluster 3) [4]; a newly defined group of low-risk GCB DLBCLs with distinct alterations in JAK/STAT and BRAF pathway components and multiple histones (cluster 4); and [5] an ABC/GCB-independent group of tumors with bi-allelic inactivation of *TP53, 9p21.3/CDKN2A* copy loss and associated genomic instability (cluster 2). Significant differences in PFS were identified in these clusters, with a significantly higher risk of relapse in cluster 5 ABC-DLBCL and cluster 3 GCB-DLBCL [15].

These recent comprehensive analyses have shed light on the previously appreciated genomic complexity of DLBCL, the limitations of gene expression-based classification systems, and challenges in adopting a uniform treatment approach. Nevertheless, they have also opened doors to experiment with novel therapeutic strategies, based on multi-genomic signatures. A randomized phase III study evaluated ibrutinib and R-CHOP in untreated non-GC DLBCL, and found that in patients age younger than 60 years, ibrutinib plus R-CHOP improved event-free survival, PFS, and OS with manageable safety [88]. Although, the genetic data from the PHOENIX trial is not published yet, these findings support the notion that patients with *MYD88L265P* and *CD79A* or *CD79 B* mutations can possibly benefit from adding ibrutinib to R-CHOP.

MYD88L265P and *CD79B* mutations and amplifications enriched in the MCD subtype, are the hallmark of extra-nodal lymphomas, including primary central nervous system lymphoma, primary testicular lymphoma, primary breast lymphoma, primary cutaneous lymphoma, and intravascular lymphoma. The MCD subtype is characterized by BCR-dependent NF-KB activity and abrogation of immune surveillance inactivating class I HLA genes or CD58 [14]. The genetic basis for the dysregulation of BCL2 in this group is gains in 18q, which increases expression of transcription factor TCF4 (E2-2), that in turn, activates IG μ and MYC [89]. The 5-year survival for the MCD subtype (using R-CHOP) is 26%.

Another high-risk group is cluster 3, which is enriched in GCB-DLBCL [15]. This group exhibits frequent inactivating mutations and/or copy loss of *PTEN* and additional mutations of *GNA13* and *HVCN1* that likely increased BCR/PI3K signaling. *BCL2* translocations are the genetic bases for the dysregulation of BCL-2 in this group. Pre-clinical studies have provided evidence supporting the combination of PI3Kα/δ and BCL-2 inhibitors and have set the stage for clinical trials using copanlisib (PI3K inhibitor with predominant α/δ activity) and venetoclax in patients with cluster 3 DLBCL [90]. Note that venetoclax, as a single agent, has limited activity in both DLBCL and FL regardless of BCL-2 status. In addition, a phase II Trial of Tazemetostat demonstrated an objective response rate of 29% in DLBCLs with *EZH2* mutation (*EZH2mut*) and 15% in DLBCLs with wild-type *EZH2* [91].

Recently, Wright et al. have segregated two additional groups "A53" and "ST2", from the genetically unassigned cases of their previous cohort [92]. The new A53 group is characterized by aneuploidy and TP53 inactivation, and the ST2 group is enriched with mutations involving *SGK1* and *TET2* genes. In addition, the EZB group was subdivided into two subgroups EZB-MYC+ (with an inferior outcome) and EZB-MYC-negative. EZB-MYC+ was found to be enriched in aberrations in MYC, and four other genes that are frequently mutated in Burkitt lymphoma [92]. Of note, not all EZB-MYC+ cases were "double hit", only 38% of these cases had a MYC abnormality, suggesting cryptic genetic abnormalities [93, 94] or other genetic mechanisms enhancing MYC function. Among the non-EZB GCB cases the DHIT signature was not associated with adverse outcome. These data further support the notion that current diagnostic modalities available in daily practice could be missing a subset of aggressive DLBCL cases that are likely to require a different treatment than R-CHOP.

It is worth mentioning that these studies, although exhaustive, endorsed contradictory data. For example, cluster 3 which contains *EZH2mut* was considered poor prognosis in one study [15], whereas in other studies *EZH2mut* were associated with better prognosis [14, 95]. Similarly, the C5 and MCD clusters were associated with poorer prognosis according 2 studies [14, 15], however, *MYD88* mutations which are enriched in these clusters, were associated with a better prognosis in another study

[95]. In addition to these conflicting observations, limitations of survival models adopted by these investigations are noted. For example, these clustering analyses did not evaluate the contribution of each additional gene to the survival prediction, nor the superiority of the cluster to the prognostic power of single genomic abnormalities within the cluster (e.g., was the poor prognosis in cluster 2 a cluster effect or simply driven by cases with *TP53mut* within the cluster). This endpoint on the other hand, is usually achieved in survival modeling using one of several criteria (e.g., AIC, Harrel C index, Brier score, etc.).

A recent study by Lacy et al. investigated the clinical value of targeted sequencing, and subsequent categorization of DLBCL cases [96]. The authors performed targeted sequencing (using a 293-gene of hematologic malignancy-designed panel) on a large "unselected" patient cohort, with clinical follow-up. Their research identified three molecular subtypes that recapitulated the studies by Chapuy et al. [15] and Schmitz et al. [14]: BCL2, NOTCH2, and MYD88, with good, intermediate, and poor prognosis, respectively. They also described a TET2/SGK1 and SOCS1/SGK1 subtypes. The latter, demonstrated a biological overlap with primary mediastinal B-cell lymphoma (PMBL) and correlated with an excellent prognosis. This study confirmed the prognostic value of genomic testing of DLBCL cases in clinical settings, suggesting the standardization of proposed subtypes, for a swift transition into clinical implementation.

Immune landscapes of DLBCL

Tumor microenvironment (TME) has been recently increasingly recognized as a biomarker for checkpoint inhibitor therapy, especially in cases of "inflamed lymphomas" (lymphomas with a prominent inflammatory component), such as classic Hodgkin lymphoma (CHL) and PMBL [97]. TME surrounding lymphoma cells, is composed of a variable number of immune cells (T-, NK-, and B-cells as well as macrophages) and stroma (blood vessels and extra-cellular matrix) [15, 98].

Immune landscapes in DLBCL appear to be heterogeneous and could be modulated by intrinsic molecular/genetic features of the neoplastic cells (discussed below), but also by other factors, such as the immunological status of the patient, previous or current therapy, and Epstein–Barr virus (EBV) infection. EBV infection seems to promote an inflamed environment [99–101] and provides a source of foreign antigen for T-cell recognition in the host [97]. In fact, EBV-positive DLBCL is notorious to express well-defined immunogenic viral antigens, in addition to PD-L1 upregulation, which seems to be particularly associated with a decreased response to frontline therapy [102, 103]. There

is some evidence suggesting that EBV infection may play a role in the transformation of follicular lymphoma into DLBCL. This occurs in part by inducing AID activity, resulting in genomic instability, in addition to generating changes in the lymphoma microenvironment [99, 104].

The TME in DLBCL could be categorized as "inflamed" (with two main subtypes: immune suppressed and immune evasion) and "non-inflamed" or "immune excluded". The immune suppressed microenvironment refers to the presence of immune cells with immunosuppressive functions or with an exhausted phenotype; whereas the immune evasion phenotype refers to the presence of certain mechanisms exploited by the tumor cells in order to escape detection by the immune system. It is worth mentioning that these variable immune landscapes are not equally represented among cases of DLBCL, as the majority of DLBCLs seem to have a "non-inflamed" landscape, and a small subset of cases might be distinguished by an "inflamed" phenotype, through the presence of certain genetic and microenvironmental features [97, 98].

Genetic signatures associated with the "inflamed" immune landscape of DLBCL

The molecular prototypes of the inflamed immune landscape are group C1 [15] and clusters BN2 and N1 [14], which seem to be dominated by the ABC subtype. C1 DLBCLs were found to harbor alterations in genes important for immune surveillance, such as inactivating mutations of *B2M, FAS, CD70*, and recurrent *PD-L1* structural variations (SVs), in addition to alterations in NF-kB pathway members BCL10 and TNFAIP3 [15]. Compounded with this, are frequent mutations seen in NOTCH2 pathway [15], that may contribute alongside with NF-kB pathway, to create an inflammatory immune landscape in this subgroup, even though the underlying mechanisms are still poorly elucidated [97]. As observed in solid tumors [105], NF-kB activation is thought to result in secretion of chemokines by lymphocytes, leading to enhancement of lymphocytic mobilization [97]. The BN2 cluster of DLBCLs [14] seems to harbor recurrent alterations similar to the C1 group of DLBCL [15], such as *BCL6* fusions, *NOTCH2, TNFAIP3*, and *BCL10* mutations, in addition to mutations of immune regulator *CD70*, which contribute to immune escape mechanisms (see below). The N1 cluster with NOTCH1 mutations was also found to harbor an immune-linked genetic signature, recapitulating the genetic map of an inflammatory TME [14]. The MCD subtype is characterized by BCR-dependent NF-kB activity and an inflammatory landscape, with potential abrogation of immune surveillance through inactivation of class *I HLA* or *CD58* genes [14].

Inflamed immune landscapes in lymphomas, as well as in other tumors, are associated with mechanisms that suppress

antitumor immune responses. These mechanisms can be divided into two main types: immune suppression or immune evasion. However, both of these mechanisms can overlap in certain cases of lymphomas.

Immune suppressed mechanisms are exemplified by the sustained inhibition of synapses of T-cells, leading to T-cell exhaustion and repression of function (Fig. 4); and immune evasion mechanisms are characterized by decreasing immunogenicity through upregulation of PD-1/PD-L1 immune evasion pathway and decreased expression of /loss of HLA expression (Fig. 5).

Mechanisms of immune suppression

In this scenario, the acquisition of an inflamed landscape leads to accumulation of high numbers of immune suppressive cells, inducing exhaustion (a state of dysfunction, where the differentiation, proliferation, and effector function of T-cells are suppressed) (Fig. 4). This is caused by sustained expression of inhibitory receptors, such as programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3 (LAG3), and T-cell IG and mucin-domain containing 3 (TIM3) on the surface of T-cells [106]. The genetic signature of the MCD group and cluster C5 DLBCLs harboring *CD79B* and *MYD88* mutations, although enriched for NF-kB activation, are notorious for the absence of genetic alterations normally associated with an increased immune capacity [14], which is in keeping with an immune suppressed landscape of DLBCL. In fact, NF-kB activation has been correlated to an inflamed microenvironment (as seen in "the inflammatory immune landscape of DLBCL" discussed above), however the

Fig. 4 Immune landscapes in DLBCL: inflamed phenotype, immune suppressed subtype. Mutations in *NFkB, NOTCH2,* and *NOTCH1* among others in DLBCL, contribute to excessive cytokine secretion, which in turn attracts T-cells, and enhances the inflammatory microenvironment. However, high numbers of suppressive immune cells inactivating class I HLA or CD58 in the tumor microenvironment, cause T-cell exhaustion.

downstream impact of this pathway seems to be heterogeneous in DLBCL. In addition, MCD DLBCLs harbor an "immune editing" capacity where the majority of these cases acquire mutation/deletion of HLA-A, HLA-B or HLA-C, and a subset acquire truncating mutations of the activator of NK-cells, CD58 [14] (Fig. 4).

Mechanisms of immune evasion

Unlike solid tumors, where genetic upregulation of PD-L1 occurs via IFN-γ production by surrounding T-cells [107], upregulation of PD-L1 expression in lymphomas is mediated by SVs either within the PD-L1 and PD-L2 loci [108–110], or within the 3′ un-translated region (UTR) of the *PD-L1* gene [111]. PD-L1 upregulation, although occasional in DLBCL, appears to be more common in EBV-positive and non-GCB DLBCL [112] and is found in C1 clusters of DLBCLs. In fact, EBV was found to enhance PD-L1 expression in CHL and other EBV-positive lymphomas through LMP1 activity in key PD-L1 driver pathways [113]. A study of 1253 patients reported PD-L1 expression by IHC in 11% of DLBCL cases [112]. PD-L1 SVs were detected in ~20–25% of DLBCL by FISH [109, 110], with a prevalence of *PD-L1* copy gains, in addition to *PD-L1* amplifications, chromosome 9 polysomy, and translocations involving *PD-L1* [110]. Expression of PD-L1 was found to be highest in DLBCL with *PD-L1* amplifications and translocations, and less frequent in cases lacking *PD-L1* SVs [110]. SVs within the 3′ UTR of the *PD-L1* gene appear to stabilize PD-L1 transcripts, leading to increased PD-L1 protein translation [111]; in addition, they are associated with CD8+ T-cell upregulation of the two key cytolytic effectors perforin and granzyme A, contributing to the "inflamed" phenotype of this subset of DLBCL [111, 114]. Patients with PD-L1-positive DLBCL have an inferior OS when compared with patients with PD-L1-negative DLBCL [112]. In addition, patients with PD-L1-negative DLBCL, with abundant PD-L1-positive non-malignant cells in the microenvironment (a phenomenon termed "microenvironmental PD-L1(+) DLBCL" or mPD-L1(+) DLBCL), have no significant difference in terms of OS when compared with mPD-L1(−) DLBCL [112].

HLA I and II expression on neoplastic cell surfaces is responsible for exposing tumor-derived peptide antigens. Loss of or decreased expression of these molecules in DLBCL have been reported to occur via genetic alterations involving mutations/deletions of *B2M* [115], deletion of 6p21.32 [116], alterations of *CD58* gene [115] and alterations in *CIITA* gene and *CREBBP* mutations [117]. The downstream effects of these genetic alterations leading to diminished/loss of HLA expression, seem to work synergistically with PD-L1 SVs to escape immune surveillance [110].
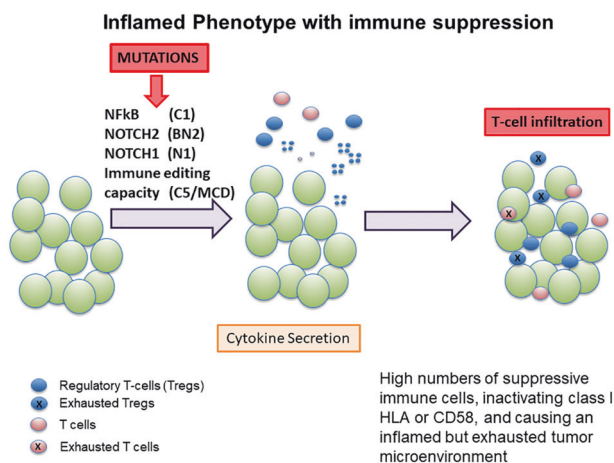
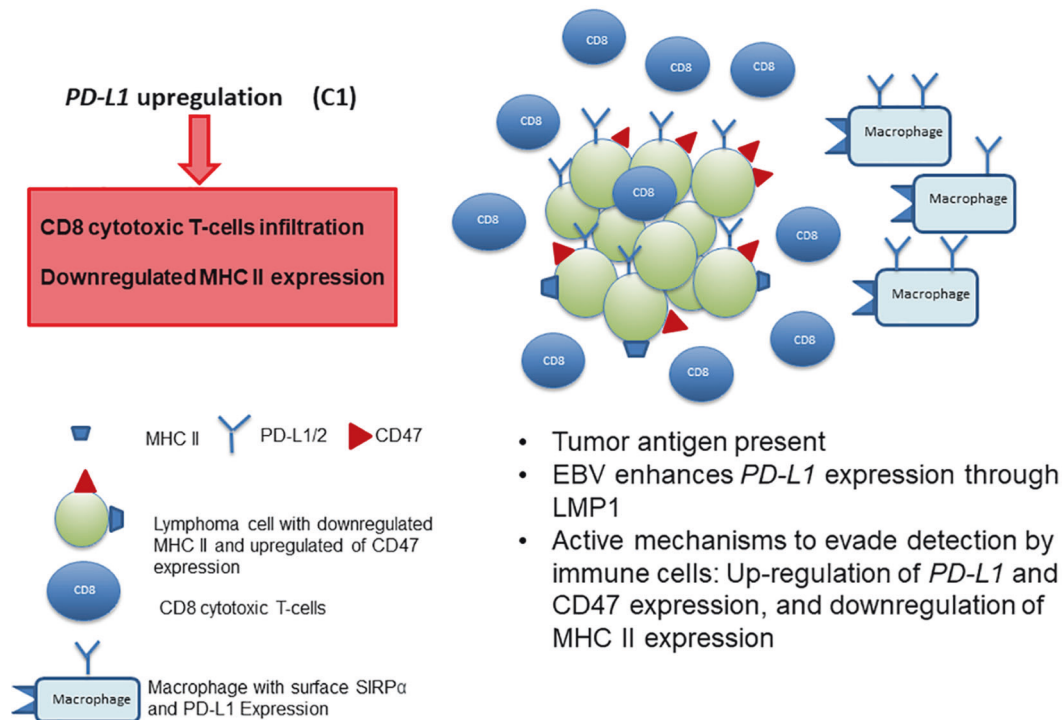## Inflamed Phenotype with Immune Evasion

Fig. 5 Immune landscapes in DLBCL: inflamed phenotype, immune evasion subtype. Active mechanisms to evade detection by the immune system include upregulation of PD-L1 and CD47 expression, and downregulation of MHC-II expression. EBV infection stimulates PD-L1 upregulation on the surface of lymphoma cells, leading to downregulation of MHC-II expression, and upregulation of CD8 cytotoxic T-cells, both of these mechanisms help neoplastic cells to escape immune cell controls. Upregulation of CD47 expression on the surface of lymphoma cells, help them escape phagocytosis via SIRPα, expressed by macrophages. Macrophages with surface PD-L1 upregulation, help neoplastic cells escape antitumor immunity.

Other mechanism of immune evasion involves tumor-associated macrophages (TAM), which constitute a complex system with two main players: pro-inflamatory M1 and pro-tumoral M2 macrophages [118, 119]. It has been shown in several studies that an increase in the M2 component of TAM, correlates with a poor prognosis in DLBCL [120, 121]. A recent investigation has shown that M2 macrophages were the most notable constituent of TME, in a series of 40 Burkitt lymphoma cases [122]. In this study, M2 macrophages demonstrated a high rate of PD-L1 expression, likely allowing tumor cells to escape immune control [122]. In addition, lymphoma cells seem to escape phagocytosis through enhanced CD47 (integrin-associated protein) expression, via interaction with SIRPα on the surface of macrophages, inhibiting phagocytosis [123, 124]. High CD47 expression in B-lymphomas was shown to portend an inferior clinical outcome in DLBCL patients treated with R-CHOP [125]. Inspired by these findings, clinical trials of CD47/SIRPα blockade therapy alone or in combination with antibodies that activate Fc-mediated phagocytosis have been initiated, with seemingly promising results [126] (Fig. 5).

Genetic signatures associated with the non-inflamed or immune excluded landscape of DLBCL

This category is notorious for scant to absent background immune cells, compounded by the absence of tumor neo-antigens (Fig. 6). These features can be the result of decreased aberrations in genes responsible for immune escape mechanisms, or due to high expression of molecular programs, precluding entry of immune cells into TME [76]. This is in addition to the high tumor proliferation rate, which creates an exclusively neoplastic milieu [97]. Immune excluded DLBCLs seem to be dominated by the GCB subtype that is enriched with the EZB group [14] or the C3/4 [15] groups. In fact, *EZH2* activating mutations have been shown to downregulate HLA expression in DLBCL [76, 127, 128]. Also, the EZB genome seems to be enriched in acquired mutations affecting the major histocompatibility complex (MCH) class II pathway genes CIITA and HLA-DMA [14]. In addition, DHL and THL GCB-DLBCL were shown to have a high incidence of mutations within chromatin-modifier genes, with a paucity
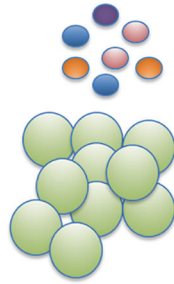
Non-inflamed or Immune Excluded Phenotype

(EZB or C3/4)

-Tumor with high proliferation rate
-Decreased mutations in genes responsible for immune escape
-High expression of genes preventing immune cells entry into TME

T-cells and other immune cells are kept outside the tumor

- Lack of neo-antigen
- Mutations in chromatin modifiers (*EZH2*, *KMTD2*, *CREBBP*, *EP300*)
- *BCL2* translocations
- Loss of 10q23 (PTEN) inactivation of *TNFRS14*

Fig. 6 Immune landscapes in DLBCL: non-inflamed or immune excluded phenotype. These tumors are characterized by low number of inflammatory cells admixed with the lymphoma cells. These features can be the result of high expression of specific molecular programs, precluding the entry of immune cells into the tumor microenvironment, in addition to inducing a high tumor proliferation rate, resulting in an exclusively neoplastic milieu. Immune excluded DLBCLs seem to be dominated by the GCB subtype, which is enriched with the EZB group or the C3/4 groups.
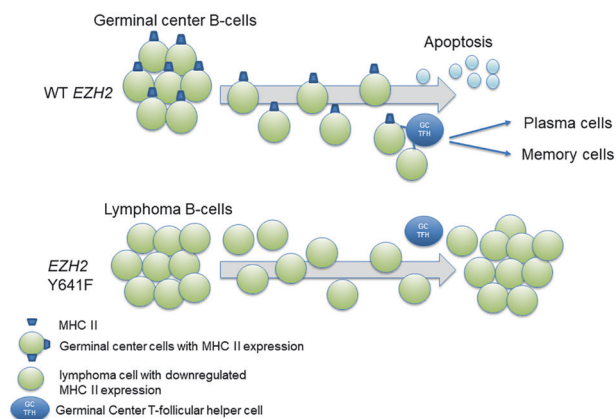
Germinal center B-cells

Apoptosis

WT *EZH2*

Plasma cells

Memory cells

Lymphoma B-cells

EZH2 Y641F

- MHC II
- Germinal center cells with MHC II expression
- lymphoma cell with downregulated MHC II expression
- Germinal Center T-follicular helper cell

Fig. 7 Mutant *EZH2* impairs dynamic expression of immune synapse genes. *EZH2* mutant centrocytes fail to productively engage T-cells. Consequently, *EZH2* mutant-germinal center B-cells entering the light zone gain a competitive advantage over wild-type *EZH2* centrocytes, and thus survive and expand regardless of their immunoglobulin status.

of infiltrating T-cells and a high incidence of low MHC-I and MHC-II expression [76].

TMEM30A mutations seem to correlate with concurrent loss of tumor-suppressor genes in chromosome 6q, and are uniquely found in DLBCL [129]. One study analyzed the biological mechanisms underlying the primary selection of B-cell lymphoma development, and detected a favorable outcome in patients with *TMEM30A*-mutated DLBCL. *TMEM30A* mutation was associated with macrophage

engulfment using CD47 blockade [129]. These findings suggest predictive value of *TMEM30A* mutation status, and related macrophage biology in the context of new checkpoint inhibitor treatments [129].

Novel combination therapies; epigenetic modulators and immunotherapy

Downregulation of MHC molecules on cell membrane reduces immune reactivity against tumors, and results in reduced efficacy of cancer immunotherapies [130]. The frequency of attenuated expression of MHC molecules by DLBCL cells is high; MHC-I is low to absent in 40–60% and MHC-II in 20–40% of DLBCL cases [115, 131, 132]. Low expression of these molecules is mediated epigenetically in most cases, and the combination of epigenetic-modulating agents with immunotherapy provides a promising pathway for future research.

Although the tazemetostat phase II study in DLBCL showed a very limited efficacy in both EZH2mut and EZH2wt DLBCL [91] it has been recently shown, that MHC-II deficient-DLBCL in murine models harbor somatically acquired gene mutations that reduce MHC-II expression, with a strong enrichment of *EZH2* mutations (mutant *EZH2 Y641*) [127]. Thus, *EZH2* mutations could impair dynamic expression of immune synapse genes inside the GC, giving rise to acquired immune escape in GCB-DLBCL [127] (Fig. 7). In the same study, EZH2 inhibitors were found to be efficient in restoring MHC expression in *EZH2*-mutated human DLBCL cell lines, providing a rationale for combining immunotherapy with epigenetic reprograming [127] (Fig. 8).

Loss of function mutations in gene encoding proteins, with established roles in histone acetylation such as CREBBP and EP300, are commonly observed in DLBCL, and result in repression of genes involved in MHC class II-mediated antigen presentation [133]. HDAC6 has been shown to up-regulate the expression of CD20, and enhance the efficacy of anti-CD20 monoclonal antibodies, such as rituximab [134]. DNA methyltransferases inhibitors (or hypomethylators) seem to increase the tumor sensitivity to immune-checkpoint inhibitors, in addition its antitumoral effect appears to be in part mediated by the stimulation of the immune TME and reactivation of endogenous retroviruses leading to upregulation of viral defense responses [135]. In addition, genes perturbed by *CREBBP* mutation are direct targets of the BCL-6–HDAC3 onco-repressor complex [136]. Accordingly, HDAC3-selective inhibitors can reverse *CREBBP*-mutant aberrant epigenetic programming, resulting in growth inhibition of lymphoma cells through induction of BCL-6 target genes such as CDKN1A and restoration of immune
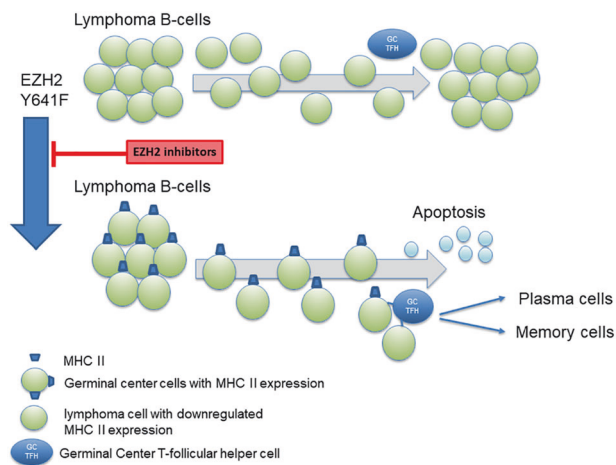
Fig. 8 EZH2 as a therapeutic target to enhance immune response. EZH2 inhibitors block *EZH2* mutant-germinal center cells, leading to upregulation of MCH II expression on the surface of lymphoma cells, and enhancing immune regulation by surrounding follicular helper T-cells.

surveillance due to induction of BCL6-repressed IFN pathway and antigen-presenting genes [136].

Although the above-mentioned findings are based on solid scientific rationale, a complete assessment is still premature and data remains in some degree speculative. Further investigations will help eliminating inaccurate speculations and notions.

Prognostic and theragnostic markers for DLBCL for the near future

Recent groundbreaking insights into the pronounced genomic heterogeneity of DLBCL have confirmed the existence of reproducible molecular subtypes of DLBCL, and identified vulnerable and potentially druggable targets. This has paved the way for a standardized application of precision medicine, extending beyond gene expression-based qualifiers. In addition to the established prognostic markers such as *MYC* rearrangements and mutation of *TP53*, we believe that the recent stratification of DLBCL according to the recently proposed molecular subtypes will guide the design and interpretation of clinical trials in the near future. The standardized identification of patients with DLBCL who belong to some of the "high risk" molecular groups, is of clinical interest and will be part of the next "phase" of prognostic and predictive biomarkers in DLBCL. The MYD88/C5/MCD cluster is a robust group that has been identified in all the recent genomic studies, and was found to show poor response to R-CHOP. The EZB-MYC+ group is also of high clinical interest, because it expands the current concept of "double

hit" lymphoma, and identifies a subset of DLBCL patients who might respond poorly to frontline therapy.

Immune landscapes in DLBCL are orchestrated by the presence of certain genetic, host and microenvironmental factors, some of which were identified in the newly elucidated genomic subgroups and clusters of DLBCL. Understanding the role of immune landscapes in lymphomagenesis (including, but not restricted to, PD-1/PD-L1 upregulation and TAM) will enable us to identify candidate patients who will benefit from targeted immunotherapy (e.g., PD-1/PD-L1 inhibitors and CD47/SIRPα inhibitors) and combinations with epigenetic-modulating agents.

Compliance with ethical standards

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