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Molecular classification and therapeutics in diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) encompasses a wide variety of disease states that have to date been subgrouped and characterized based on immunohistochemical methods, which provide limited prognostic value to clinicians and no alteration in treatment regimen. The addition of rituximab to CHOP therapy was the last leap forward in terms of treatment, but regimens currently follow a standardized course when disease becomes refractory with no individualization based on genotype. Research groups are tentatively proposing new strategies for categorizing DLBCL based on genetic abnormalities that are frequently found together to better predict disease course following dysregulation of specific pathways and to deliver targeted treatment. Novel algorithms in combination with next-generation sequencing techniques have identified between 4 and 7 subgroups of DLBCL, depending on the research team, with potentially significant and actionable genetic alterations. Various drugs aimed at pathways including BCR signaling, NF-κB dysfunction, and epigenetic regulation have shown promise in their respective groups and may show initial utility as second or third line therapies to patients with recurrent DLBCL. Implementation of subgroups will allow collection of necessary data to determine which groups are significant, which treatments may be indicated, and will provide better insight to clinicians and patients on specific disease course.

KEYWORDS

DLBCL, molecular diagnostics, molecular classification, signaling pathway, targeted therapy

1 Introduction

Diffuse large B-cell lymphoma (referred to as DLBCL) is characterized by a consistent range of cellular precursors and nuclear morphology, namely large B lymphoid cells with nuclei exceeding normal lymphocyte size, but spans many immunologic and genetic subtypes without much further classification (Stein et al., 2008). Immunohistochemistry in combination with various algorithms (which can differ from lab to lab) have allowed for basic classification of DLBCL into two groups, activated B-cell like type (ABC) and germinal center B-cell like type (GCB) which has provided prognostic insight into disease course without yielding much into targeted therapies for these distinct subtypes (Cerami et al., 2012; Tilly et al., 2015). In addition, this stratification system is largely a phenotypic description of DLBCL as opposed to a genetic description, so treatment responses are not fully explained by these subtypes (Wright et al., 2020). Clinically relevant stratification of DLBCL is largely non-existent and a classification such as a combination of CD10, BCL6, and IRF4/MUM1 expression, so-called "Hans classifier" was proposed to be useful in predicting long or short term survival (Hans et al., 2004).

The International Consensus Classification of Mature Lymphoid Neoplasms, which aims to provide standardized diagnostic criteria for lymphoid malignancies to pathologists, geneticists, scientists, and clinicians recognizes the utility of cell-of-origin (COO) designation in DLBCL, but also acknowledges that the system has shortcomings (Campo et al., 2022). Stratification by purely COO studies is considered insufficient to fully capture the genetic diversity of these tumors, especially relating to patient outcomes and treatment options, and essentially represents only the end result of faulty or mutated genetic pathways. Further classification may be warranted for DLBCL occurring in extranodal locations or immune privileged sites such as the central nervous system or testis. Inclusion of this extranodal branch of DLBCL in future classification systems may be warranted because these malignancies tend to share similar genetic alterations, such as a high prevalence of *MYD88*^{L265P} and *CD79B* mutations, which are also defining characteristics of MCD/C5 genetic subgroups, which are to be expanded upon further in this review.

The fifth edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms (WHO-HAEM5) exists to provide global definitions and classification systems for lymphoid tumors, and is intended for use by pathologists, clinicians, and research scientists (Alaggio et al., 2022). Characterization of DLBCL, otherwise known in the referenced paper as "Large B-cell lymphomas", are defined by morphology and must be carefully and meticulously differentiated from other malignancies such as blastoid variant of mantle cell lymphoma or lymphoblastic lymphoma, which can appear similar. In this globally accepted system for the identification and diagnosis of DLBCL the heterogeneity of DLBCL is greatly emphasized, and the recommendation for further rendering of ABC/GCB subtypes is made due to the lack impact that this system has on clinical outcomes for patients. DLBCL that does not fall into ABC or GCB categories is listed as DLBCL NOS (DLBCL not otherwise specified) and this alone has over 150 identified genetic drivers that occur in various combinations that ultimately lead to neoplasia and disease. The utility of next-generation sequencing (NGS) with novel clustering algorithms is acknowledged, and patterns have begun to emerge such as the similarity of genetic subtypes to follicular lymphoma or mantle cell lymphoma, which may also imply an overlap in possible treatment, and may suggest the possibility of genetic founder effects that initiate the cascade of mutations. WHO-HAEM5 overall emphasizes the importance of new subtypes for diagnosis and treatment, but awaits data from clinical trials before attempting to publish novel diagnostic subclasses of DLBCL.

National Comprehensive Cancer Network (NCCN) guidelines direct practitioners to standardized R-CHOP therapy (rituximab, cyclophosphamide, doxorubicin hydrochloride, vinicristine sulfate, and prednisone) as first line treatment regardless of presentation or cellular markers, and refractory cases are treated uniformly with further R-CHOP therapy at higher dosage, second line therapy in combination with hematopoietic cell transplant, chimeric antigen receptor T-cell (CAR T-cell) therapy, clinical trials, or finally palliative care (Tilly et al., 2015). The second line therapies are dependent on whether or not the patient and clinician intend on proceeding to hematopoietic stem cell transplant, as well as other confounding factors such as presence of poor left ventricular function. The introduction of rituximab, a chimeric anti-CD20 monoclonal antibody, was the most recent and last leap forward in treatment of DLBCL, and was added to previous first line therapies to create the newest standard of care treatment R-CHOP (Coiffier, 2007). Trials conducted using regimens other than R-CHOP have failed to demonstrate significant advantage over the current first line therapy (Coiffier, 2007).

Identification of genetic markers that alter disease course is essential not only for implementation of effective treatment, but also to indicate which patients may require less potent treatment or less invasive surveillance in looking for relapse. New therapies derived from NGS provided data are aimed at disrupting signaling pathways or modulating immune response, however the impact of these therapies can only be fully realized when we are able to categorize DLBCL into subtypes based on internal disease mechanism, as opposed to outward morphology or presence of basic cell markers. These internal mechanisms, and sorting the influential from the irrelevant, are the basis for future treatment modalities and the cessation of indiscriminate therapy based on patient response. In this paper we will begin by exploring the various diagnostic criteria for subtyping of DLBCL on a molecular level, and the genetic alterations that change basic signaling pathways and cascades that determine disease progression and outcome. Targeted therapies aimed at these pathways will be investigated, and lastly the clinical implications and research still needed will be discussed.

2 Molecular diagnosis and classification of DLBCL

DLBCL can currently be divided into 3 subtypes based on cell of origin (COO), ABC, GCB, and Unclassified, which yield moderate prognostic value and decidedly limited clinical value due to the standard progression of treatment regimens across all three types (Schmitz et al., 2018). These subtypes are based primarily on which stage in B cell development these malignant cells most resemble morphologically and genetically (ABC/GCB), or the absence of identifying characteristics (Unclassified) which is the broadest subtype (Campo et al., 2011; Rosenquist et al., 2016; Rosenquist et al., 2017). While these subtypes do share high incidences of similar genetic mutations within their respective group (e.g., B-cell receptor or NOTCH abnormalities), these groups are insufficient for the implementation of precision treatment and prediction of disease course, especially in disease that is non-responsive to traditional R-CHOP therapy. Heterozygosity among DLBCL within ABC/GCB/Unclassified groups is the primary barrier to treatment with a uniform regimen like R-CHOP and explains the need for a narrower and more well-defined set of groups with targeted treatment options.

NGS has allowed for the analysis of extremely large DLBCL samples in order to differentiate impactful pathogenetic sequences from those that do not significantly alter disease course, as well as provide a meaningful way of typing cells more accurately than the current standard (ABC/GCB grouping based off variable immunochemistry) (Mansouri et al., 2022). A study conducted by Schmitz et al. subdivided DLBCL into 4 groups (EZB, BN2, MCD, N1) based on co-occurrence of predefined sequences and mutations in an effort to look at mutations as a "constellation" of abnormalities that defines the subtype and thus forms the specific disease state (Figure 1A) (Schmitz et al., 2018). These four subtypes were demonstrated to have distinctly different progression-free survival (PFS) and overall survival (OS) associated with them, as well as more predictable disease course such as trends towards extra-nodal

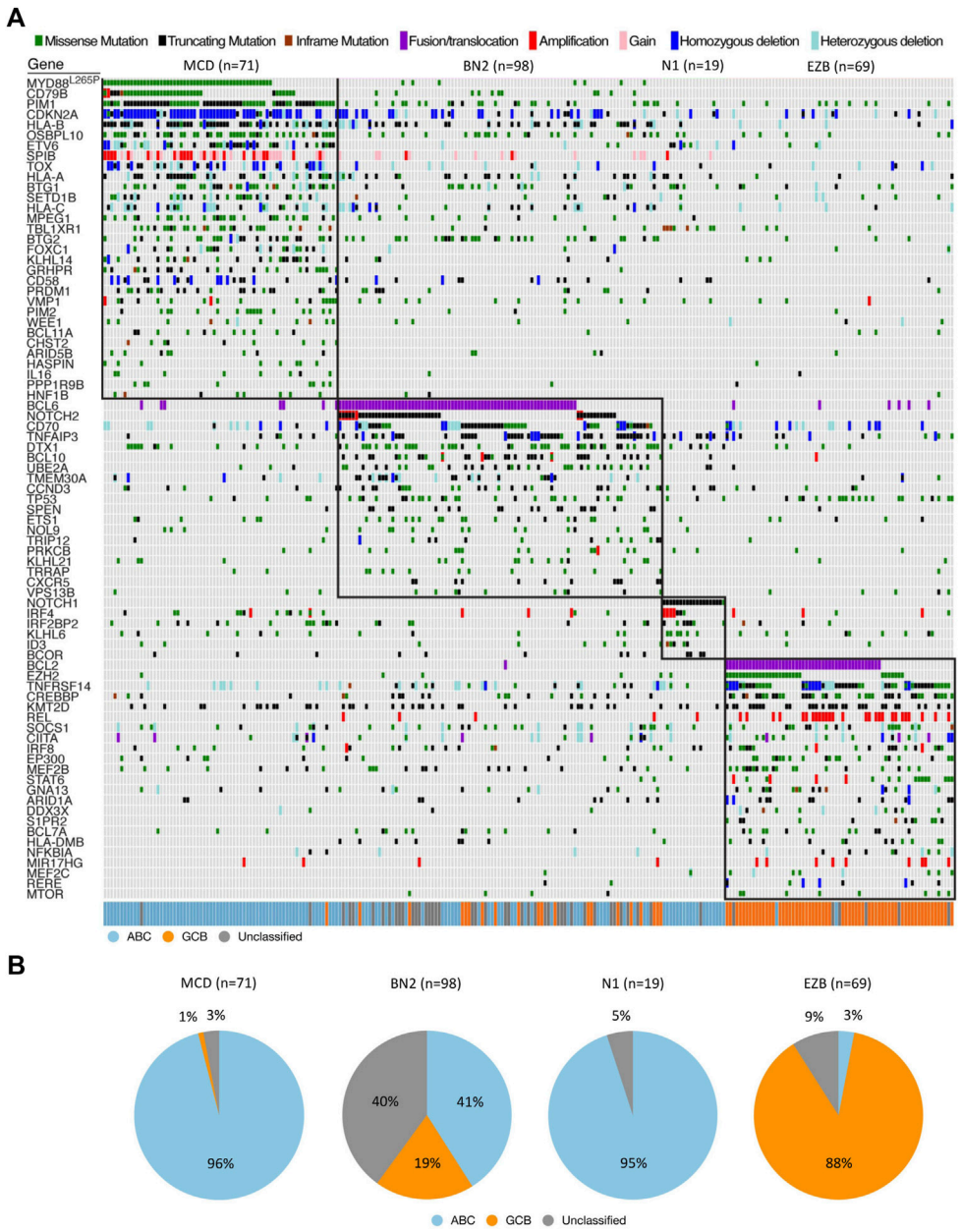

FIGURE 1
Genetic features of DLBCL. **(A)** Landscape of DLBCL genetic subtypes based on genetic alteration. Relationship between genetic subtypes (e.g., MCD, BN2, N1, EZB) and gene expression subtypes (e.g., ABC, GCB, Unclassified) is also shown. **(B)** Distribution of gene expression subtypes within genetic subtypes. All data were extracted from the results of the whole exome sequence and RNA-seq published by Schmitz et al. (2018). Adapted with permission from Schmitz et al. (2018). Copyright 2018, Massachusetts Medical Society.

involvement as seen in the MCD subtype (Puente et al., 2015; Schmitz et al., 2018). These delineations are meaningful not only prognostically, but differing response to treatment has been observed among the groups, such as the increased susceptibility of the MCD subtype to BCR inhibitors (Wright et al., 2020).

2.1 Subgroup by cell of origin (COO)

ABC, GCB, and Unclassified DLBCL are divided based on stage of development they most resemble after some genetic mutation disrupts typical progression and causes malignant transformation. The cells of these subtypes develop their morphologic and clinical characteristics based on the stage of development in which they accumulate enough genetic variation to become malignant, and differentiation either ceases or continues on a path not seen by healthy and functional B cells (Schmitz et al., 2018). The ABC subtype is thought to have progressed through the germinal center and is committed to plasmablastic differentiation, while GCB cells are thought to originate from light zones in the germinal center (Pfreundschuh et al., 2008). Clinical outcomes of GCB lymphomas are widely recognized as superior to ABC malignancies when traditional

R-CHOP therapy is utilized, however the risk stratification has not led to improvements in treatment outcome. 30%–50% of patients with DLBCL will not respond to this therapy, and only 10% with disease refractory to this treatment will be cured with second line salvage treatment or bone marrow transplants (Feugier et al., 2005; Pfreundschuh et al., 2006; Pfreundschuh et al., 2008; Gisselbrecht et al., 2010; Friedberg, 2011; Coiffier and Sarkozy, 2016). The heterogeneity of treatment outcomes, even among ABC, GCB, and Unclassified subtypes likely results from the specific pathways with altered regulation, expression, or end products that are not defined by classical subtyping.

2.1.1 Activated B-cell like type (ABC)

Cells currently falling under the ABC classification to tend to express common mutations and translocations, such as *PRDM1* truncations or homozygous deletions only seen in the ABC subtype, that have given insight into their behavior patterns (Pasqualucci et al., 2006; Mandelbaum et al., 2010; Schmitz et al., 2018). Identification of these defining pathways and association with a specific disease course, albeit in mice, further backs the strength of disease grouping in prognostics and treatment options (Mandelbaum et al., 2010; Miao et al., 2019). These cells show increased incidence of "chronic active" BCR signaling which is characterized by BCR clustering and autoreactive self-antigens as opposed to tonic signaling which is antigen independent and exhibits a lack of BCR clustering, as seen in GCB. *MYD88* mutations conferring extranodal involvement, TNFAIP3 inactivation leading to uncontrollable NF-κB expression, and *NOTCH1* mutations are seen almost exclusively in this subtype. These mutations however are not seen in a majority of cells expressing the ABC phenotype so they can hardly be called ABC defining traits, but are far more strongly associated with this subtype than the GCB or Unclassified types.

2.1.2 Germinal center B-cell like type (GCB)

GCB cells are affected by the master regulator BCL6 similarly to ABC cells, but also are affected by more unique mutations. These possess an association with *REL* amplifications promoting lymphomagenesis, an almost exclusive presentation of t(14;18)(q32; q21) translocations leading to BCL2 activation and overexpression, and *CREBBP* mutation affecting the histone acetyltransferase domain leading to epigenetic dysregulation (Iqbal et al., 2004; Kusumoto et al., 2005; Kridel et al., 2012; Visco et al., 2013; Ennishi et al., 2017; Miao et al., 2019). The incidence of these mutations among all patients diagnosed with GCB DLBCL is relatively low and again can hardly be called defining to the disease type. The presence of these mutations in a GCB DLBCL and the association of GCB type with superior response to first line R-CHOP therapy supports the somewhat obvious notion that different (dysregulated) pathways respond differently, or not at all, to a given treatment.

One of the biggest problems with the accepted stratification of DLBCL is the disparity in treatment response even in the presence of seemingly identical focal mutations in both ABC and GCB, such as *TP53* deletion or *MYC* mutations (Jia et al., 2012; Xu-Monette et al., 2012; Cao et al., 2016; Xu-Monette et al., 2016). This observation coupled with the vast number of possible mutations suggests not that we need groups based on individual pathway alterations, but the need for grouping based on sets of mutations that can be found together and that can be targeted therapeutically (Mansouri et al., 2022).

2.2 Subgroup by genetic alteration and signaling pathway

The current ABC and GCB subgroups provide relatively little utility because they do not take into account the numerous changes that can occur within the genome that are not observable with immunohistochemical staining techniques. In order to develop targeted treatments and better predict disease course, we must take a closer look at which pathways specifically are causing the dysplastic growth of these cells, where these pathways have gone awry, and if they are even impactful to the overall progression of the malignancy. We will now identify the pathways that have been deemed influential by various research teams, and that help define the proposed subgroups (Table 1).

2.2.1 Schmitz's classification: BN2, EZB, MCD, N1

A classification system developed by Schmitz et al. (2018) has sought to link certain groupings of mutations with disease course, severity, and response to traditional R-CHOP therapy as well as suggest other potential therapies. Their view of genetic anomalies was not to view each mutation as an independent and unrelated event, but to try and group mutations that commonly occurred together and characterized a distinct disease course. Independent mutations would be nearly impossible to keep track of and treat individually at this stage in our knowledge and practice of medicine, so groupings like these encompassing multiple class defining mutations could prove to be actionable. The four groups of DLBCL they established through use of their algorithm were "MCD" defined by *MYD88* and *CD79B* mutations, "BN2" defined by *BCL6* fusions and *NOTCH2* mutations, "N1" defined by *NOTCH1* mutations, and "EZB" defined by *EZH2* and *BCL2* translocations. ABC cells in the MCD category were observed to have a superior response to ibrutinib, which halts B-cell proliferation through action as a Bruton's tyrosine kinase (BTK) inhibitor, supporting the notion that better characterization of genetic phenomena and heterogeneity can lead to more effective and targeted treatment (Shaffer et al., 2006; Puente et al., 2015; Chapuy et al., 2016). Mutations found more commonly in ABC or GCB subtypes respectively further add defining features and explanation to their course, such as N1 subtype appearing in ABC 95% of the time, or the observed co-occurrence of *EZH2* mutations with *BCL2* translocations in GCB type lymphomas (Figure 1B) (Miao et al., 2019). The newly defined subtypes also observed significant progression-free survival (PFS) differences, with best outcomes going to BN2 and EZB types (Schmitz et al., 2018). Interestingly these subtypes, BN2 and EZB, were most commonly seen in the current GCB classification which is already accepted as having superior response and survival rate to ABC type malignancies (Cerami et al., 2012; Gao et al., 2013; Valls et al., 2017; Schmitz et al., 2018). The alignment between the potential new classification types and the currently accepted system adds a layer of confidence in exploring the subtypes proposed by Schmitz et al. in terms of prognostic indication, and development of treatments targeted at the pathways identified in their algorithm.

2.2.2 Chapuy's classification: C1-C5

Chapuy et al. have developed another classification system through use of whole exome sequencing (WES) that divides DLBCL cells into C1, C2, C3, C4, or C5 depending on pattern of gene expression with defining genetic drivers, or C0 if a genetic driver could not be identified (Chapuy et al., 2018). Cluster 5 exhibited a

TABLE 1 Molecular classification of DLBCL. *Prevalance data were extracted from the results published by Wright et al. (2020).

Molecular classification				
Wright	Schmitz	Lacy	Chapuy	Genetic alteration (% prevalance*)
BN2	BN2	NOTCH2	C1	*BCL6* (72.8%), *NOTCH2* (41.8%), *TNFAIP3* (51.6%), *DTX1* (50.0%), *CD70* (41.3%), *BCL10* (39.6%), *UBE2A* (30.4%), *TMEM30A* (26.7%), *KLF2* (21.7%), *SPEN* (21.7%)
A53	—	—	C2	*TP53* (86.8%), *B2M* (34.2), *TP53BP1* (27.0%), *CNPY3* (23.7%), *ING1* (15.8%), *NFKBIZ* (15.8%), *TP73* (13.2%)
EZB-MYC+ EZB-MYC−	EZB	BCL2	C3	*BCL2* (68.4%), *EZH2* (44.7%), *TNFRSF14* (66.2%), *KMT2D* (53.9%), *CREBBP* (52.7%), *REL* (34.3%), *FAS* (30.1%), *IRF8* (28.9%), *EP300* (27.8%), *MEF2B* (26.3%), *CIITA* (25.0%), *ARID1A* (22.9%), *GNA13* (22.5%), *STAT6* (21.1%), *PTEN* (20.0%)
ST2	—	TET2/SGK1 SOCS1/SGK1	C4	*TET2* (48.1%), *DUSP2* (44.4%), *ZFP36L1* (40.7%), *ACTG1* (37.0%), *SGK1* (37.0%), *ITPKB* (33.3%), *NFKBIA* (33.3%), *EIF4A2* (29.6%), *JUNB* (29.6%), *STAT3* (29.6%), *BCL2L1* (25.9%), *CD83* (25.9%), *DDX3X* (25.9%), *SOCS1* (25.9%), *CD83* (25.9%), *P2RY8* (22.2%), *RFTN1* (22.2%)
MCD	MCD	MYD88	C5	*MYD88* (66.2%), *CD79B* (50.0%), *PIM1* (92.5%), *HLA-B* (73.8%), *BTG1* (70.0%), *CDKN2A* (62.0%), *ETV6* (55.0%), *SPIB* (51.9%), *OSBPL10* (51.2%), *TOX* (48.1%), *BCL2* (48.1%), *BTG2* (43.8%), *MPEG1* (43.8%), *HLA-A* (43.0%), *HLA-C* (42.5%), *SETD1B* (41.8%), *KLHL14* (41.2%), *TBL1XR1* (35.0%), *GRHPR* (33.8%), *PRDM1* (32.5%), *CD58* (31.6%), *TAP1* (26.6%), *PIM2* (25.0%), *FOXC1* (21.2%), *IRF4* (20.0%)
N1	N1	—	—	*NOTCH1* (100%), *IRF2BP2* (43.8%), *ID3* (25.0%), *BCOR* (25.0%), *EPB41* (18.8%), *IKBKB* (18.8%), *ALDH18A1* (18.8%)

consistent 18q gain, and is notable because 8 of the 9 patients in the Chapuy sample that had testicular involvement fell into the C5 cluster and 1 of 2 patients in the sample with CNS involvement fell into this cluster (Wright et al., 2003; Monti et al., 2005). This correlation is exciting for two reasons, first it shows a relation between disease course and genetic drivers in DLBCL which has the potential to aid in targeted screening for patients with identified mutations (e.g., 18q deletion) and in selecting more appropriate therapies. Secondarily the C5 group displaying increased extranodal involvement is significant because genes observed in the C5 group (e.g., *MYD88*) overlap heavily with the genes observed in the Schmitz et al. subgroup MCD, where extranodal involvement was also noted to be significant (Table 1). While the grouping of genes is slightly different between MCD and C5, the overlap and mutually identified association with specific disease course emphasizes the utility of being able to subtype DLBCL beyond what is currently used. Subtype C1 shared alterations in certain pathways (e.g., NOTCH2) with low grade marginal zones lymphomas, and alterations in other NOTCH2 and BCL6 pathways were defining characteristics of this group (Li and Durbin, 2009; Chapuy et al., 2013; Scott et al., 2014; Chapuy et al., 2016; Staiger et al., 2017; Chapuy et al., 2018; Papageorgiou et al., 2021). C3 which was characterized by *BCL2* and chromatin modifier mutations, C4 was defined by mutations in four linker and four core histone genes, and these two pathways are mentioned together because they both represent subtypes of GCB type DLBCL (Chapuy et al., 2018). These subtypes are of interventional significance because they both alter the function of common pathways such as PI3K, but do so through different mechanisms (Chapuy et al., 2018). This is yet another example of why therapies must be tailored to the underlying mechanism of disease state, not just the presentation of disease. C2 was defined by markers including *TP53* mutation and loss of *CDKN2A* and *RB1* which alter chromosomal stability and cell cycle (Chapuy et al., 2018). Cluster zero (C0) lacked any defining characteristics or identifiable genetic homogeneity and represents a significant lack of understanding in disease pathology (Chapuy et al., 2018).

2.2.3 Lacy's classification: NOTCH2, BCL2, TET2/SGK1, SOCS1/SGK1, and MYD88

Research conducted by Lacy et al. (2020) sought to investigate their own set of subgroups using methods "potentially applicable in routine clinical medicine" and came up with 5 distinct groups. Their study aimed not only to establish groups with potentially workable mutations, but also to compare overlap between their groups to subtypes identified in studies Schmitz et al. and Chapuy et al. (2018) (Table 1). The first group, "MYD88" cluster, was defined by *MYD88* mutation among others and contained the majority of observed primary CNS lymphomas and primary testicular lymphomas, similarly to MCD and C5 groups (Schmitz et al., 2018; Lacy et al., 2020). The "BCL2" group predominantly had characteristic t(14;18) translocation and other mutations in BCL2 pathways (Lacy et al., 2020). "SOCS1/SGK1" had mutations common to primary mediastinal B-cell lymphoma, and was observed to represent a subdivision of the C4 cluster described by Chapuy et al. (2018) (Lacy et al., 2020). The "TET2/SGK1" was largely GCB in origin and had characteristic mutations including *TET2*, *SGK1*, and *KRAS*, and was postulated to represent another subdivision of the C4 cluster (Chapuy et al., 2018). The final subtype described here which corresponded to the C1 subtype was the "NOTCH2" subtype which included mutations in *NOTCH2*, *BCL10*, *CD70*, and showed a strong correlation between *NOTCH2* mutation and *BCL6* rearrangement (Chapuy et al., 2018; Schmitz et al., 2018; Lacy et al., 2020). These subtypes seem to provide some prognostic ability with 5-year OS rates for MYD88, SOCS1/SGK1, BCL2, TET2/SGK1, and NOTCH2 groups observed at 42.0%, 64.9%, 62.5%, 60.1%, and 48.1% respectively (Lacy et al., 2020). This study demonstrated significance not only for itself, but helped cement the significance of studies conducted by Schmitz et al. and Chapuy et al. stemming from the overlap of their respective subtypes in terms of genetic anomaly identified and observed disease course, especially in the context of different algorithms and research parameters utilized between the studies.

2.2.4 Wright's classification: BN2, A53, EZB-MYC+, EZB-MYC−, ST2, MCD, N1

Another key classification system using an algorithm referred to as "LymphGen" developed by Wright et al. (2020) intended on developing a more clinically useful stratification system based partially on work done by Schmitz et al. and Chapuy et al. This algorithm again recognizes genetic "constellations" rather than individual abnormalities and divides patients into 7 groups based on the prevalence of defined features. These groups are "MCD" characterized by BCR-dependent NF-κB immune evasion, "N1" characterized by NOTCH1 signaling and altered B cell differentiation, "A53" characterized by TP53 inactivation and aneuploidy, "BN2" characterized by NOTCH2 signaling with BCR dependent NF-κB immune evasion and a loss of CD70, "ST2" characterized by JAK/STAT3 signaling with NF-κB activation, and EZB which was further subdivided as being MYC+/− ("EZB-MYC+" and "EZB-MYC−") and had chromatin modification conferring anti-apoptosis as well as PI3 kinase signaling and S1PR2-GNA13 inactivation (Wright et al., 2020). The LymphGen algorithm, using these criteria among other genetic markers, used genetic information gathered from malignant cells to then score the cells on the presence of these various mutations and assign them to the appropriate group based on assigned confidence intervals (Wright et al., 2020). The benefit to this algorithm was the ability to not only define subtype based on presence of these traits, but also to identify cells that had a probability of being part of a core, extended or genetically composite group where probability of belonging to a subtype was >90%, 50%–90%, or if the cell was a core member of more than one subtype, respectively (Wright et al., 2020). Using the algorithm, 63.1% of the cohort was able to be classified, greater than the 46.6% classified by Schmitz et al. (Wright et al., 2020). This is an especially high number when considering that to fall into one of the 7 subtypes with acceptable confidence for the algorithm, cells must acquire multiple mutations that seem as though they statistically should not occur together as often as they do if they were independent of each other. The ability to group these cells together may stem from a founder effect where a cell acquires an initial genetic mutation that may be relatively more common, and only certain secondary mutations confer continued survival for the cell. MYC overexpression would normally lead to cell death if not also paired with a *BCL2* translocation that prevents cell death (Evan et al., 1992). A sort of natural selection such as this confers the ability of these algorithms to become of clinical use in the near future since these mutations likely do not occur in isolation (e.g., *BCL2* and *MYC* mutations codependency), but are dependent on each other for the survival of the cell inextricably linking the altered pathways.

3 Genetic alterations in DLBCL

Understanding which genetic anomalies tend to occur together and the associated prognosis is an essential primary step in establishing a new categorization system for DLBCL, but is far from the end product. To develop effective treatments we must thoroughly understand the pathways that have undergone alteration, and establish whether or not they are even influential in overall disease progression. Equally important is establishing where in a specific pathway the disruption occurs, because mutations downstream of drug targets may render the drug useless, even if it acts on the appropriate pathway. By taking a closer look at which pathways tend to undergo alteration and where alterations tend to take place, we will establish not only the extreme complexity of DLBCL, but can begin to identify potential drug targets for later discussion of therapeutic indications.

3.1 Major signaling pathways affected by genetic alteration in DLBCL

Until now we have discussed pathways that have been identified as influential in disease state and developing criteria for new subtypes. We will now take a closer look at some of the most commonly mutated pathways, including BCR signaling, PI3K-AKT-mTOR signaling, BCR dependent NF-κB activation, NF-κB signaling, TLR signaling, and the BCL2 anti-apoptotic family (Figure 2). These pathways are related in their ability to evade apoptotic pathways, promote cell proliferation and gene expression, and confer lymphomagenesis. The ability to target not only faulty pathways, but specific faulty mutations within pathways, will pave the way for targeted DLBCL treatment, and identification of influential mutations is the first step in that direction.

3.1.1 B-cell receptor (BCR) signaling

BCR signaling is involved in regulation of B cell survival, development, and differentiation, and can be chronic active or tonic in DLBCL, and both overexpression and overactivation are implicated in lymphomagenesis (Rawlings et al., 2017). Chronic signaling resembles antigen-dependent (autoreactive self-antigens) active BCR signaling in non-malignant B cells and is characterized by BCR clustering (Davis et al., 2010; Young and Staudt, 2013; Young et al., 2015; Havranek et al., 2017). Tonic BCR signaling is antigen-independent and clustering is not observed (Davis et al., 2010). ABC cells are characterized by chronic active BCR signaling, GCB DLBCL display tonic signaling (Davis et al., 2010; Havranek et al., 2017). During normal signaling after the BCR is bound by an appropriate ligand, CD79A and CD79B form a heterodimer and following dual phosphorylation of the intracellular immunotyrosine-based action motifs (ITAMs) of CD79A/CD79B, SYK kinase is recruited and activated which then activates BTK (Bruton's tyrosine kinase) which leads to downstream activation of BCR signaling, notably mTOR and NF-κB (Miao et al., 2019). Mutations in any part of this pathway can potentially be problematic and lead to overactivation, such as loss of negative feedback ITAM mutation of *CD79B* which is detected in near 20% of ABC DLBCL cases (Davis et al., 2010). B cell receptors can be positively regulated by mutation or amplification of *CD79B* and *CD79A*, and negatively regulated by inhibitors including LAPTM5, LYN, PTPN6, GRB2, PRKCD, DGKZ, SLA, and MAP4K1 (Lenz et al., 2008a; Lam et al., 2008; Affymetrix® White Paper, 2009; Dobin et al., 2013; Anders et al., 2015; Seshan, 2017; NCI Genomic Data Commons, 2016). Loss of function of negative regulators of BCR signaling leads to unchecked activation, or a loss of negative feedback, of the BCR and was present in 38.5% of cases of DLBCL (Schmitz et al., 2018). Loss of function of negative regulators was more prevalent in *MYD88*

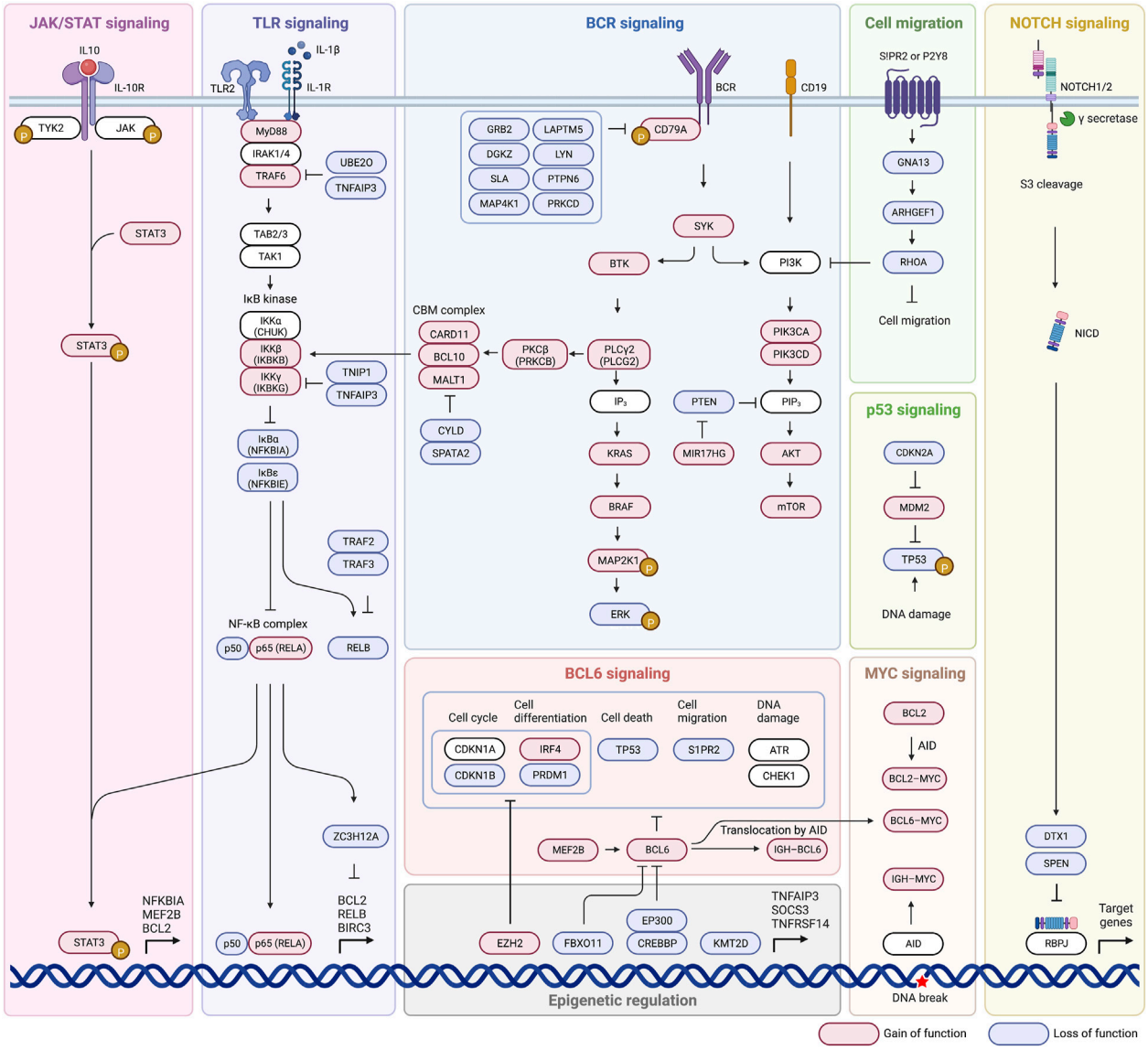

FIGURE 2
Major signaling pathways affected by genetic alterations in DLBCL.

with *CD79A*/*CD79B* activating mutations, than in *MYD88* lacking *CD79A*/*CD79B* alterations (56% vs 36.4%) which is of potential clinical utility because aggressive lymphomas with $MYD88^{L265P}$ and *CD79B* mutations have a response to ibrutinib (Bruton's tyrosine kinase is downstream of BCR) and presumably have chronic active BCR signaling (Shaffer et al., 2006; Puente et al., 2015; Chapuy et al., 2016; Schmitz et al., 2018). The most common mutation affecting BCR and TLR signaling is *MYD88*, 18%–27% of cases, which directly affects TLR signaling and indirectly influences BCR signaling because MYD88, TLR9, and BCR form a multiprotein supercomplex which leads to promotion of downstream NF-κB and mTOR activation (Phelan et al., 2018).

3.1.2 PI3K-AKT-mTOR signaling

PI3K can indirectly activate NF-κB pathway and was genetically altered in 34.3% of cases of DLBCL, and is also able to directly activate mTOR mechanisms (Kent, 2002). Activation of mTOR inappropriately promotes cell proliferation and metabolism that contributes to tumor initiation and progression (Tian et al., 2019). Normally, activation through the BCR or CD19 leads to activation of the PIK3CD/PIK3CA heterodimer (BCR does this indirectly through intermediate activation of PIK3AP1), which goes on to activate PIP3 leading to activation of PDPK1, then activation of an AKT1/AKT2 heterodimer, and final activation of mTOR (Schmitz et al., 2018; Miao et al., 2019). Activating events involving PI3K signaling subunits (PIK3CA, PIK3CD, PIK3AP1, PDK1, AKT1/2) can lead to downstream AKT activation (Miao et al., 2019). Activation of MIR17HG which functions to inhibit PTEN, a negative regulator of PIP3, also leads to overactivation of this pathway and overexpression of mTOR (Miao et al., 2019). Inactivating mutations of negative PIP3 regulators *PTEN* and *INPP5D* are also seen in up to 15% and 10% of cases respectively

and lead to unchecked mTOR expression (Schmitz et al., 2018). BCR co-receptor CD19 and the BCR itself (through SYK kinase) are able to activate PI3K signaling, which leads to activation of AKT which activates downstream mTOR promoting cell survival, again showing the large role BCR signaling plays in promotion of DLBCL (Uddin et al., 2006; Young and Staudt, 2013). Activating mutations of PI3K subunits (*PIK3CA* amplification/activating mutation) are identified in 6% of ABC DLBCL but not GCB, others (*PTEN* deletion) are present in both ABC and GCB (9%–11%) (Lenz et al., 2008a; Pfeifer et al., 2013; Chapuy et al., 2018; Schmitz et al., 2018; Wang et al., 2018).

3.1.3 BCR-dependent NF-κB activation

Normally, BCR stimulation caused by antigen binding activates BTK signaling through SYK activation, which activates PLCλ2 and PKCβ, PKCβ then phosphorylates CARD11 which recruits BCL10 and MALT1 to form a complex (CBM complex) that inactivates negative regulator CYLD/SPATA2 complex and inactivates RELB (Schmitz et al., 2018). ZC3H12A is a more independent regulator of NF-κB pathway-dependent transcripts and enhances mRNA decay of anti-apoptotic gene transcripts including *BCL2L1*, *BCL2A1*, *RELB*, *BIRC3*, and *BCL3* (Skalniak et al., 2009; Lu et al., 2016). Inactivating mutations of *ZC3H12A* are seen in up to 10% of cases, the consequence of which is anti-apoptotic and pro-lymphomagenesis (Skalniak et al., 2009; Lu et al., 2016; Schmitz et al., 2018). Failure of the NF-κB pathway to produce adequate IL-1β, which ZC3H12A is dependent on for activation, may also lead to inadequate mRNA degradation and anti-apoptotic characteristics (Skalniak et al., 2009). Mutations that cause overactivation of BCR signaling such as activating mutations of *CD79A*/*CD79B* or *SYK* which overstimulate BTK/PLCG2 causing activation of PRKCB and the CBM complex lead to an overactive NF-κB pathway (Schmitz et al., 2018). *CARD11* mutations, which occur in both ABC and GCB type, impair auto-inhibition seen in wild type proteins which lead to continued activation of NF-κB (Lenz et al., 2008b; Lamason et al., 2010; Wilson et al., 2012; Bohers et al., 2014; Schmitz et al., 2018). Inactivating mutations are also seen in the negative regulators of the CBM complex themselves, including inactivation of CYLD/SPATA2 complex which functions as the inhibitor of the CBM complex in a negative feedback loop (Schmitz et al., 2018). Inactivation of negative regulators of RELB is also seen in up to 15% of cases, specifically inactivation of the TRAF2/3 complex, and overactivation of RELB is thought to indicate dismal outcome after immunochemotherapy because RELB confers DLBCL cell resistance to DNA damage induced apoptosis, preventing genotoxic agent doxorubicin from having the desired effect (Schmitz et al., 2018; Eluard et al., 2022). Negative regulators of inactivation signaling enzymes and adaptors that promote BCR-dependent NF-kB activation are aberrant in 44.9% of cases (Schmitz et al., 2018). 66.2% of cases also had mutations in other regulators of NF-κB such as *TLR2* and *ZC3H12A* which negatively regulate the stability of NF-κB messenger RNA (mRNA) (Nicorici et al., 2014). In the BN2 subtype 2 components of BCR-dependent NF-κB pathway were altered in 47% of cases, protein kinase C beta (PKCB) and BCL10 (Schmitz et al., 2018). Signatures of BCR-dependent NF-κB activation were highest in MCD and BN2 (Schmitz et al., 2018).

3.1.4 NF-κB signaling (IκB kinase-dependent NF-κB activation)

NF-κB signaling may also be stimulated through activation of the IKBKG/IKBKB complex, which is only partially dependent on the BCR pathway and may be activated through various means (Schmitz et al., 2018). Activating mutations of positive regulators, *TLR2* and *MYD88*, or inactivation of negative regulators TNIP1/TNFAIP3, result in the ability of IκB kinase (IKBKG/IKBKB) to inhibit the NFKBIA/NFKBIE complex (Schmitz et al., 2018; Miao et al., 2019; Ma and Malynn, 202012). Inhibition of this complex causes release of p65/p50 and REL pathways, which may also adopt activating mutations themselves, and final overexpression of NF-κB expression (Schmitz et al., 2018; Miao et al., 2019). Genetic alterations of NF-κB negative regulators were a prominent feature of subtype BN2, those affecting negative regulators TNFAIP3 or TNIP1 were found in 55% (Schmitz et al., 2018). Mutations in positive regulator *MYD88* are also a defining trait of the MCD subtype and are a prominent source of gene overexpression (Schmitz et al., 2018).

3.1.5 Toll-like receptor (TLR) signaling

Toll-like receptor 2 (TLR2) signaling in its standard state is mediated by MYD88, an adapter protein which assists in signaling, in complex with IRAK1/4 (Ngo et al., 2011). Mutation of *MYD88* promotes assembly of a complex composed of IRAK1 and IRAK4 which enhances IRAK4 kinase activity and IRAK1 phosphorylation. Hyperphosphorylated IRAK1 causes activation of TRAF6, which activates TAK1, which can then accomplish downstream NF-κB and JAK/STAT signaling through the IκB kinase pathway (Ngo et al., 2011; Schmitz et al., 2018; Miao et al., 2019). Activating mutations of *TLR2*, *MYD88*, and *TRAF6* are observed and can stimulate this pathway in absence of a proper TLR ligand (Schmitz et al., 2018). Activating mutations of *MYD88* increase activity of both NF-κB and JAK/STAT pathways (Rosenquist et al., 2017). Inactivating mutations of negative regulators UBE2O and TNFAIP3, which normally inhibit TRAF6, are also observed to cause overstimulation of downstream NF-κB pathway (Miao et al., 2019).

3.1.6 Anti-apoptotic BCL2 family

In healthy B cells, apoptosis is induced through a lack of affinity of BCL2 for a specific antigen, so constitutive BCL2 activation through mutation would result in B cells that can avoid apoptotic programs in the absence of proper antigen recognition (Miao et al., 2019). Various *BCL2* mutations occur and are noted in various DLBCL subtypes, such as the t(14;18)(q32;q21) translocation seen in 34%–44% of GCB DLBCL (Iqbal et al., 2004; Visco et al., 2013; Ennishi et al., 2017). Translocations such as this move *BCL2* near genes that cause overactivation, or constitutive activation of BCL2 and promote survival (Kridel et al., 2012). Somatic hypermutation involving promoter and coding regions of *BCL2* is seen in about 35% of DLBCL cases, with the majority being in the GCB subtype (Schuetz et al., 2012). These two examples indicate that while activation/mutation of *BCL2* is common across many DLBCL's, the root cause is not the same and may drive the need for multiple approaches to treating the same faulty pathway. Different mutations also result in differential response to BCL2 inhibitors, which is a consideration in measuring response to BCL2 inhibitors (Schmitz et al., 2018). Overactivation of other BCL2 family members

such as BCL2L1 and MCL1 can also impair intrinsic apoptotic pathways and lead to continued cell survival and proliferation (Schmitz et al., 2018; Miao et al., 2019).

3.2 Alterations in epigenetic regulation that contribute to the development of lymphoma

Previously discussed are mutations within genes themselves, but equally important are changes in epigenetic regulators that alter expression of these genes. Greater epigenetic heterogeneity is associated with poor clinical outcome (De et al., 2013; Jiang and Melnick, 2015) and inhibitors of these mechanisms such as DNA methyltransferase and histone methyltransferase inhibitors may be a source of therapeutic intervention in DLBCL (Jiang and Melnick, 2015). Each subtype may express epigenetic dysregulation in relatively characteristic ways, such as EZB type displaying *EZH2* mutations or inactivation of KMT2D (Schmitz et al., 2018). We will now take a closer look at disruptions in regulation caused by EZH2, KMT2D, and EP300 and CREBBP.

3.2.1 EZH2

EZH2 is an important mediator of negative transcriptional activity, achieved through trimethylation of Lys27 of histone H3 subunit, referred to as H3K27 (Velichutina et al., 2010; Beguelin et al., 2013; Caganova et al., 2013). Overactivation of EZH2 leads to excessive activation of cell cycle regulators and promoters of plasma cell differentiation, such as CDKN1A, CDKN1B, CDKN2A, PRDM1, IRF4, or XBP1 (Miao et al., 2019). These problems culminate in defective germinal center formation in GCB cells, cells that can proliferate unchecked by the cell cycle regulators, protection from AID-dependent genotoxic damage-induced apoptosis, and restricted differentiation (Velichutina et al., 2010; Beguelin et al., 2013; Caganova et al., 2013). *EZH2* mutation alone is not enough to create an environment conducive to the development of DLBCL, however in mice it has been shown that *EZH2* mutation coupled with overexpression of BCL2 may be able to propagate the development of DLBCL (Cattoretti et al., 2005; Miao et al., 2019).

3.2.2 KMT2D

KMT2D is a histone monomethyltransferase responsible for regulation of transcription of tumor suppressor genes such as *TNFAIP3*, *SOCS3*, or *TNFRSF14*, so inactivation of this gene will lead to decreased tumor suppressor activity (Ortega-Molina et al., 2015; Miao et al., 2019). Nearly 30% of DLBCLs show mutation in this gene, commonly a non-sense or frameshift, but some cases of DLBCL show decreased KMT2D activity in the absence of mutation indicating other epigenetic regulation may be relevant (Morin et al., 2011; Ortega-Molina et al., 2015; Schmitz et al., 2018). KMT2D inactivation is also implicated in disruption of cell cycle regulators CDK6 and BCL2, which promote malignant cell survival (Zhang et al., 2015).

3.2.3 EP300 and CREBBP

Among multiple histone lysine acetylation sites, H3K27 acetylation is related to active transcription while deacetylation represses transcription. EP300 and CREBBP are involved in H3K27 acetylation to promote transcription of tumor suppressor genes such as *PRDM1*, *IRF4*, *CIITA*, *CD74*, or *HLADR*, as well as acetylation of BCL6 and p53 which results in inhibition and activation of transcription respectively (Miao et al., 2019). *CREBBP* mutation reduces expression of these genes essential to plasma cell differentiation and immune responses against lymphomagenesis, which can be found in about 20% of patients with DLBCL and more commonly in the GCB than ABC subtype (Pasqualucci et al., 2011a; Lohr et al., 2012). *CREBBP* mutations have been observed in mice to promote substantial increases in the number of germinal center B cells which promotes development of MYC-driven lymphoma (Hashwah et al., 2017). Deficiencies in CREBBP also reduce expression of genes responsible for germinal center exit, antigen presentation and immune response, promoting lymphomagenesis (Jiang et al., 2017). *EP300* is mutated in roughly 10% of DLBCLs of both ABC and GCB types, and effects are similar phenotypically to *CREBBP* mutations (Pasqualucci et al., 2011b).

3.3 Alterations in other pathways

Various other pathways are involved in the continued survival, proliferation, and immune evasion of malignant DLBCL cells. Subtype N1 is based off alterations in NOTCH signaling, germinal center homing pathways and migration are disrupted (S1PR2 and GNA13) in 38% of EZB type cases. BCL6 signaling disruption is also found in commonly in EZB subtype. *TP53* mutations prevent cell death, and *MYC* mutations are highly associated with MCD and BN2 type (Davis et al., 2010; Schmitz et al., 2018). Evasion of immune surveillance is seen across numerous DLBCL subtypes, and we will now take a closer look at these pathways.

3.3.1 NOTCH signaling

NOTCH genes (*NOTCH1* and *NOTCH2*) code for a series of receptors involved in specialization of cells, cell growth, differentiation, and apoptosis (Notch1 Gene MedlinePlus, 2015). Mutations in *NOTCH1* are a defining characteristic of the N1 subtype, and *NOTCH2* mutations in combination with BCL6 fusions are defining of the BN2 subtype (Schmitz et al., 2018). Mutations in NOTCH1/2 signaling can be activating type in the NOTCH receptor itself or activating mutations of downstream intracellular messengers such as PEST, which can activate nuclear gene transcription (Lee et al., 2009; Arcaini et al., 2015). Inactivating mutations of *SPEN* and *DTX1*, negative regulators of NOTCH signaled transcription, could also lead to overexpression of genes (Schmitz et al., 2018; Miao et al., 2019).

3.3.2 Cell migration

GCB cells under normal conditions should exist only within appropriate germinal centers but are found in circulation and extranodal sites in DLBCL (Montesinos-Rongen et al., 2011; Pham-Ledard et al., 2012; Pham-Ledard et al., 2014a; Pham-Ledard et al., 2014b; Kraan et al., 2014; Oishi et al., 2015; Chapuy et al., 2016; Fukumura et al., 2016; Taniguchi et al., 2016; Cao et al., 2017; Franco et al., 2017; Zheng et al., 2017; Zhou et al., 2018; Miao et al., 2019). Migration of these cells can likely be traced back to inactivation or under-activation of the sphingosine 1-phosphate receptor-2 (S1PR2) (Green et al., 2011; Muppidi et al., 2014; Miao et al., 2019). Under malignant conditions, S1PR2 is inactivated, resulting in a failure of activation of downstream GNA13, which then is unable to activate ARGHEF1, which then cannot activate RHOA, causing

a lack of inhibition of cell migration (Muppidi et al., 2014; Rosenquist et al., 2017; Miao et al., 2019). *GNA13* and *ARHGEF1* normally encode for mediators that control the growth of GCB cells and confine them to germinal centers, so a lack of these mediator's results in their systemic distribution and uncontrolled migration (Morin et al., 2011; Muppidi et al., 2014). The homing pathway encoded by *GNA13* and *ARHGEF1* is disrupted in 38% of EZB subtype cases, showing the importance of this specific pathway (Schmitz et al., 2018).

3.3.3 BCL6 signaling

BCL6 fusions are commonly associated with *NOTCH2* mutations, which make up the defining characteristics of the BN2 subtype (Schmitz et al., 2018). BCL6 in its normal functional capacity is responsible for recruiting histone deacetylase 3 (HDAC3) which deacetylates BCL6-bound transcriptional enhancers, rendering them inaccessible for transcription (Hatzi et al., 2013; Miao et al., 2019). Alterations in the first-noncoding exon of BCL6 also interfere with negative autoregulation, further increasing activity of the gene (Miao et al., 2019). BCL6 regulates plasma cell differentiation (IRF4, PRDM1), cell migration (S1PR1), DNA damage response (ATR, CHEK1), the cell cycle (CDKN1A, CDKN1B), and cell death (TP53) (Miao et al., 2019). Activating mutations of *BCL6* (or activators such as *MEF2B*) or inactivating mutations of BCL6 inhibitors (*EP300*, *CREBBP*, *FBXO11*) lead to suppression of these processes previously mentioned, and promotion of germinal center formation and disruption of plasma cell formation (Cattoretti et al., 2005; Pasqualucci et al., 2011b; Miao et al., 2019).

3.3.4 p53 signaling

p53, encoded for by *TP53*, plays a critical role in activation of genes involved in regulation of cell death and cell cycle progression, including *BAX* and *CDKN1A* (Miao et al., 2019). Inactivation of p53 through deletion of *TP53* is seen in 8%–24% of DLCBL (Jardin et al., 2010) but inactivation can occur through numerous other mechanisms. $p14^{ARF}$ (CDKN2A) normally inhibits MDM2, preventing inactivation of p53 by MDM2, but $p14^{ARF}$ can develop an inactivating mutation leading to constitutive inhibition of p53 by MDM2 (Schmitz et al., 2018; Miao et al., 2019; Lenz et al., 2008a; Ma and Malynn, 202012). Activating mutations of *MDM2* or *MDM4* (another inhibitor of p53) can also lead to continued inactivation of p53, and failure of *CDKN1A*/*BAX* mRNA transcription (Miao et al., 2019). Inactivation of p53 also causes failure of proper cell cycle progression (limited BTG1, BTG2, CDKN1A expression), failure of apoptotic pathways (limited FAS, BCL2 expression), and failure of adequate DNA repair mechanisms (limited BRCA2, ATM, PRKDC expression) (Rosenquist et al., 2017).

3.3.5 MYC signaling

MYC is a proto-oncogene at the center of multiple second messenger pathways and regulates transcription of genes involved in cell growth and proliferation, apoptosis, metabolism, DNA replication, and protein biosynthesis (Dang, 2012; Karube and Campo, 2015). Cells with gene expression characteristic of MYC, and characteristic of proliferation, are highly associated with the MCD and BN2 subtypes (Schmitz et al., 2018). DLBCL with rearrangements involving *MYC* and *BCL2*/*BCL6*, which upregulate *MYC* gene expression, are referred to as double hit or triple hit lymphomas, and are associated with inferior progression free survival and overall survival in patients receiving traditional R-CHOP therapy (Hummel et al., 2006; Savage et al., 2009; Valera et al., 2013; Rosenquist et al., 2017). Interestingly however, *MYC* rearrangements are not predictive of poorer prognosis in patients who receive the DA-EPOCH-R regimen (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, rituximab) suggesting the possible targetability of this pathway (Lai et al., 2018). Another study found MYC partner to be prognostically significant, with *IGH-MYC* translocations having the worst prognosis of all *MYC* translocations, likely because *IGH-MYC* translocations place *MYC* adjacent to immunoglobulin enhancers which leads to constitutive MYC expression (Copie-Bergman et al., 2015; Karube and Campo, 2015). One of the causes of *MYC* translocations with *IGH* or *BCL6* is thought to be mediated by activation-induced cytidine deaminase (AID), which normally is responsible for somatic hypermutation and class switch recombination, but can also target and create breaks in *BCL6* and *MYC* (Shen et al., 1998; Miao et al., 2019). Mutations resulting from incorrect AID-related hypermutation, especially in regions not coding for immunoglobulin genes, has been reported in more than half of DLBCL patients (Gordon et al., 2003; Deutsch et al., 2007; Miao et al., 2019). In addition to activating *MYC* translocations leading to downstream gene upregulation, inactivating mutations of the negative regulator MAX-gene associated protein (MGA) are also implicated in improper MYC expression (Miao et al., 2019). Not only are the MYC aberrations themselves relevant clinically as indicated by trials with DA-EPOCH-R protocol, but being able to further subtype these based on MYC partner or mutation location may provide further prognostic significance (Copie-Bergman et al., 2015; Karube and Campo, 2015; Xu-Monette et al., 2016).

3.3.6 Evasion of immune surveillance

Immune editing and evasion of immune surveillance is a hallmark of the DLBCL, especially the MCD subtype and therefore the ABC type, with 76% of MCD acquiring mutation or deletion of *HLA-A*, *HLA-B*, or *HLA-C* (Chang et al., 2016). Inactivating alterations to MHC class I molecules, through augmentation or deletion of components HLA-A/B/C or β2 microglobulin (B2M) is common among DLBCL in both GCB and ABC types, and results in failed expression of surface MHC class I and subsequent failure to activate cytotoxic $CD8^+$ T cells (Challa-Malladi et al., 2011). GCB types occasionally (10% of cases) also display disruptions in the MHC class II molecules, and thus failure to activate $CD4^+$ T cells, through mutations in *HLA-DMA*/*HLA-DMB* or inactivating mutations in the MHC class II transactivator *CIITA* (Steimle et al., 1994; Schmitz et al., 2018). Natural killer (NK) cells display the CD2 receptor and are activated by the CD58 ligand, so mutations or deletions in *CD58* (21% of all DLBCL and in ABC 68% of the time) cause loss of this extracellular ligand and subsequent failure of NK-mediated cytolysis (Miao et al., 2019). $CD4^+$ and $CD8^+$ T cells also display the CD2 receptor, and activation would also be affected by loss of the CD58 ligand on the tumor cell (Miao et al., 2019). *CD274*/*PDCD1LG2* (PD-L1/PD-L2) gains, amplifications, and translocations are another mechanism by which DLBCL evades destruction by T cells, because binding of this ligand to PD-1 receptors on $CD8^+$/$CD4^+$ T cells prevents activation (Georgiou et al., 2016). Another deleterious mutation of DLBCL cells that can cause failure to activate

immune cells is a loss of CD70, which would normally bind to CD27 on the $CD4^+$ and/or $CD8^+$ T cells to cause activation.

4 Targeted therapeutic strategies in DLBCL

Having explored the various proposed ways to subtype DLBCL, some of the mechanisms behind the transformation of these malignant cells, and the various pathways that are associated with disease, it is now appropriate to delve in to targeted therapeutics. Acknowledging that DLBCL is a group of heterogenous disease processes that result in similar disease state and cellular phenotype, it is understandable that different therapies may be needed to target not only different pathways, but also the same pathway in different ways depending on how it was altered along its mechanism. While these various subtyping methods may not be entirely clinically useful or relevant yet, it is in our best interest to begin conceptually examining pharmaceuticals that could lead to outcomes superior to traditional R-CHOP therapy when applied to these experimental subgroups. We summarize drugs targeting BCR signaling, the PI3K-AKT-mTOR pathway, NF-κB signaling, BCL2 signaling, epigenetic pathways, MYC signaling, and finally immune evasion (Figure 3; Table 2).

4.1 Targeting BCR signaling

Three targets of the BCR signaling pathway that are actionable by current drugs, are PKCβ, SYK, and Bruton's tyrosine kinase (BTK). Enzastaurin is a selective PKCβ inhibitor which would inhibit signal transduction and ultimate pathway activation, but efficacy has yet to be shown with this drug, and clinical failures have been attributed to mutations further down the pathway than at PKCβ (Robertson et al., 2007; Crump et al., 2016; Hainsworth et al., 2016). This drug may be of clinical use in patients with mutations specifically affecting PKCβ, so being able to detect mutations here would be essential in utilizing this treatment. SYK inhibitor (SYKi) entospletinib has shown promise in clinical trials following BTK or PI3Kδ inhibitors, with a response rate of 69% (Sharman et al., 2016). Fostamatinib is another SYKi but has shown little clinical benefit, and likely caused observed side effects (diarrhea, fatigue, nausea, hypertension, cytopenias) because it is a non-selective agent (Friedberg et al., 2010). Another promising agent in this pathway is ibrutinib, a Bruton's tyrosine kinase inhibitor, and overall response rate in one monotherapy trial of refractory DLBCL with ibrutinib was 40% in ABC type and 5% in GBC type (Wilson et al., 2012; Intlekofer and Younes, 2014). This therapy is shown to disrupt the My-T-BCR complex with CARD11-BCL10-MALT1 complex and mTOR, with complete resistance in the ABC subtype provided by *CARD11* gene alterations (Lenz et al., 2008b; Phelan et al., 2018). MYD subtype in combination with *CD79A* and *CD79B* mutations increased susceptibility to ibrutinib, 80% of responses had *MYD88* mutation with concomitant *CD79B* mutation, while the wild type CD79A/CD79B provided protection from this intervention (Wilson et al., 2012; Wilson et al., 2015). One study showed addition of ibrutinib to traditional R-CHOP therapy increased event free survival and overall survival of patients under 60 years of age with non-GCB DLBCL, a notable finding as this is the first time addition of an agent to R-CHOP therapy has improved event free and overall survival of patients (Miao et al., 2019; Younes et al., 2019).

4.2 Targeting PI3K-AKT-mTOR

The PI3K-AKT-mTOR is another commonly active pathway in DLBCL with treatments that have shown promise in limited trials. CUDC-907 is a small molecule that inhibits PI3K (class Iα, β, and δ) and HDAC (class I and II), that showed a response rate of 64% in patients with DLBCL concurrent with MYC alteration (Oki et al., 2017). Other drugs targeting PI3K are pilaralisib (Brown et al., 2015; Bechter et al., 2016), buparlisib (Zang et al., 2014; Stewart et al., 2022), and copanlisib (Paul et al., 2017; Bojarczuk et al., 2019). AKT inhibitor MK-2206 showed promise in preclinical models, but failed to show results in any of the patients treated as part of a phase II trial (Oki et al., 2015). Two drugs showing some clinical significance are the mTOR inhibitors everolimus and temsirolimus, with positive responses observed, and one patient achieved a durable and complete response to the everolimus for years (Iyer et al., 2012; Milowsky et al., 2013; Witzens-Harig et al., 2021; Major et al., 2022). The patient was noted as the only participant in the trial with mutations in both *TSC1* and *NF2*, which were deemed significant to the dramatic efficacy of this drug (Iyer et al., 2012; Milowsky et al., 2013). Another cohort consisting of 24 patients who had not received any prior treatment for their DLBCL were given everolimus added to R-CHOP-21 induction therapy (R-CHOP given over 21 days) and demonstrated no disease progression or relapse after 24 months in all 24 patients (Johnston et al., 2016; Witzig et al., 2017). Further trials with the R-CHOP-21 plus everolimus will be needed to establish this as a superior modality to traditional R-CHOP therapy, but preliminary responses show promise (Johnston et al., 2016; Witzig et al., 2017).

4.3 Targeting NF-κB signaling

Lenalidomide acts on the NF-κB pathway by targeting the E3 ubiquitin ligase component of cereblon, and shows substantial activity in patients with relapsed or refractory DLBCL alone or in combination with other regimens (Wiernik et al., 2008; Hernandez-Ilizaliturri et al., 2011; Witzig et al., 2011; Zinzani et al., 2011; Hitz et al., 2013; Wang et al., 2013; Zhang et al., 2013; Feldman et al., 2014; Hitz et al., 2016; Martin et al., 2016; Czuczman et al., 2017; Ferreri et al., 2017). The greatest effects have been seen in patients with non-GCB or ABC DLBCLs (Wiernik et al., 2008; Hernandez-Ilizaliturri et al., 2011; Witzig et al., 2011; Zinzani et al., 2011; Hitz et al., 2013; Wang et al., 2013; Feldman et al., 2014; Hitz et al., 2016; Martin et al., 2016; Czuczman et al., 2017; Ferreri et al., 2017), and the addition of lenalidomide to CHOP treatment in patients with novel DLBCL seems to negate the negative prognostic implications of non-GCB DLBCL (Nowakowski et al., 2015). Lenalidomide has also been shown to be effective as a maintenance therapy and prolong PFS in patients aged 50–80 who respond to R-CHOP (Reddy et al., 2017; Thieblemont et al., 2017). Another mechanism for the utility of lenalidomide may be realized, aside from acting as a sole inhibitor of the NF-κB pathway, is through synthetic lethality (Kaelin, 2005). Synthetic lethality can be explained simply by the

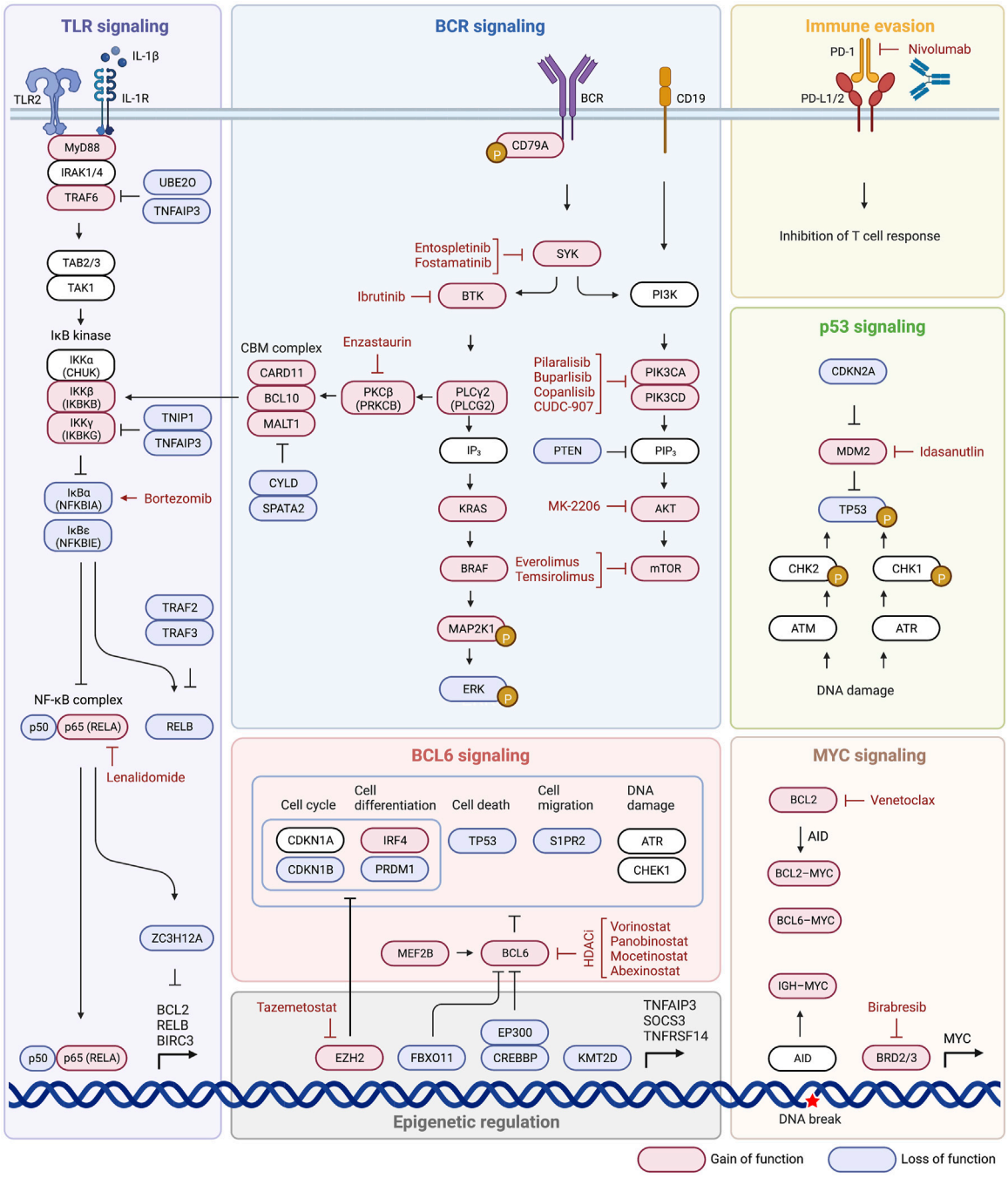

FIGURE 3
Targeted therapeutic strategies and potential inhibitors in DLBCL.

concept of redundancy, targeting of one gene or pathway alone has little to no effect on the cell while targeting both genes or pathways will result in cell death (Kaelin, 2005). This is seen in MYD88 DLBCLs where *MYD88* mutations promote NF-κB signaling, encouraging cell proliferation and survival, while also promoting the production of interferon-beta (IFNβ) which is cytotoxic to DLBCL cells (Yang et al., 2012). IFNβ is negatively regulated by interferon regulatory factor 4 and Spi-B in an inhibitory circuit that is reinforced by chronic BCR stimulation, so the cytotoxic effects of IFNβ are not seen (Yang et al., 2012). Lenalidomide can induce degradation of the inhibitory factors Interferon regulatory factor 4 (IRF4) and IFNβ, and ibrutinib is able to inhibit BCR signaling, resulting in increased IFNβ (through decreased degradation) and a synthetic lethality effect (Yang et al., 2012). This approach to synthetic lethality could prove to be clinically significant in trials, but identification of these synergistic pathways is trailing behind and must be further explored with approaches such as RNA interference screening (Kaelin, 2005). Another drug acting on the NF-κB path is bortezomib which downregulates NF-κB through inhibition of proteasomal degradation of IκBα, but is has failed to show significant efficacy as a monotherapy or in addition to R-CHOP therapy (Goy et al., 2005; Chen et al., 2011; Offner et al., 2015; Leonard et al., 2017).

TABLE 2 Potential therapeutic agents in DLBCL.

Pathway	Target	Therapeutic agent	Reference
NF-κB signaling	NFKBIA	Bortezomib	Chen et al. (2011); Goy et al. (2005); Offner et al. (2015); Leonard et al. (2017)
	RELA (p65)	Lenalidomide	Zhang et al. (2013); Czuczman et al. (2017)
BCR signaling	SYK	Entospletinib	Sharman et al. (2016)
	SYK	Fostamatinib	Friedberg et al. (2010)
	BTK	Ibrutinib	Wilson et al. (2012); Wilson et al. (2015); Younes et al. (2019)
	PRKCB	Enzastaurin	Robertson et al. (2007); Crump et al. (2016); Hainsworth et al. (2016)
PI3K-AKT-mTOR	PIK3CA/PIK3CD	Pilaralisib	Brown et al. (2015); Bechter et al. (2016)
	PIK3CA/PIK3CD	Buparlisib	Zang et al. (2014); Stewart et al. (2022)
	PIK3CA/PIK3CD	Copanlisib	Paul et al. (2017); Bojarczuk et al. (2019)
	PIK3CA/PIK3CD	CUDC-907	Oki et al. (2017)
	AKT	MK-2206	Oki et al. (2015)
	mTOR	Everolimus	Johnston et al. (2016); Witzig et al. (2017)
	mTOR	Temsirolimus	Major et al. (2022); Witzens-Harig et al. (2021)
Epigenetic pathway	EZH2	Tazemetostat	Radford et al. (2016); Italiano et al. (2018); Vincent Ribrag et al. (2018); Sarkozy et al. (2020)
	BCL6	Vorinostat (HDACi)	Siddiqi et al. (2020)
	BCL6	Panobinostat (HDACi)	Assouline et al. (2016)
	BCL6	Mocetinostat (HDACi)	Batlevi et al. (2017)
	BCL6	Abexinostat (HDACi)	Evens et al. (2016)
	BCL2	Venetoclax	Davids et al. (2017); de Vos et al. (2018); Morschhauser et al. (2021); Liu et al. (2018)
MYC signaling	BRD2/3	Birabresib	Delmore et al. (2011); Mottok and Gascoyne. (2015); Li et al. (2019); Amorim et al. (2016)
p53 signaling	MDM2	Idasanutlin	Gu et al. (2019)

4.4 Targeting BCL2 signaling

BCL2 expression is one of the most influential alterations in DLBCL and is already prognostically significant, as DLBCL with *MYC* and *BCL2*/*BCL6* mutations are termed double hit or triple hit lymphomas and are associated with a worse prognosis (Xu et al., 2013). Venetoclax is a BCL2 inhibitor that was shown to have a complete response in 12% of patients when used as a monotherapy, and an overall response rate of 41% when used in combination with bendamustine plus rituximab (Davids et al., 2017; de Vos et al., 2018). In patients with confirmed *BCL2* mutations venetoclax had a superior overall response rate when compared to R-CHOP in the matched population, suggesting the potential of venetoclax to improve outcomes in patients receiving R-CHOP (Morschhauser et al., 2021). One of the possible genes responsible for variable BCL2 inhibitor response is *PMAIP1*, *in vitro* studies have shown that DLBCL cells with amplifications of this gene are more sensitive to drugs like venetoclax (Liu et al., 2018). This further backs the notion that understanding the exact alteration in specific pathways plays a key role in treatment selection and response.

Another mechanism of therapeutic interest may be the inhibition of AID which functions as the driver of somatic hypermutation in B cells (Muramatsu et al., 2000). AID terminally leads to conversion of cytosine residues into uracil residues in single-stranded DNA, and is able to target not only immunoglobulin variable genes or switch recombination sequences, but also transcriptionally active genes such as *BCL6* and *MYC* (Robbiani and Nussenzweig, 2013). AID-mediated off-target mutations and subsequent double-stranded breaks are thought to be associated with oncogenic transformation (Lieber, 2016). Particularly, AID-dependent class-switch recombination is associated with *IGH-MYC* and *IGH-BCL6* translocations because breaks involve the switch region of *IGH* which is characteristic of AID (Lenz et al., 2007). Prevalence of mutations in *BCL2*, *SGK1*, *PIM1*, and *IGLL5* associated with AID were noted in a 2018 study, and predominance of single nucleotide substitutions help to confirm this association between mutations and overactive AID (Chapuy et al., 2018). Possible future therapies may aim to downregulate or completely inhibit the off-target mutations mediated by AID, and reduce the genetic burden on cells and prevent new, treatment-resistant prodigy cells from appearing and propagating.

4.5 Targeting epigenetic pathways

Gene regulation is another great target for manipulation since DLBCL displays frequent disruptions in histone-modifying enzymes and the general activity of genes (Jiang et al., 2017). Tazemetostat is an EZH2 inhibitor with an encouraging safety profile and response in both EZH2 wild-type and mutant relapsed/refractory (R/R) DLBCL, with responses up to 60% in R/R DLBCL (Radford et al., 2016; Italiano

et al., 2018; Vincent Ribrag et al., 2018; Sarkozy et al., 2020). This drug was shown in one trial to be implicated in complete remission among all patients who received 8 complete cycles of tazemetostat in combination with traditional R-CHOP therapy (Sarkozy et al., 2020). Histone deacetylase inhibitors (HDACi) such as vorinostat (Siddiqi et al., 2020), panobinostat (Assouline et al., 2016), mocetinostat (Batlevi et al., 2017), and abexinostat (Evens et al., 2016) show potential benefit in certain patients with B-cell lymphoma when combined with other chemotherapies. Use of HDACi is proving useful especially in CREBBP-mutant cells, to restore acetylation of histones at transcriptional enhancer regions to enhance expression of tumor suppressor genes (Jiang et al., 2017). HDACi's are also of interest in individuals with elevated MYC concurrent with elevated BCL2 levels, and can lead to induction of apoptosis through acetylated BCL6 accumulation (Bereshchenko et al., 2002; Duan et al., 2005; Kurland and Tansey, 2008; Zhang et al., 2012). Patients treated with panobinostat who had R/R DLBCL showed response rates as high as 67% when *MEF2B* mutations were also present, as opposed to 18% in those with wild type, indicating the potential benefit of these therapies (Assouline et al., 2016). Further studies including patients with p53 dysregulation may also be warranted, as these HDACi's also increase levels of acetylated (wild-type) p53 which stimulates pro-apoptotic pathways (Dai and Gu, 2010). Additionally, MDM2 antagonist, idasanutlin, showed potent anti-tumor activity in both ABC and GCB cell lines and idasanutlin could be used as a novel drug in the clinical setting of DLBCL (Gu et al., 2019).

4.6 Targeting MYC signaling

The ability to block overactive *MYC* transcription is of interest because of the genes' involvement in not only overall pathogenesis, but also its association with relapse and refractory DLBCL (Mondello et al., 2017). The bromodomain and extra-terminal (BET) protein family (BRD2, BRD3, BRD4, and BRDT) enhance *MYC* transcription by binding acetylated histones. BET inhibitors (BETi) interfere with BET-mediated *MYC* transcription through disruption of bromodomain-containing proteins which normally organize transcriptional machinery (Delmore et al., 2011; Mottok and Gascoyne, 2015; Li et al., 2019). Birabresib specifically is a drug of interest, and functions through binding to the BRD2 and BRD3, limiting the transcription of *MYC* among other oncogenes (Amorim et al., 2016). The suppression of these other oncogenes may require further exploration to understand the true mechanism of this drug, a phase II clinical trial with birabresib showed a 18% response and 12% complete response among eligible participants, without any apparent correlation with MYC expression (Amorim et al., 2016).

4.7 Targeting immune evasion

Evasion of DLBCL immune surveillance is accomplished through various mechanisms, but PD-L1 (*CD274*) and PD-L2 (*PDCD1LG2*) dysregulation is one of the most notable from a therapeutic standpoint. This pathway is targetable by antibodies that bind and block the PD-L1/2 ligand on the tumor cell, preventing it from binding PD-1/2 receptor and disabling immune escape (Miao et al., 2019). One such anti-PD-L1 antibody that can be administered is nivolumab, which has shown some promise in studies with ORR of 36% in patients with R/R DLBCL (Lesokhin et al., 2016). Failure to respond has been attributed to the relatively small number of patients in the study (16% and 3% respectively) who had 9p21.1 (*CD274*/*PDCD1LG2*) low level copy gains and amplifications (Ansell et al., 2019). An alternate study (KEYNOTE-013) that measured response in patients with and without *CD274* mutations showed a 50% response in patients with copy number gains, amplifications, and translocations of *CD274*, as opposed to a 9% response in those who did not have these mutations (Justin Kline et al., 2018). These results suggest what was proposed, that *CD274* mutations may be predictive of response to anti-PD-L1 antibodies (Justin Kline et al., 2018). A third study consisting of 4 patients with R/R primary CNS DLBCL and 1 patient with primary testicular DLBCL showed a response to nivolumab in 5/5 patients, and 3 patients that remained in continuous remission (Nayak et al., 2017). The success of this trial was attributed to *CD274*/*PDCD1LG2* gains, amplifications, and translocations that are commonly seen in patients with primary CNS DLBCL (60%) and primary testicular DLBCL (60%) and further emphasizes the importance of these PD-1 ligands in predicting PD-1 antibody treatment success (Chapuy et al., 2016; Nayak et al., 2017).

5 Conclusion and future perspectives

DLBCL encompasses a wide array of disease mechanisms and presentations that have historically been and are currently, lumped into essentially two prognostic groups all with the same therapeutic indication. Various novel ways to delineate cases of DLBCL have been proposed by groups such as Schmitz et al., 2018, Chapuy et al., 2016, Lacy et al., 2020, and Wright et al., 2020. These systems have used various algorithms to group DLBCL by "constellations" of genetic alteration instead of examining and grouping each alteration individually, because it is the sum of the parts that leads to the specific disease state and that may point to specific treatment regimen. These groups each had varying amounts of overlap in their classification systems, which may indicate the strength of the proposed subtypes as far as prevalence in the DLBCL population and impact of the established genetic constellation. Mutations in pathways regulating BCR signaling, the PI3K-AKT-mTOR signaling pathway, BCR-dependent NF-κB signaling, NF-κB signaling, TLR signaling, and the BCL2 family are among the most influential when it came to subdividing DLBCL cases into new subgroups. The end result of these pathways, whether it be overstimulation of pro-growth factors or inhibition of apoptotic pathways, leads to the same phenotypic result of continued cell growth and survival, yet cannot be treated as a singular problem. Even therapies directed specifically at these pathways may continue to fail if they treat steps further up the cascade than the mutation actually lies, so having the ability to identify and target multiple steps in these pathways will be a key factor in extending overall survival for these patients. Dysregulation of genes involved in epigenetic regulation such as *EZH2*, *KMT2D*, *EP300*, and *CREBBP* may also result in aberrant pathway activation or inactivation in many DLBCL cases, so exploring treatments directed at regulation of these pathways may also prove to be impactful on outcomes. Other pathways of interest with potential therapeutic interventions are the NOTCH signaling pathway, malignant cell migration resulting from S1PR2 inactivation,

BCL6 signaling, p53 signaling, MYC signaling, and immune evasion through mutations in various receptors and ligands such as PD-L1 overexpression. Therapies currently showing promise include ibrutinib in targeting BCR signaling, everolimus in the PI3K-AKT-mTOR pathway, lenalidomide in targeting NF-κB signaling, venetoclax in BCL2 signaling, tazemetostat in EZH2 epigenetic regulation, birabresib in targeting MYC signaling, and nivolumab in targeting immune evasion.

In order to progress in the treatment of DLBCL, a new classification system must first be implemented as part of NCCN guidelines that will allow physicians to provide better prognostic information, and which may also indicate which second line therapies may have superior response when first line R-CHOP fails. Availability of NGS for use in patients with DLBCL will also need to be increased, as these groupings rely on this in combination with novel algorithms to appropriately place a malignancy in its respective group. Implementation of these groups will also greatly supplement data concerning tracking of disease course by group and response to therapies, because currently the population of DLBCL that has had their disease subtyped or genomically sequenced is relatively low and limited to experimental groups. With larger quantities of data we will be able to further differentiate which mutations are impactful on disease course, and which mutations may indicate a specific or targeted treatment. This will also allow us to increase the specificity of the subgroups and establish a more concrete stratification system. There is little doubt that a newly implemented subtyping system for DLBCL will need revision over time as data is collected, and the standard of care R-CHOP therapy will not be replaced overnight. Currently implementation of these groups will provide us with much needed and much lacking data and will give providers and patients the ability to supplement second- or third-line therapies with regimens targeted to their specific subtype or mutation which has never previously been done. Providing one more therapeutic option before making the move to palliative care is enough justification alone to support the use of new groups and targeted therapies and is step-one on the road to creating a new standard of care for these patients.

Author contributions

TN conceived and carried out study design, literature search, data collection, data interpretation, generation of figures, writing of the manuscript. GS carried out data interpretation and writing of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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