NEW INSIGHTS INTO THE COMPLEXITY OF TUMOR IMMUNOLOGY IN B-CELL MALIGNANCIES: PROGNOSTIC AND PREDICTIVE BIOMARKERS AND THERAPY

EDITED BY: Jérôme Paggetti, Martina Seiffert and Etienne Moussay
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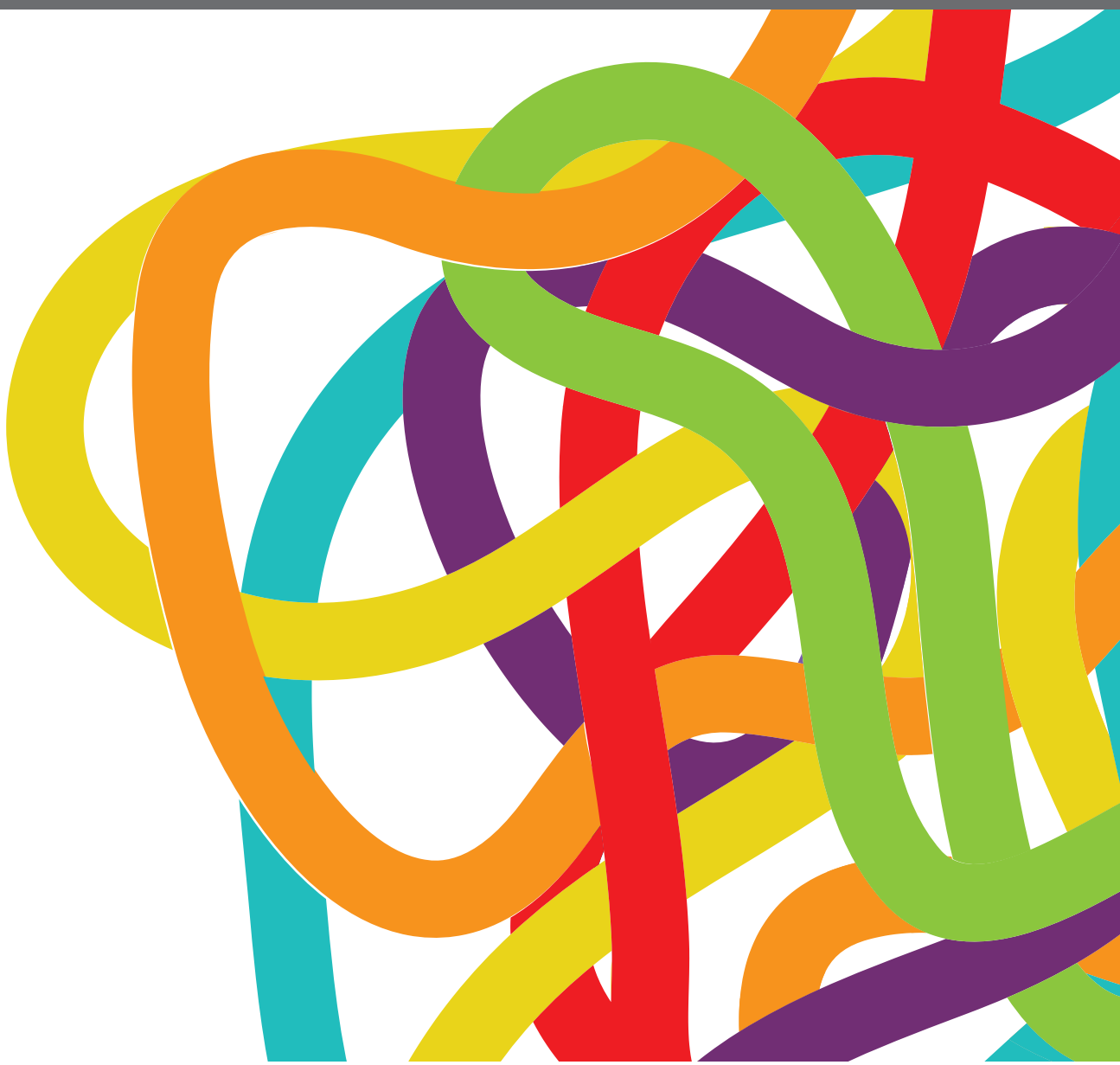

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NEW INSIGHTS INTO THE COMPLEXITY OF TUMOR IMMUNOLOGY IN B-CELL MALIGNANCIES: PROGNOSTIC AND PREDICTIVE BIOMARKERS AND THERAPY

Topic Editors:
Jérôme Paggetti, Luxembourg Institute of Health, Luxembourg
Martina Seiffert, German Cancer Research Center (DKFZ), Germany
Etienne Moussay, Luxembourg Institute of Health, Luxembourg

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EDITORIAL
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Editorial: New Insights Into the Complexity of Tumor Immunology in B-Cell Malignancies: Prognostic and Predictive Biomarkers and Therapy

Etienne Moussay[1]*, *Martina Seiffert*[2] and *Jérôme Paggetti*[1]

[1] Tumor-Stroma Interactions, Department of Cancer Research, Luxembourg Institute of Health, Luxembourg, Luxembourg,
[2] Molecular Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany

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Editorial on the Research Topic

New Insights Into the Complexity of Tumor Immunology in B-Cell Malignancies: Prognostic and Predictive Biomarkers and Therapy

The importance of the tumor microenvironment (TME) in sustaining tumor growth is nowadays well described in hematologic malignancies. The aim of the Research Topic is to summarize for the reader some recent advances related to biomarkers and therapies in B-cell leukemias and lymphomas including multiple cell types, soluble factors, and extracellular vesicles (EV) found in the TME. Specifically, Ondrisova and Mraz describe the role of the TME in triggering pro-proliferative and anti-apoptotic signals for chronic lymphocytic leukemia (CLL) cells and the respective contributions of T cells, stromal cells, and monocytes/nurse-like cells in the secretion of soluble factors. They review the current therapeutic interventions with a focus on ibrutinib and idelalisib, and discuss the associated resistance mechanisms and strategies to overcome them.

Advances for the development of new targeted therapies focused in recent years on B-cell receptor signaling and induction of apoptosis. In addition to ibrutinib and idelalisib, venetoclax (Bcl-2 inhibitor) completes the arsenal of targeted therapies for B-cell malignancies. Bcl-2 inhibition is currently used as first-line therapy and for the treatment of relapsed/refractory CLL and acute myeloid leukemia patients. In this topic issue, Vereertbrugghen et al. show the sensitivity of hairy cell leukemia cells to venetoclax *in vitro*, independently of the disease form (classic, variant, and VH4-34). However, the protection of stromal cells observed in co-culture points to the need to target both leukemic cells and the microenvironment.

Several solid cancers, including from pancreas, breast, and lung, show important signs of inflammation. Since many years, a high systemic immune-inflammation index (SII), associated with neutrophil, platelet, and lymphocyte counts, is reported as a poor prognostic marker. Wang et al. perform a retrospective study on 224 patients to evaluate the power of SII in diffuse large B-cell lymphoma (DLBCL). They compare SII with the pretreatment neutrophil-lymphocyte ratio (NLR) and the platelet-lymphocyte ratio (PLR) reported to be associated with disease outcome. In multivariate analyses, a high SII correlates with a poor overall survival, demonstrating its suitability as an accurate prognostic factor.

The TME is a very plastic entity, being under the influence of the tumor and therefore being constantly remodeled. In this topic, Menzel et al. extensively describe the angiogenic process as a

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Edited and reviewed by:
Alessandro Isidori,
AORMN Hospital, Italy

***Correspondence:**
Etienne Moussay
etienne.moussay@lih.lu

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major component of TME remodeling in lymph nodes (LN). The authors describe the vasculature as a major contributor to inflammation and cancer development in multiple lymphoma subtypes. Very interestingly, although crucial for anti-tumor immune response, immune cells also contribute to the production of neoangiogenic factors. The endothelium being crucial for effective immune infiltration, the authors discuss the careful design of therapeutic strategies to limit neoangiogenesis without hindering the success of emerging cellular immunotherapies. The myeloid compartment has also recently gained interest in lymphoma. Ferrant et al. precisely describe the diversity of myeloid derived suppressor cells of monocytic and polymorphonuclear origins, their phenotypes and their functional roles and identify S100A9^{high} circulating myeloid cells as promising diagnostic and prognostic biomarkers in DLBCL.

Tumor-derived EV, also called exosomes, contribute as well to the plasticity of the TME, by transferring RNA and protein to target cells and by affecting cellular signaling through direct ligand-receptor interactions. Due to their characteristics and content, EV can be used as potential biomarkers to allow an early detection of tumor cells in an organism. Gargiulo et al. review the advances in the field of EV diagnostics, how EV contribute to immune evasion, and finally discuss recent strategies using engineered EV as a valuable therapeutic tool against cancer and specifically B-cell malignancies.

The easy detection of surface receptors makes them highly appreciated for biomarker development. The transmembrane glycoprotein CD200 is overexpressed by CLL cells and plays an important role in disease development and progression. D'Arena et al. describe CD200 structure, physiologic functions and its immunosuppressive role towards T cells in cancer. Based on an extensive review of the literature on CD200 expression in patients with B-cell malignancies, the authors discuss the relevance of CD200 for the diagnosis of CLL and how it helps to discriminate CLL from similar entities (e.g. mantle cell lymphoma), and its potential prognostic role.

The recurrent mutations in the splicing factor SF3B1 are associated with a defect in DNA damage response (DDR) and the generation of cryptic transcripts in CLL which are subject to degradation *via* nonsense-mediated mRNA decay (NMD). Using a cohort of treatment naïve CLL patients, Leeksma et al. study the DDR response to irradiation in SF3B1 mutated CLL patients and highlight the influence of prior treatments in their previous findings based on a mixed treatment cohort. In addition, they detect cryptic transcripts in CLL cells and activation of NMD, and therefore suggest that NMD modulatory agents can benefit patients with mutant SF3B1. Understanding the interactions between cancer cells and the TME is crucial for efficient targeting. In an effort to identify ligand-receptor pairs associated with B-cell precursor acute lymphoblastic leukemia, Wu et al. analyze RNA-sequencing data for survival analysis and prognostic model construction. The authors identify 57 ligand-receptor pairs in the autocrine network of B cells and 29 other pairs related to the communication with myeloid cells, some of which could be linked with survival outcomes. The analysis of large cohorts is valuable for the design of future therapeutic interventions. The *IGHV* mutational status has a high prognostic value and contributes to the determination of optimal treatments in CLL patients. In this issue, Bagnara et al. perform deep DNA-sequencing in CLL leukemic clones and confirm the high intraclonal *IGHV-IGHD-IGHJ* diversification. But surprisingly, the presence of subclones was similar among *IGHV* mutated and unmutated CLL patients. The expansion of subclones appears downstream of the dominant clone. The authors then intensely discuss the mechanisms involved in targeting the immunoglobulin loci and the different microenvironmental inputs in the CLL LN leading to dysregulated B-cell functions and aggressive disease.

This Research Topic brings together critical reviews and original research articles describing the TME in B-cell malignancies, reporting on the use of new genetic and non-genetic biomarkers having prognostic and diagnostic values, and suggesting their use to optimize the response to therapies for patients.

AUTHOR CONTRIBUTIONS

All authors edited the Research Topic. EM wrote the editorial. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Diagnostic and Therapeutic Potential of Extracellular Vesicles in B-Cell Malignancies

Ernesto Gargiulo[1], Pablo Elías Morande[1,2], Anne Largeot[1], Etienne Moussay[1†] and Jérôme Paggetti[1*†]*

[1] Tumor-Stroma Interactions, Department of Oncology, Luxembourg Institute of Health, Luxembourg, Luxembourg, [2] Instituto de Medicina Experimental (IMEX)-CONICET-Academia Nacional de Medicina, Buenos Aires, Argentina

Extracellular vesicles (EV), comprising microvesicles and exosomes, are particles released by every cell of an organism, found in all biological fluids, and commonly involved in cell-to-cell communication through the transfer of cargo materials such as miRNA, proteins, and immune-related ligands (e.g., FasL and PD-L1). An important characteristic of EV is that their composition, abundance, and roles are tightly related to the parental cells. This translates into a higher release of characteristic pro-tumor EV by cancer cells that leads to harming signals toward healthy microenvironment cells. In line with this, the key role of tumor-derived EV in cancer progression was demonstrated in multiple studies and is considered a hot topic in the field of oncology. Given their characteristics, tumor-derived EV carry important information concerning the state of tumor cells. This can be used to follow the outset, development, and progression of the neoplasia and to evaluate the design of appropriate therapeutic strategies. In keeping with this, the present brief review will focus on B-cell malignancies and how EV can be used as potential biomarkers to follow disease progression and stage. Furthermore, we will explore several proposed strategies aimed at using biologically engineered EV for treatment (e.g., drug delivery mechanisms) as well as for impairing the biogenesis, release, and internalization of cancer-derived EV, with the final objective to disrupt tumor–microenvironment communication.

Keywords: extracellular vesicles, exosome, CLL, leukemia, lymphoma, myeloma, EV-based therapy

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Edited by:
Cyrus Khandanpour, University Hospital Münster, Germany

Reviewed by:
Shaji Kumar, Mayo Clinic, United States
Ingo Ringshausen, University of Cambridge, United Kingdom

***Correspondence:**
Jérôme Paggetti
jerome.paggetti@lih.lu
Etienne Moussay
etienne.moussay@lih.lu

†These authors share senior authorship

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INTRODUCTION

Extracellular vesicles (EV) are vesicular components released by every cell of an organism. This broad group consists of small EV or exosomes (sEV; 30–150 nm) characterized by an endocytic origin, microvesicles (MV; 100 nm–5 μm) shed from the plasma membrane, and apoptotic bodies (<5 μm) derived from membrane disintegration (1). The study of EV is a process in constant

Abbreviations: ALL, acute lymphocytic leukemia; ALV, artificial lipid vesicles; BTK, Bruton tyrosine kinase; CLL, chronic lymphocytic leukemia; ctDNA, circulating tumor DNA; CTX, cyclophosphamide; DC, dendritic cell(s); DLBCL, diffuse large B-cell lymphoma; EBV, Epstein–Barr virus; EGFR, epidermal growth factor receptor; EV, extracellular vesicle(s); HL, Hodgkin lymphoma; HM, hematological malignancy; HS, heparan sulfate; ICP, immune checkpoint; IFN-γ, interferon-γ; IL6ST, interleukin 6-signal transducer; MDR, multiple drug resistance; MSC, mesenchymal stem cell(s); MM, multiple myeloma; MV, microvesicle(s); NK, natural killer(s); NV, nanoparticle(s); PG, proteoglycans; sEV, small extracellular vesicle(s); shRNA, small hairpin RNA; SIRPα, signal regulatory protein α; SMART, synthetic multivalent antibodies retargeted; TGF-β(1), transforming growth factor-β(1); TNFR1, tumor necrosis factor receptor 1.

evolution, where EV characteristics, as well as nomenclature and isolation, are in continuous improvement (2–5).

Extracellular vesicles can be virtually found in every biofluid, which makes them relatively easy to recover and analyze (6). In the early years of EV-focused research, their synthesis and release were considered as a mechanism to remove harming material from the cell (7). Nowadays, this notion has been replaced by considering the mechanism as an active way to transfer material to targeted cells (1). Indeed the most common and accepted role of EV is intercellular communication through ligand–receptor interactions and the transfer of molecular cargoes.

The composition of EV is extremely heterogeneous. Depending on their nature, origin, physiological context, and parental cells, EV contain and transfer distinct elements to targeted cells, such as proteins, nucleic acids, lipids, metabolites, and organelles (8–10). The interest in understanding the role of EV in cancer is due to their unique ability to re-educate and attenuate the normal activity of healthy cells across the whole body. In line with this, B-cell malignancy-derived EV have been shown to interact with the surrounding microenvironment, leading to its profound remodeling. EV-based communication assists cancer development by stimulating tumor cell proliferation and migration (11), by modifying distant microenvironment to generate a pre-metastatic niche (12), and by strategically inhibiting tumor immune surveillance and anti-tumor response (13, 14).

EV DIAGNOSTIC AND THERAPEUTIC POTENTIAL

Due to a complex and parental-cell-dependent molecular cargo and to their presence in every biological fluid, EV represent an innovative tool for the design of diagnostic and therapeutic strategies in B-cell malignancies. In the sections below, we will explore the multiple potential uses of EV: (i) EV as biomarkers, (ii) EV as therapeutic targets, (iii) EV in immune evasion and use in immunotherapy, and (iv) biologically engineered EV (**Figure 1** and **Table 1**).

EV as Biomarkers

Given that EV are continuously released by all the cells and that they can be recovered from every fluid in the body, they clearly represent a potential source of biomarkers (15, 16). Compared with most common biomarkers obtained from liquid biopsies, such as circulating tumor DNA (ctDNA), EV represent a more reliable source of information and can be easily incorporated in the diagnostic routine. Specifically, ctDNA represents only a minimal fraction of plasma-cell-free DNA (17, 18); thus, despite its high specificity, the starting amount is already challenging (17, 19). Furthermore, ctDNA is often affected by high fragmentation and low stability (20, 21). EV are very stable and, depending on the time of use, can be stored at different temperatures for extended periods of time (several weeks at 4°C, several months at −80°C, and several years in liquid nitrogen) (22). Cancer cells generally produce high amounts of EV that mirror the characteristics—as a sort of fingerprint—of the parental cancer cells (23–25). Indeed cargo composition (e.g., microRNA and proteins) and transmembrane molecules (tumor antigens or immune cell markers) allows the identification of the parental cell and its physiological state (24, 26). Analysis of EV isolated from patients was confirmed to be an important tool in the detection of cancer development at an early stage and to stratify patients based on intrinsic tumor characteristics and for following patients in remission (16, 27, 28).

In line with this, we have shown that the plasma of a patient affected by chronic lymphocytic leukemia (CLL) is highly enriched in multiple key microRNAs (e.g., miR-21, miR-155, and miR-146a) which can potentially affect multiple pathways involved in the pathogenesis and the progression of the disease. MicroRNA expression profile can also be used to stratify CLL patients in view of a more personalized treatment and follow-up (29). Importantly, we and others found these same microRNAs and some additional ones (miR-148a and Let-7g) also in CLL-derived EV, demonstrating how these vesicles can indirectly provide information on cellular state (30, 31). Van Eijndhoven and colleagues detected a higher level of EV carrying miR-24-3p, miR-127-3p, miR-21-5p, miR155-5p, and Let7a-5p in the plasma of Hodgkin lymphoma patients compared with controls. Upon treatment, patients showing remission had a stable reduction of these miRNAs compared with relapse patients who displayed their progressive accumulation (32). Interestingly, a further study performed on a large multiple myeloma (MM) cohort described that the progression-free survival and the overall survival of patients could be successfully followed by monitoring the plasma levels of miR-18a and Let7b, which were found to be highly enriched in MM-derived EV (33).

Beyond miRNA content, RNA packed in plasma EV has the potential to be used to define tumor mutation landscape, such as BRAF V600E for melanoma, with the aim to detect early-stage disease or define the best clinical approach for certain patients (34). This strategy may be transposed to mutated RNA contained in circulating EV from B-cell malignancies, with the attempt to perform a personalized patient follow-up and therapy.

EV reflect parental cell also through the presence of cellular markers on the EV membrane and in their protein cargo (35). Different studies show that CLL-derived EV from a patient's plasma present typical markers of leukemic cells (e.g., CD19, CD20, CD5, and CD37) and major histocompatibility complex (e.g., HLA-A, HLA-B, and HLA-C) (36, 37). Despite this correlation, it is essential to highlight that not all markers or their possible combinations are always present on EV released by tumor cells. Indeed CLL cells can release a subset of EV carrying CD52 but only few CD19 molecules; this particular marker combination was shown to be characteristic of CLL patients with advanced disease (38). Another study by Harshman and colleagues showed the presence of typical MM cell (CD38, CD138, and CD147) and treatment resistance (CD44)-associated markers on MM-derived EV isolated from a patient's serum (39, 40).

In sum, EV represent a faithful snapshot of the parental cell that synthesized them. The follow-up of proteins

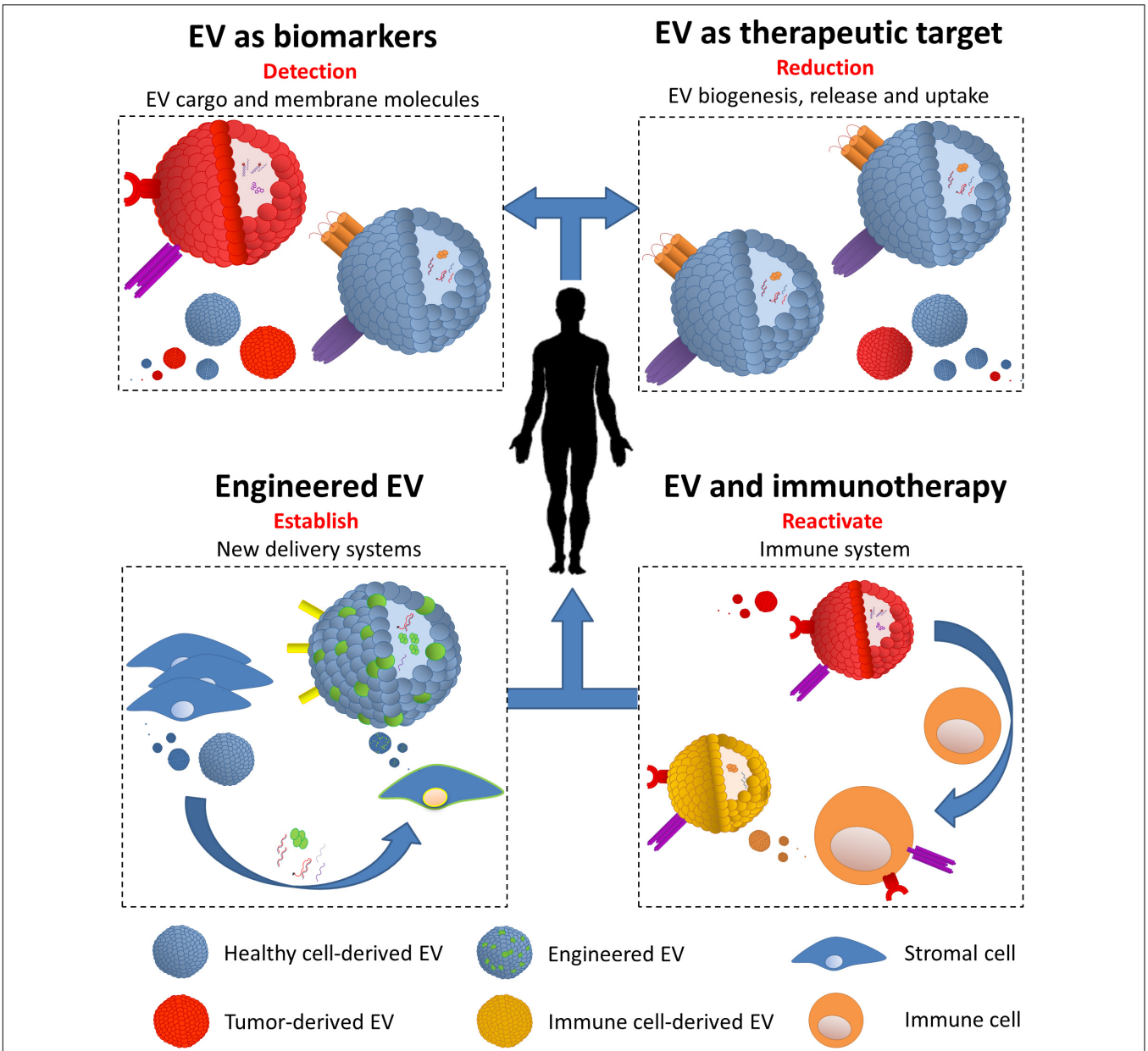

FIGURE 1 | Extracellular vesicles (EV) diagnostic and therapeutic potential. (i) EV as biomarkers: multiple physiological characteristics allow EV to be easily collected from different biological sources (e.g., plasma and urine). Definition of content and carried molecules is essential to determine their origin, thus detecting tumor in different stages of the disease and in post-treatment follow-ups. (ii) EV as therapeutic targets: biogenesis, release, and uptake are all key steps which can be targeted in order to decrease the circulating amount and the function of tumor-derived EV. Beyond this, targeting active EV components (e.g., immune checkpoint) decreases their impact on various aspects on targeted cells. (iii) EV and immunotherapy: tumor cells have the ability to re-educate the immune system via the release of EV, thus able to affect the function of multiple immune cells. Despite this, tumor-derived EV can be strategically used to build a strong immune response by the endogenous immune cells (e.g., dendritic cells) and use their EV to diminish cancer cell functions. (iv) Engineered EV: together with nanoparticles, EV can be modified to carry specific molecules (e.g., shRNA and chemotherapy drugs) and directed to cancer and its microenvironment cells. Engineered EV can be produced by transformed cell lines (e.g., mesenchymal stem cells) and directed against tumor cells.

and RNAs carried by circulating EV in B-cell malignancy patients constitutes a promising source of predictive biomarkers. The low invasiveness of their isolation could allow regular sampling, and by monitoring EV content, the evolution of tumors within patients can be followed, either before or after anti-cancer treatment, allowing clinicians to select the best course of action for each specific case.

EV as Therapeutic Targets

Biogenesis, release, and internalization of EV are tightly regulated processes that involve multiple putatively targetable protein

TABLE 1 | Overview of the diagnostic and the therapeutic potentials of extracellular vesicles in B-cell malignancies.

EV Role	Target	B-Cell malignancy	References
Biomarker	miR-21, miR-155, miR-146a, miR-148a, and Let-7g	CLL	(29–31)
	miR-24-3p, miR-127-3p, miR-21-5p, miR155-5p, and Let7a-5p	HL	(32)
	miR-18a and Let7b	MM	(33)
	CD19, CD20, CD5, CD37, CD52, and MHC	CLL	(36, 37)
	CD38, CD138, CD147, and CD44	MM	(39, 40)
Target	EV release (indomethacin)	DLBCL	(42)
	EV uptake (low molecular weight heparin)	CLL	(30)
	TGF-β1 (shRNA)	ALL	(44, 71)
Immune modulator	T lymphocytes (DC-derived EV) and DC (poly I:C)	ALL	(58)
	TGF-β1 (shRNA)	ALL	(44, 71)
Vaccination	DC (DLBCL-derived EV)	DLBCL	(78)
	T lymphocytes (DC-derived EV)	ALL	(64)
Carrier	c-Myc (siRNA)	Lymphoma	(70)
	TGF-β1 (shRNA)	Lymphoma	(44, 71)
	CD21 (Gp350)	CLL	(72)
	Apoptosis (Apo2L/TRAIL)	MM	(73)

CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; MM, multiple myeloma; ALL, lymphocytic leukemia; HL, Hodgkin lymphoma.

complexes. Impairing the pro-tumoral effect of EV can be achieved by acting through two main axes. On one hand, the autocrine signals, where EV bind to and modify neoplastic cells themselves, could be disrupted (41). On the other hand, the role of EV in the cross-talk between cancer cells and the supportive microenvironment could be targeted. This can have a major impact on decreasing tumor survival, proliferation, and migration (42).

In B-cell malignancies, Koch and colleagues demonstrated that treating several diffuse large B-cell lymphoma (DLBCL) cell lines with indomethacin, a non-steroidal anti-inflammatory drug, suppressed EV release and strongly decreased tumor progression. They further demonstrated that a decreased EV release guarantees a more potent effect of cytostatic drugs, such as anthracyclines and anthracenediones, both *in vitro* and *in vivo* (42). Another strategy implies the reduction of EV uptake by blocking key factors, e.g., heparan sulfate proteoglycans (HSPGs), on target cells (41). HSPGs have been suggested to act as a receptor for EV internalization. In line with this, we demonstrated that pre-treatment of EV with low molecular weight heparin, a HS analog, strongly decreases the uptake of CLL-derived EV by target cells (30).

Several studies have demonstrated how EV have a strong impact on the immune system, allowing a more efficient tumor immune evasion (13). One of the characteristics associated with this phenomenon is the presence of immune checkpoint (ICP) ligands on tumor-derived EV. Chen et al. (43) recently showed an increase in PD-L1 molecule on melanoma derived-EV upon interferon-γ stimulation. Considering that PD-L1 and, potentially, other ICP molecules are present on EV derived from B-cell malignancies characterized by a strong immune-suppressed microenvironment (e.g., CLL), therapies such as recombinant blocking antibodies can represent an effective solution in targeting immune-suppressive EV. The same strategy can also be extended to other EV molecules; for instance, targeting leukemia-derived EV bearing transforming growth factor-β1 (TGF-β1) has been shown to improve anti-leukemia immunity (44).

Molecules carried by tumor-derived EV are not the only source of harming. EV released by tumor microenvironment B-cells carry CD39 and CD73, two surface molecules able to hydrolyze ATP released by dying cancer cells to adenosine and effectively hijack CD8 T cell immune activity by binding the A2A adenosine receptors (45). In line with this, it is possible to speculate that B-cell malignancy-derived EV may have a similar effect, if not greater, given their higher concentration in the tumor milieu. The study of Zhang et al. (45) shows that a decrease of B-cell-derived EV bearing CD73 and CD39 can be achieved by deregulating the docking protein RAB27A. This has been performed using an inactivated Epstein–Barr virus-mediated siRNA, but it is also possible to generate EV (e.g., derived from modified stroma cell lines) carrying RAB27A siRNA and specifically deliver it to tumor cells.

Targeting B-cell malignancy EV represents an interesting strategy in the treatment of cancer. These approaches imply obvious risks and limitations, such as possible drug resistance and off-targets, which can ultimately lead to decreased efficacy of the therapy.

Role of EV in Immune Evasion and Use in Immunotherapy

The immune system is one of the main players involved in cancer cell recognition and elimination (46–48). However, cancers deploy numerous strategies to repress the immune system during disease development and, thus, effectively evade immune surveillance. The concept behind immunotherapy is to re-activate the patient's immune system in order to recognize and remove cancer cells. One example of the many mechanisms involved in immunoevasion is the use of EV to fully re-educate

the immune microenvironment, causing a domino effect and ultimately leading to overall immune repression and cancer development (13).

Being released by neoplastic cells, EV represent an important source of selective antigens that educate naïve immune cells (49, 50). As for solid tumors (51–53), leukemia-derived EV are loaded with antigens and several immunogenic molecules, such as TGF-β and IL-6, capable to impair dendritic cells (DCs) to build a specialized immune response against neoplastic cells (54–57). EV structure and characteristics allow them to specifically drive DC-dependent immunization; in fact, stimulation of DCs with only leukemic cell lysate results in failure to build an appropriate immune response (55, 56).

Immunotherapy can also be used in parallel with chemotherapy. Guo and colleagues combined leukemia-specific DC-derived EV with cyclophosphamide and polyinosinic-polycytidylic acid sodium salt (poly I:C) (58). This combination is based on the ability of DC-derived EV to stimulate T lymphocyte proliferation and enhance their cytotoxic activity against leukemia, while poly I:C acts on DC maturation. This strategy led to leukemia cell suppression *in vitro* and increased survival of tumor-bearing mice (58).

Targeting tumor-derived EV surface molecules is also a valuable strategy to strongly reduce their activity on immune cells. As we have previously mentioned, TGF-β1 is enriched in EV released by a wide range of tumors and, acting on various immune cells such as DCs, is one of the main causes of immune escape (57). EV released by acute lymphocytic leukemia (ALL) L1210 cell line carry high levels of TGF-β1. By using small hairpin RNA (shRNA), Huang and colleagues knocked down TGF-β1 in the ALL cell line, thus removing it from their EV and reconstituting DC maturation and activity *in vivo*. Additionally, the same group demonstrated that pulsed DCs were able to increase T cell proliferation and enhance cytotoxic activity against ALL cells (44).

Dendritic cells are not the only immune cells that can be used to re-establish a proper cytotoxic activity against B-cell malignancies. Another valid target is natural killer (NK) cells, whose activity is repressed by molecules, such as NKG2D ligands, exposed on hematological malignancy (HM)-derived EV (59, 60). One particular study presented a double role of the NKp30 ligand BAG6 in CLL. First, high levels of soluble BAG6 can be detected in the plasma of CLL patients, being one of the causes for the defect in NK cytotoxicity observed in these patients (61). At the same time, BAG6 is released *via* EV by healthy cells in response to cellular stress and has a protective activity by enhancing NK cytotoxicity (60). Based on this, it is possible to hypothesize that treatment with $BAG6^+$ EV derived from stressed cells may be a reliable opportunity to maintain (and possibly to re-establish) NK activity during cancer development.

Despite their evident activation effects on the immune system, the use of EV-based vaccination is still under discussion, although several studies already suggest how EV can be used to build an effective immune response before tumor cells arise. In line with this, DC cells pulsed with DLBCL-derived EV have been shown to stimulate T lymphocyte expansion and, by consequence, increase anti-lymphoma immunity in mice (62). These results are in accordance with other studies where leukemia-derived EV can be used to build anti-leukemia immunity, with evident results both *in vitro* and *in vivo* (54, 63). Finally, Qazi and colleagues showed that EV antigens alone are enough to induce memory T lymphocytes through B-cell activation (64).

Altogether these findings position EV as potent immune modulators that can become a major tool for immunotherapy and vaccine design.

Engineered EV

The ability of EV to bind to specific receptors on both tumor and microenvironment cells through surface molecules makes them an interesting tool to transfer exogenous cytotoxic and inhibitory molecules for therapeutic purposes (65). Furthermore, EV represent a useful method to deliver anti-tumor drugs due to their ability to retain stable concentrations of the components loaded in them as well as a natural accumulation in vascular sites, such as inflammation and wounds, tumor, and infection areas (66–69).

Lunavat and colleagues successfully engineered EV-like nanoparticles (NV) that contain siRNA and showed that silencing c-Myc by this approach efficiently activates poly (ADP-ribose) polymerase-dependent apoptotic pathways in treated λ820 lymphoma cells (70). More recently, shRNA strategy has been used to silence TGF-β1 in lymphoma cells, forcing them to release TGF-β1-depleted EV. By removing this strong antitumor–immune surveillance inhibitor, the authors achieved an increase in the response of the immune system against leukemic cells (71). In line with this, engineered EV were also used to transfer specific antigens, with the purpose of re-activating or enhancing the immune system. Stromal cells transfected with the Epstein–Barr virus (EBV) protein gp350 release $gp350^+$ EV that specifically interacts with CD21 on B-cells. $Gp350^+$ EV were further engineered to carry CD154. By treating CLL patients' cells, the authors achieved the internalization of EV in leukemic cells and a strong immunogenic effect, leading to a dual activation of tumor-associated and EBV-specific T cells (72).

Further strategies based on EV-like nanostructures have been explored in B-cell malignancies. Artificial lipid vesicles (ALV) decorated with bioactive Apo2 ligand/TNF-related apoptosis-inducing ligand were tested on lymphoma and MM cell lines. These novel ALV showed a consistent pro-apoptotic effect in multiple HM cell lines while sparing normal cells (such as $CD4^+$ and $CD8^+$ T cells) *in vitro* and with no sign of toxicity *in vivo* (73).

Engineered EV can be broadly produced *in vitro* using a wide range of cell lines. Mesenchymal stem cells (MSCs) have been shown to be a valuable tool for the production of EV with high tropism toward tumor cells. In line with this, MSCs can be modified to release engineered EV with various downstream applications and means (74).

As mentioned before, ICPs on EV surface are important targets for immunotherapy. As alternative to monoclonal antibodies, EV released by modified MSCs can be used as antagonists for delivering immune checkpoint blockade proteins (e.g., ICP receptors or monoclonal antibodies) (75). A further well-known immune escape strategy applied by tumors is the

overexpression of CD47, also carried by tumor-derived EV, which interacts with the signal regulatory protein α (SIRPα) on phagocytic cells, limiting their activity (76). The production of EV bearing SIRPα molecules has been shown to successfully hamper the tumor "don't-eat-me" signal in favor to macrophage phagocytosis (77).

Recently, a new class of engineered EV has been tested, with the aim to enhance T cell activity against tumor cells. Synthetic multivalent antibodies retargeted (SMART) EV bear monoclonal antibodies against CD3 and cancer cell-associated epidermal growth factor receptor (EGFR). These characteristics allow them to act as a bridge directing T cytotoxic cells toward tumor cells, thus inducing crosstalk and enhancing antitumor response (78). SMART EV may have a potential application on MM due to its high level of EGFR, which guarantee proliferation and resistance to conventional therapies (79, 80). Furthermore, the same strategy could be applied for other B-cell malignancies, such as CLL and lymphoma, by targeting highly enriched surface molecules.

EV are also able to epigenetically reprogram target cells and to completely change their phenotype in a short time (81, 82). A potential strategy in the re-activation of the immune system can be to inhibit the molecules responsible for this deep change as well as creating engineered EV carrying specific cargo components (e.g., siRNA and shRNA) that are able to lift the immune cell exhaustion by rewiring the epigenetic landscape.

An interesting application for engineered EV is to use them as an instrument for decoy of cytokines (e.g., pro-inflammatory cytokines). This strategy actively restrains tumor effect on microenvironment cells (e.g., reducing inflammation). An example comes from a recent preprint where stroma-derived EV were engineered to bear tumor necrosis factor receptor 1 and interleukin 6-signal transducer. The authors reported striking effects concerning inflammation reduction and survival of experimental mouse models (83). A possible application of this strategy in B-cell malignancies would be to decoy important cytokines related to immune suppression, such as IL-10 and TGF-β1 (44, 84).

Various studies have successfully used EV as a drug delivery system. The rationale to generate EV containing specific drugs is based on cell ability to encapsulate exogenous material and release either actively (e.g., microvesicles budding) or by cellular death consequence (apoptotic bodies). Based on this, EV released by drug-treated tumor cells have been used to deliver specific chemotherapeutic agents to untreated tumor cells (85). EV-based drug delivery has been tested on various cancer models, including multiple drug resistance (MDR). In each case, EV shielded the therapeutic agents and delivered them to tumor cells, demonstrating a higher cytotoxic effect compared with its administration alone (85, 86). Furthermore, Osterman et al. (87) have shown how EV can be used to encapsulate toxic drugs, such as curcumin, and be specifically directed to cancer cells, highly reducing any side effect. Despite the fact that no EV-based drug delivery has been established for B-cell malignancies, strategies to encapsulate chemotherapeutic agents should be explored. One of the many possibilities could be to encapsulate the ceramide supplement C6 in EV to target MM cells. Indeed Chang and colleagues showed *in vitro* how C6 ceramide treatment leads to increased apoptosis and block the proliferation of MM cells by upregulating miRNA clusters such as miR-202 and miR-16 (88).

Engineered EV, as well as artificial NV, are powerful and plastic tools to be deployed against B-cell malignancies. As mentioned above, it is evident that one of the multiple advantages lies in the possibility to combine this strategy with other well-established approaches, such as those aiming at reactivating the host immune system. Furthermore, using engineered EV with a tropism toward specific cells may improve the targeting of tumor EV. Loading shRNA into these delivery systems has the potential to reduce tumor EV production, release, and uptake. Finally, it is important to consider the high affinity of engineered EV for tumor cells as a strategic tool to deliver chemotherapy drugs, thus reducing any off-target side effects.

ONGOING CLINICAL TRIAL USING EV

Several clinical trials involving EV are currently ongoing. In cancer, the vast majority are applied to solid tumors. The aims are various: to establish novel sources and standardized methods to isolate EV from patients (NCT03821909), to deepen the characterization of cancer EV as predictive biomarkers (NCT03830619), to develop EV-based vaccination (NCT01159288), and finally to further improve EV-based treatment (NCT03608631).

In reference to B-cell malignancies, the ExoReBly project (NCT03985696) has the overall aim to characterize DLBCL-derived EV from patients' samples. The rationale of this clinical trial is based on the fact that half of the patients subjected to immunotherapy—typically with the aim to stimulate immune activity against CD20—fails to show any benefit. The major hypothesis is that the presence of a high amount of CD20 and PD-L1, present on DLBCL-derived EV, acts as a decoy target for rituximab antibody and as a strong immunosuppressive signal, respectively, leading to therapeutic resistance. Apart from the characterization of DLBCL-derived EV, the project aims to use these EV as a marker of response to therapy and disease outcome. Interestingly, we made a similar observation with CLL-derived EV presenting high levels of CD20 on their surface and being able to act as a decoy for rituximab, potentially highlighting a conserved mechanism among B-cell malignancies (30).

With over 70 clinical trials, EV are gaining more attention in view of practical and clinical applications. A small percentage of these clinical trials take in consideration HM, and only one is being developed in B-cell malignancies. Nevertheless, every year, new information concerning EV impact on cancer progression is generated, which strongly suggests that more applications for HM will arise.

CONCLUSION

The path to decipher the complex characteristics of EV composition in B-cell malignancies, as well as the biological role that they accomplish in the behavior of tumors, has shown

important advances in the last decade. The increasing amount of novel data recently generated reflects a continuous interest for studying EV within the community, due in part to their potential as a tool to improve cancer diagnosis and therapy. It is now well established that EV can mirror the tumor cells they originate from and can carry key targetable molecules strongly related to cancer biology, like immune checkpoints among many others. Consequently, several additional important discoveries herein summarized pinpoint a central role attributed to EV in B-cell driven diseases diagnosis and follow-up and for putative novel treatment strategies.

However, these reports remain in the area of translational research but are not yet successfully translated to clinical applications for patients with B-cell malignancies. While the treatment of solid cancers using an EV-derived rationale is already ongoing in advanced clinical trial phases, this is neither the case for the diagnosis and the follow-up nor for any treatment in B-cell-originated neoplastic diseases. The fact that EV-based approaches work properly in other chronic neoplastic and non-neoplastic diseases represents a motivation to fully enhance initiatives that use this rationale in HM in the near future. Thus, the upcoming decade will hopefully shed light into the clinical applicability of EV as a powerful tool for patients with B-cell neoplastic disorders.

AUTHOR CONTRIBUTIONS

EG wrote the manuscript and created the figure. PM and AL helped in writing the manuscript. EM and JP finalized the manuscript and supervised the team. All authors contributed to the article and approved the submitted version.

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REVIEW
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Genetic and Non-Genetic Mechanisms of Resistance to BCR Signaling Inhibitors in B Cell Malignancies

Laura Ondrisova[1,2] *and Marek Mraz*[1,2*]

[1] Molecular Medicine, CEITEC Masaryk University, Brno, Czechia, [2] Department of Internal Medicine, Hematology and Oncology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czechia

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***Correspondence:**
Marek Mraz
marek.mraz@email.cz

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The approval of BTK and PI3K inhibitors (ibrutinib, idelalisib) represents a revolution in the therapy of B cell malignancies such as chronic lymphocytic leukemia (CLL), mantle-cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), or Waldenström's macroglobulinemia (WM). However, these "BCR inhibitors" function by interfering with B cell pathophysiology in a more complex way than anticipated, and resistance develops through multiple mechanisms. In ibrutinib treated patients, the most commonly described resistance-mechanism is a mutation in *BTK* itself, which prevents the covalent binding of ibrutinib, or a mutation in *PLCG2*, which acts to bypass the dependency on BTK at the BCR signalosome. However, additional genetic aberrations leading to resistance are being described (such as mutations in the *CARD11*, *CCND1*, *BIRC3*, *TRAF2*, *TRAF3*, *TNFAIP3*, loss of chromosomal region 6q or 8p, a gain of Toll-like receptor (TLR)/MYD88 signaling or gain of 2p chromosomal region). Furthermore, relative resistance to BTK inhibitors can be caused by non-genetic adaptive mechanisms leading to compensatory pro-survival pathway activation. For instance, PI3K/mTOR/Akt, NFkB and MAPK activation, BCL2, MYC, and XPO1 upregulation or PTEN downregulation lead to B cell survival despite BTK inhibition. Resistance could also arise from activating microenvironmental pathways such as chemokine or integrin signaling *via* CXCR4 or VLA4 upregulation, respectively. Defining these compensatory pro-survival mechanisms can help to develop novel therapeutic combinations of BTK inhibitors with other inhibitors (such as BH3-mimetic venetoclax, XPO1 inhibitor selinexor, mTOR, or MEK inhibitors). The mechanisms of resistance to PI3K inhibitors remain relatively unclear, but some studies point to MAPK signaling upregulation *via* both genetic and non-genetic changes, which could be co-targeted therapeutically. Alternatively, drugs mimicking the BTK/PI3K inhibition effect can be used to prevent adhesion and/or malignant B cell migration (chemokine and integrin inhibitors) or to block the pro-proliferative T cell signals in the microenvironment (such as IL4/STAT signaling inhibitors). Here we review the genetic and

non-genetic mechanisms of resistance and adaptation to the first generation of BTK and PI3K inhibitors (ibrutinib and idelalisib, respectively), and discuss possible combinatorial therapeutic strategies to overcome resistance or to increase clinical efficacy.

Keywords: B cell malignancies, ibrutinib, resistance, adaptation, targeted therapy, B cell receptor, BCR inhibitor

INTRODUCTION

The BCR signaling pathway plays a central role in the onset and progression of mature B cell malignancies, such as chronic lymphocytic leukemia (CLL), mantle-cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), or Waldenström's macroglobulinemia (WM). Activating mutations in the BCR signaling pathway are commonly found in DLBCL, FL, or WM (1). Though these mutations are usually missing in CLL and MCL, BCR signaling is constitutively activated and is a key player in their pathogenesis (2–5). Introducing "B cell receptor (BCR) inhibitors" in recent years has marked a revolution in treating B cell malignancies since many patients are responsive to the inhibitors of BCR-associated kinases BTK or PI3K, such as ibrutinib and idelalisib, respectively. They are now widely used as a first-line treatment or to treat relapsed/refractory diseases. However, the patient response to them varies across B cell malignancies in clinical trials as well as in real-world setting, and a large percentage of patients develop resistance or have to stop the therapy due to toxicities associated with these inhibitors' long-term use (6–14).

In this review, we summarize the genetic and non-genetic mechanisms of resistance and adaptation to the first generation of BTK and PI3K inhibitors (ibrutinib and idelalisib, respectively), and discuss possible therapeutic strategies to overcome resistance or increase clinical efficacy by using combinatorial therapeutic strategies. We also discuss the complexity of the mechanisms of action of "BCR inhibitors" and how this affects the choice of potential combinatorial therapy.

BCR SIGNALING AND ITS CROSS-TALK WITH OTHER PATHWAYS

Cell-surface immunoglobulin does not have any kinase activity itself. It is non-covalently connected to disulphide-linked heterodimers Igα and Igβ (CD79A, CD79B). After recognition and antigen binding, BCRs start to aggregate and change their conformation, which concludes in phosphorylation of tyrosine-based activation motifs (ITAMs) on Igα and Igβ's cytoplasmic domains. This phosphorylation, mediated by Src-family kinase LYN, creates a docking site for spleen tyrosine kinase (SYK) (15). The activated SYK then phosphorylates the B cell linker protein (BLNK), an adaptor protein helpful in recruiting other molecules such as Bruton tyrosine kinase (BTK). BCR stimulation also leads to phosphorylation of co-receptor CD19 and PI3K adaptor BCAP by LYN and SYK, which afterwards activates phosphoinositide 3-kinase (PI3K) leading to PIP3 generation (16, 17) (**Figure 1**).

PIP3 helps to recruit GRB2-associated-binding protein 1 (GAB1), 3-phosphoinositide-dependent protein kinase 1 (PDK1, also known as PDPK1), protein kinase B (PKB, Akt), and BTK to the plasma membrane *via* their pleckstrin homology (PH) domain. Here, Akt is phosphorylated on S473 by mTORC2 which also facilitates Akt phosphorylation on T308 by PDK1 leading to full Akt activation (18). PI3K signaling is further positively regulated by the adaptor protein GAB1, which recruits additional PI3K molecules generating more PIP3 (19, 20). On the other hand, the amount of PIP3 is negatively balanced by the activity of phosphatases such as SHIP1, SHP1, and PTEN.

PIP3 is also needed for optimal BTK activation, since it helps to translocate BTK to the cell membrane and *via* the interaction with its PH domain, it allows the activation of BTK's kinase activity (21). For full BTK activation after the recruitment to the cell membrane, phosphorylation at two sites is needed. Firstly, BTK gets phosphorylated by SYK or LYN at tyrosine Y551, which then leads to autophosphorylation at Y223 (22, 23). Fully activated BTK phosphorylates phospholipase Cγ2 (PLCγ2). PLCγ2 hydrolyses PIP2 into secondary messengers inositol triphosphate (IP3), which controls intracellular Ca^{2+} levels, and diacylglycerol (DAG) which, *via* protein kinase Cβ (PKCβ) activation, induces cRaf-MEK-Erk pathway activation. PKCβ also activates CARD11, which then forms a complex with MALT1 and BCL10 to activate TAK1 (24). Afterwards, TAK1 phosphorylates IKKβ which initiates the NFκB pathway (25). Apart from this, PKCβ plays a role in negative feedback regulation of BCR signaling by removing BTK from the plasma membrane by phosphorylating BTK on S180 (26). Non-redundant negative regulation is also mediated by LYN kinase, since mouse B cells with LYN knockout have a surprisingly stronger BCR signaling suggesting that LYN has a specific role in negatively regulating the pathway (27). BCR signaling propensity is also affected by levels of cell-surface molecules that act as docking sites for positive or negative BCR pathway regulators, which include molecules such as CD19, CD22, and CD32. Recently, we have shown that a notorious therapeutic target in B cell malignancies, CD20, is also a positive BCR signaling regulator (28). When CD20 is silenced, response to BCR stimulation is weaker, as underscored by the lower phosphorylation of BCR-associated kinases and impaired calcium flux (29, 30). Moreover, an additional layer of regulation involves small non-coding RNAs (microRNAs) that influence both the positive and negative regulation of BCR signaling propensity (20, 31–38).

BCR signaling is activated in the lymphatic tissue microenvironment and is closely intertwined with the pathways responsible for the cell homing and adhesion (5). BCR activation affects adhesion *via* integrin VLA4 formed by

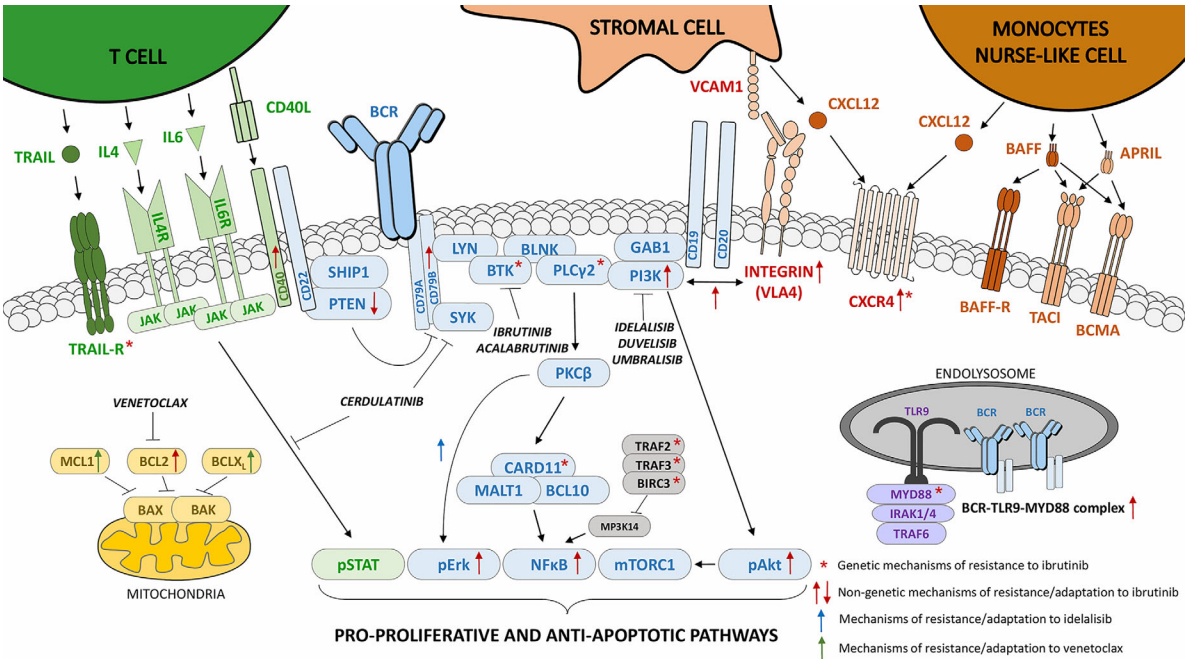

FIGURE 1 | The genetic and non-genetic mechanisms of resistance to BTK or PI3K inhibition in B cell malignancies. The "*" indicates genetic mechanisms of resistance to ibrutinib (BTK inhibitor), the red arrows indicate non-genetic mechanisms of resistance/adaptation to ibrutinib, the blue arrows indicate mechanisms of resistance/adaptation to idelalisib (PI3K inhibitor), and the green arrows indicate mechanisms of resistance/adaptation to venetoclax (BH3-mimetic [BCL2 inhibition]).

CD49d and CD29 (integrin β1); together BCR and VLA4 provide B lymphocytes with adhesion and enhanced signaling (39). CD49d activation causes SYK phosphorylation and, on the other hand, BCR stimulation leads to VLA4 activation (40–42). BCR stimulation also increases chemotaxis towards chemokines such as CXCL12 produced in the microenvironment. Binding of CXCL12 to its receptor CXCR4 activates PI3K, MAPK, and STAT3, and leads to actin polymerization and cell migration (43–45). In CLL, cell-surface IgM levels and BCR signaling is increased by the IL4 produced by T cells which also activates the JAK1-3/STAT6 pathway and upregulates the levels of anti-apoptotic proteins from BCL2 family, resulting in partial malignant B cell protection from the effects of "BCR inhibitors" (46, 47). The importance of the microenvironment can be well illustrated in CLL, where malignant B cells are dependent on constant re-circulation between the peripheral blood and lymph nodes, where they are supported by pro-survival signals from mesenchymal stromal cells, monocyte-derived nurse-like cells, and T lymphocytes (29, 43, 48–50). The supportive stromal cells produce not only chemoattractants CXCL12 and CXCL13 but also BAFF, APRIL, CD31, and plexin B1 which protect CLL cells from spontaneous and induced apoptosis by activating BCR and NFκB signaling (43, 49, 51, 52). Kinases of the BCR pathway BTK and PI3Kδ together with JAK are also involved in T cell dependent proliferation induced by CD40L and IL21, which can be inhibited by ibrutinib, idelalisib or JAK inhibitor (53).

Overall, there is crosstalk between the BCR, chemokine signaling and cell adhesion pathways. Therefore, the success of "BCR inhibitors" lies not only in inhibiting the BCR pathway itself but also in inhibiting other processes. In CLL and some lymphomas, BTK/PI3K inhibition results in malignant B cells egressing from the lymph nodes, causing transient lymphocytosis in patients (8, 54, 55).

MECHANISTIC EFFECTS OF IBRUTINIB ACTION

Ibrutinib is an orally administered small-molecule inhibitor targeting BTK. It binds to BTK covalently, selectively, and irreversibly, inhibiting its phosphorylation and enzymatic activity. BTK is an important kinase in BCR signaling needed for B cells to properly develop (56, 57). Inhibiting it with ibrutinib leads to a loss of pro-survival signals from BCR activation by ligands, and also impairs the "tonic" BCR signals that sustain B cell survival. BTK inhibition decreases cell proliferation as well as interferes with the activation of downstream molecules in BCR pathway such as PLCγ2, Akt and Erk irrespective of BCR stimulation (58–61). As BTK is not only involved in BCR signaling (see above), ibrutinib also disrupts CXCR4 internalization, impairs migration toward CXCL12 and also indirectly decreases total BTK levels (62). Ibrutinib further disrupts signaling from CXCR5 and integrins, molecules that allow B lymphocyte migration and adhesion

(63, 64). Altogether, ibrutinib inhibits BCR stimulation, B cell proliferation, and migration toward homing chemokines such as CXCL12 and CXCL13. It also blocks BCR-dependent CCL3 and CCL4 chemokine release in CLL and decreases CCL4, CCL22, and CXCL13 levels in the serum of ibrutinib-treated MCL patients (54, 61). As mentioned above, inhibiting the adhesion and homing capacity causes transient lymphocytosis in CLL and MCL patients (54, 55). In most of the patients, this resolves within 8 months after starting therapy (55). CLL cells together with non-malignant immune cells after ibrutinib treatment are of a quiescent phenotype as shown by the expression of the genes involved in senescence and/or cell quiescence (30, 55, 65). Apart from the mentioned mechanisms of ibrutinib action, it also affects the microRNAs' expression, resulting in higher levels of several tumor suppressors and inhibition of cell proliferation (66, 67).

Ibrutinib has been approved for therapy of CLL, MCL, WM, and marginal zone lymphoma (MZL). Though it is a potent drug, not all patients are responsive to ibrutinib and a significant number of them acquire resistance to the treatment or discontinue the therapy due to toxicities that are most likely caused by ibrutinib off-target inhibition of molecules such as BLK, JAK3, EGFR, and several TFK members (for a list of off-targets of ibrutinib and other BTK inhibitors see **Supplementary Table 1**). In the following sections, we will summarize the genetic mechanisms of ibrutinib resistance, the non-genetic mechanisms of adaptation/resistance by activating compensatory pro-survival pathways and describe possible solutions to different types of ibrutinib resistance (**Figure 1**).

GENETIC MECHANISMS OF IBRUTINIB RESISTANCE

Genetic mechanisms of primary or acquired resistance to ibrutinib have been widely studied and recurrent mutations associated with resistance have been described in B cell malignancies (**Table 1**, **Figure 1**). Whole-exome sequencing revealed mutations in BCR-involved proteins BTK and PLCγ2 in ~80% of CLL patients with acquired resistance to ibrutinib (7, 96), however, some studies have reported a much lower frequency of these mutations (97, 98). The most common mutation in *BTK* is a C481S point mutation which interferes with the binding of ibrutinib to BTK (7, 68). Other mutations in the *BTK* gene were also found in ibrutinib-resistant patients and have been suggested to affect either ibrutinib binding to BTK or

TABLE 1 | Recurrent mutations in ibrutinib-resistant patients and possible therapeutic strategies to overcome them.

Mutated gene/aberration	Disease	Mechanism	Possible therapeutic strategy	Ref.
BTK	CLL, MCL, WM, MZL	reversible binding of ibrutinib	third-generation BTK inhibitors, PROTAC-BTK, inhibitors of LYN and SYK	(7, 68–77)
PLCG2	CLL, MCL, WM, MZL	BTK-independent activation	inhibitors of RAC2, LYN, and SYK	(7, 68–71, 78, 79)
CARD11	CLL, MCL, WM, DLBCL, FL	↑ NFκB	proteasome or MALT1 inhibitor	(12, 71, 80–83)
BIRC3, TRAF2, TRAF3	MCL	↑ NFκB	MP3K14 inhibitor	(84, 85)
CCND1	MCL	cell cycle progression	unknown	(86)
CDKN2A and *MTAP* co-deletion	MCL	cell cycle progression	PRMT5 inhibitor	(87)
SMARCA2, *SMARCA4*, *ARID2*	MCL	disruption of SWI-SNF complex; ↑ BCL_{XL}	BCL_{XL} inhibitor	(88)
$MYD88^{mt}/CD79B^{wt}$	DLBCL	MYD88-dependent and BCR-independent subtype	SYK or STAT3 inhibitor	(9, 89, 90)
KLHL14	DLBCL	↑ MYD88-TLR9-BCR super-complex	inhibition of BCR-dependent NFκB activation/mTOR inhibitors	(91)
TNFAIP3	DLBCL	↑ NFκB	unknown	(82)
2p+	CLL	↑XPO1	XPO1 inhibition (selinexor)	(92)
Del 8p	CLL	Loss of *TRAIL-R*, insensitivity to TRAIL-induced apoptosis	unknown; possibly venetoclax	(93)
Del 6q	WM	↑ MYD88/NFκB, loss of regulators of apoptosis	unknown	(94, 95)
Del 8p	WM	↑ TLR/MYD88, loss of *DOK2*, *BLK* and *TNFRSF10A/B*	unknown	(94)

↑ represents pathway/gene activation or upregulation.
"Del" stands for deletion of a chromosomal region.

BLNK binding to BTK, altogether leading to PLCγ2 activation even in the presence of ibrutinib (93, 99–101) (**Supplementary Table 2**). *PLCG2*, a gene coding for PLCγ2, seems to mostly harbor gain-of-function mutations when PLCγ2 can be activated by RAC2 or SYK and LYN without BTK (7, 78, 79, 100) (**Supplementary Table 2**). As mutations in *BTK* and *PLCG2* occur early before relapse on ibrutinib, they may serve as a biomarker to indicate a need to change the therapy before disease progression (102). They can also co-occur, which may bring complications to solving the resistance with next-generation BTK inhibitors (103). Apart from the mentioned mutations, resistance to ibrutinib in CLL has also been associated with chromosomal aberrations such as 8p deletion and gain in the 2p region (92, 93). The 8p region contains a gene for TRAIL receptor and its deletion results in insensitivity to TRAIL-induced cell death (93). On the other hand, 2p gain causes exportin-1 overexpression (XPO1), regulating the transport of proteins between the nucleus and cytoplasm (92). Additionally, CLL patients that progress or develop Richter's transformation on ibrutinib recurrently harbor mutations of tumor-suppressor *TP53*, splicing factor *SF3B1*, or NFκB pathway regulator *CARD11*, however, whether these genetic aberrations may directly impact response to ibrutinib during Richter's transformation remains unclear (80). Though, despite good initial response in most of CLL patients, it has been shown that mutations in *TP53* are responsible for a worse prognosis in a long-term ibrutinib treatment and they also partially protect CLL cells *in vitro* from ibrutinib-induced apoptosis and inhibition of proliferation (104–107). This might be related to the recently described role of p53 in the negative regulation of BCR signalling (31, 32).

Mutations in *BTK* and *PLCG2* have also been found in MCL and WM patients with acquired ibrutinib resistance as well as in one MZL patient (69–71). List of the most common *BTK* and *PLCG2* mutations is provided in the **Supplementary Table 2**. Though CLL cells on ibrutinib have a decreased NFκB binding to DNA elements, activating an alternative NFκB pathway by genetic changes is another mechanism responsible for ibrutinib resistance, mostly in MCL (65). This is caused by mutations in *BIRC3*, *TRAF2*, or *TRAF3*, whose absence leads to MP3K14 enzyme stabilization and constitutive activation of alternative NFκB pathway (**Figure 1**) (84, 85, 108, 109). Recurrent mutations in MCL patients who have relapsed on ibrutinib have also been found in *CARD11*, a protein responsible for BCR-induced NFκB activation, or in *CCND1*, a cyclin that promotes G1-S cell cycle progression (24, 81, 86). *CDKN2A* and *MTAP* co-deletion was also observed in ibrutinib-resistant MCL tumors (87). Additionally, loss and/or mutations in the SWI-SNF chromatin remodeling complex lead to the upregulation of anti-apoptotic BCL_{XL} and cause a primary or acquired resistance to the combination of ibrutinib and venetoclax in MCL (88).

In Waldenström's macroglobulinemia (WM), mutations in *CARD11* also lead to ibrutinib resistance, and in WM patients the ibrutinib resistance may be accompanied by 6q and 8p chromosome region deletions that expand from pre-existing clones or emerge during treatment (71, 94). These chromosomal regions contain important signaling pathway regulators. The 6q region loss involves negative regulators of MYD88/NFκB (*TNFAIP3, HIVEP2, TRAF3IP2, IRAK1BP1*), an inhibitor of BTK (*IBTK*), and regulators of apoptosis (*FOXO3, BCLAF1, PERP*). The genes deleted on 8p include *DOK2*, a TLR/MYD88 signaling inhibitor, *BLK*, another target of ibrutinib that is important for B cell proliferation and differentiation, and *TNFRSF10A/B*, a gene encoding for TRAIL receptor (94, 95). Common mutations in WM are WHIM-like mutations in *CXCR4* and L265P mutations in *MYD88*, a mediator of Toll-like receptor signaling (110). In WM, $MYD88^{L265P}$ activates NFκB *via* BTK and IRAK1/4, making these cells sensitive to ibrutinib (110, 111). WHIM-like mutations in *CXCR4* are responsible for impaired CXCR4 internalization upon stimulation and result in constant Akt and Erk activation. However, mutations in *MYD88*, which occur in 90% of WM patients, seem to have a more profound effect on the WM cell survival than $CXCR4^{WHIM}$, as MYD88 inhibition could not be rescued by the $CXCR4^{WHIM}$ mutation (112). These facts might explain why, even though WHIM-like mutations promote ibrutinib resistance *in vitro*, $MYD88^{wt}$/$CXCR4^{wt}$ patients have a lower response rate to ibrutinib therapy than $MYD88^{L265P}$/$CXCR4^{wt}$ or $MYD88^{L265P}$/$CXCR4^{WHIM}$ patients (13, 113).

In DLBCL, ibrutinib seems to be more effective in patients with an activated B cell-like DLBCL (ABC-DLBCL) subtype rather than in patients with germinal center B cell-like DLBCL (GC-DLBCL) due to constitutively active BCR signaling in ABC-DLBCL. However, even amongst ABC-DLBCL, complete or partial response was only detected in 37% of patients (9, 82), and a phase III clinical trial confirmed the benefit of adding ibrutinib to R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone) therapy only in younger patients with non-GC-DLBCL (114). As for resistance, it has been shown that DLBCL patients carrying mutations in *MYD88* and simultaneously having wild-type *CD79B* are primarily resistant to ibrutinib. As other combinations ($MYD88^{mt}$/$CD79B^{mt}$, $MYD88^{wt}$/$CD79B^{mt}$, $MYD88^{wt}$/$MYD88^{wt}$) are sensitive to ibrutinib, there is a possibility of an MYD88-dependent but BCR-independent ABC-DLBCL subtype (9). These findings might be explained by the formation of a multiprotein super-complex consisting of MYD88, TLR9 and BCR. It activates the NFκB pathway and is found in ABC-DLBCL tumors that respond to ibrutinib (**Figure 1**) (115). On the other hand, cells with inactivated *KLHL14*, a negative BCR component regulator often mutated in DLBCL, induce the NFκB pathway by activating the MYD88-TLR9-BCR super-complex, which partially protects them from ibrutinib-induced cell death (91). The role of knockout of the *KLHL14* tumor suppressor was demonstrated in rescuing the ABC-DLBCL cell line from apoptosis when IgM and CD79A were knocked down, which is a manipulation normally lethal to the DLBCL cells (91). Lastly, the above mentioned mutations in *CARD11* and inactivating mutations in *TNFAIP3*, a negative NFκB regulator, were also found in DLBCL patients not responding to ibrutinib treatment (82).

The genetic mechanisms of resistance, especially the *BTK* mutations that develop during ibrutinib therapy, are a clear indication of the on-target effects of the utilized small molecular inhibitors. The other mutations in the BCR signaling pathway members such as *PLCG2* or *CARD11* also demonstrate that malignant B cells try to gain or lose gene activity in order to overcome the inhibition of the key BCR-associated kinase (**Table 1**). However, it has been shown that cancer cells can also utilize non-genetic mechanisms to bypass the inhibition of a key pathway (116–120). In the following section we will review these potential mechanisms for ibrutinib/BTK-inhibition based therapy, and suggest the implications for combinatorial therapeutic strategies (**Table 2**).

NON-GENETIC MECHANISMS OF ADAPTATION TO IBRUTINIB

Patients resistant to ibrutinib that harbor the above-mentioned mutations often have these mutations subclonally in a relatively small fraction of surviving malignant B cells. Malignant non-mutated cells that co-exist with BTK^{C418S} mutated cells might be protected by the mutated cells though this has only been conceptually demonstrated in $MYD88^{mt}$ WM and ABC-DLBCL. Mutated cells showed Erk1/2 activation, which led to pro-survival chemokine release and protection of BTK^{wt} cells (147). Even if this could explain the survival of the non-mutated sub-populations of cells, there are still ibrutinib-resistant patients (>20% of ibrutinib-resistant patients in CLL) that do not show any genetic mutations responsible for the resistance (96). This, together with slow cell apoptosis during ibrutinib therapy, suggest that there are non-genetic mechanisms of resistance and cells are able to partially adapt to ibrutinib and BTK inhibition (**Table 2**, **Figure 1**). Collectively, it seems that malignant B cells activate BTK-independent compensatory survival pathways under ibrutinib treatment, mainly PI3K/mTOR/Akt pathway and adhesion. Activating the NFκB pathway also induces some degree of ibrutinib resistance. This was described mainly by genetic mechanisms as mentioned before, but the non-canonical NFκB pathway together with MAPK signaling can also be activated and thus protect the cells from ibrutinib in MCL cells by activating CD40 (148, 149).

Activating the PI3K pathway is a well-known mechanism that rescues BCR deficient mature B cells from apoptosis (150, 151). It is therefore not surprising that more and more studies see a similar mechanism in malignant B cells by which they overcome BTK inhibition. Activated Akt has been observed in ibrutinib-resistant CLL and DLBCL cell lines, together with downregulated FoxO3a and PTEN levels (**Figure 1**) (121). Activating Akt/MAPK *via* CD79B overexpression has been sufficient to induce ibrutinib resistance in primary ABC-DLBCL (152). Ibrutinib also synergizes with CRISPR-Cas9 knockout of PI3Kδ (91). PI3K pathway activation was observed in B cell lymphoma patient-derived xenograft models with acquired resistance to ibrutinib and the growth of these tumors was blocked by combination of ibrutinib and idelalisib (122).

However, activation of PI3K/mTOR/Akt signaling after ibrutinib has been best described in MCL. Activated Akt and Erk levels, but not BTK, correlate with the response to ibrutinib in MCL cell lines and furthermore, ibrutinib-responsive patients have dephosphorylated Akt as opposed to non-responsive patients (69, 153). Ibrutinib in MCL strengthens cell adhesion *via* integrin dimer VLA4 formed by β1-integrin and CD49d and activates the PI3K/Akt pathway in this way (**Figure 1**). Zhao *et al.* described this phenomenon by showing that ibrutinib-resistant cells have a higher β1-integrin expression that helps to form an ILK-Rictor complex that activates a pro-survival mTORC2/Akt pathway. This was disrupted by ibrutinib in combination with dual mTOR1/2 inhibitor AZD8055 or dual PI3K-mTOR1/2 inhibitor BEZ235 (123). Here, it is also worth mentioning that ibrutinib-resistant MCL samples upregulate the mTOR signaling pathway (as well as genes involved in cell cycle regulation and MYC targets) compared to ibrutinib-sensitive cells (87). A similar compensatory survival mechanism was seen by Guan and colleagues who demonstrated that stromal cells protect MCL cells from ibrutinib-induced death *via* their interaction with VLA4. A combination of ibrutinib with VLA4 blockage or with an inhibitor of PI3K catalytic p110α subunit disrupted the interaction and overcame the resistance (124).

In CLL, the cells lose the ability to adhere to fibronectin almost completely and partly to stromal cells when treated with ibrutinib *in vitro* for a short period of time (63, 154). Interestingly though, BCR stimulation activates VLA4 in CLL cells exposed to ibrutinib for an extended time *via* a BTK-independent

TABLE 2 | Non-genetic mechanisms of resistance/adaptation to ibrutinib and possible therapeutic strategies to overcome them.

Mechanism of resistance/adaptation	Disease	Possible therapeutic strategy	Ref.
↑ PI3K-Akt pathway	CLL, MCL, DLBCL	PI3K, mTOR, or XPO1 inhibitor	(92, 115, 121–132)
↑ JAK-STAT	CLL	Dual SYK/JAK-STAT inhibitor (Cerdulatinib)	(133)
↑ MYC	MCL	HSP90 inhibitor	(134)
↑ MAPK pathway	CLL, MCL, DLBCL	MEK inhibitor	(125, 135, 136)
↑ BCL2	CLL, DLBCL	BCL2 inhibitor (venetoclax)	(30, 125, 129, 137–143)
Metabolic reprogramming	CLL, MCL	OXPHOS inhibitor, inhibitor of fatty acid oxidation	(87, 144)
Integrin-mediated protection	CLL, MCL	VLA4 inhibition (FAK inhibitor)	(45, 145)
Resistant cancer stem cells	MCL	Wnt pathway inhibitor	(146)

↑ *represents pathway/gene activation or upregulation.*

manner involving PI3K. Also, higher CD49d levels in patients prevent ibrutinib-induced lymphocytosis and cause a lower nodal response. This translates into a shorter progression-free survival in ibrutinib treated patients (42). Analogically, it has been described that ibrutinib induces CXCR4 expression on cell-surface, which might translate to a paradoxically increased responsiveness of these cells to chemokine ligands, however, it is likely that the response to chemokines in this context is not completely physiological since BTK is involved in the chemokine pathway (30, 155). Time to progression can also be predicted by cell-surface IgM levels prior to ibrutinib treatment, suggesting another non-genetic mechanism of ibrutinib resistance in CLL, potentially similar to the CD79B overexpression in DLBCL (**Figure 1**) (152, 156). In CLL, the mechanism might be explained by BCR signaling bypassing BTK upon BCR crosslinking as ibrutinib is not able to properly inhibit Ca^{2+} mobilization and Erk1/2 phosphorylation when surface IgM levels are high (156). Moreover, cell-surface IgM levels rise during ibrutinib treatment in CLL patients, although this might depend on the time since the start of therapy (30, 157).

Activating the MAPK pathway might also be one of the compensatory mechanisms for BTK inhibition, as noted in a recent study. Upregulation of the genes involved in MAPK was observed by Forestieri et al. in residual CLL cells after ibrutinib treatment and in addition, acquired mutations in *BRAF*, *NRAS*, and *KRAS* were found in a fraction of patients (135). This might explain the observed synergy between MEK inhibitors with "BCR inhibitors" in B cell lymphomas (125, 136).

Besides the described pathways, other ibrutinib resistance mechanisms are also possible and fit the biology of B cells. It has been shown that MYC acts as a key downstream BCR effector, and its over-expression can rescue the absence of BCR activity in some B cells (158, 159). Indeed, upregulation of MYC has been observed in ibrutinib-resistant MCL cell lines and this resistance can be reversed by inhibiting HSP90 (134). Protection from ibrutinib can also be provided by cells in the microenvironment (133, 160, 161). On the other hand, CLL cells resistant to BTK inhibition recover the ability to produce and respond to IL4 and require less T cell help for growth (162). Lymphoma relapse can potentially also arise from cancer stem cells described in MCL and FL. Their quiescent phenotype, together with high ABC-transporter activity gives them general drug-resistant properties (163–166). Indeed, the MCL-initiating cells were found to be resistant to ibrutinib and could be eliminated by inhibiting Wnt signaling pathway whose genes were overexpressed in these cancer stem cells (146).

TARGETING IBRUTINIB RESISTANCE

There are several potential ways to overcome ibrutinib resistance such as i) in cases with specific *BTK* mutations using third generation BTK inhibitors which do not target C481, or PROTAC mediated BTK degradation, ii) using different molecular targets once a patient is resistant to ibrutinib, or iii) preventing the resistance by a more rapid B cell elimination that would lower the chance of developing resistance or activating compensatory survival pathways (**Figure 1**, **Tables 1** and **2**). Additionally, time-limited or more selective treatment would likely lower toxicities in patients as up to 40% of CLL patients discontinue ibrutinib therapy, which is caused mostly by the toxicities (11, 167).

Unfortunately, second-generation BTK inhibitors such as acalabrutinib, zanubrutinib, or tirabrutinib are not able to overcome the resistance caused by BTK^{C481S} since they bind to the same protein region as ibrutinib. Their advantage is their higher selectivity with less off-targets (**Supplementary Table 1**) and lesser toxicities than ibrutinib, making them more feasible for patients intolerant to ibrutinib (168–172). Acalabrutinib has recently been approved for CLL and MCL and zanubrutinib for MCL (171, 173). The solution to BTK^{C481S} mutations could be the use of non-covalent third-generation BTK inhibitors that are able to inhibit the kinase's activity independently of C481S (**Supplementary Table 1**). BTK inhibitors such as fenebrutinib (GDC-0853), LOXO-305, or vecabrutinib are currently in the early phases of clinical testing even for patients with BTK^{C481S} (72–74). ARQ 531 has shown better efficacy than ibrutinib in murine models resembling Richter transformation, targets CLL not only with *BTK* but also *PLCG2* mutations and has off-target activity against kinases in Erk signaling and kinases in the Src family (75). The off-targets of various BTK inhibitors are summarized in the **Supplementary Table 1**. A different but also promising therapeutic strategy is provided by PROTAC which degrades its target with E3 ligase. PROTAC-induced BTK degradation is highly selective and effective in treating BTK^{C481S} ibrutinib-resistant mouse models (76, 77). CLL cells with R665W and L845F mutations in *PLCG2* are sensitive to RAC2 or SYK and LYN inhibition (78, 79). Inhibiting RAC2 or its binding partner VAV1 is synergistic with ibrutinib also in DLBCL just like inhibiting STAT3 or SYK together with ibrutinib in *MYD88* mutated DLBCL (89–91).

Another approach would be combining ibrutinib with compensatory survival pathway inhibitors such as PI3K/mTOR/Akt or NFκB (**Table 2**). As mentioned before, ibrutinib synergizes with PI3K/mTOR/Akt pathway inhibitors in MCL, CLL and DLBCL (121–126). A combination of ibrutinib and umbralisib, a next-generation PI3Kδ inhibitor, was studied in a clinical trial in CLL and MCL patients with promising results (127). It has been shown that ibrutinib increases CLL-cell sensitivity to mTOR inhibitors as well as proteasome and PLK1 inhibitors (128). Targeting mTOR combined with ibrutinib was also suggested in ABC-DLBCL by Phelan et al. as mTORC1/2 inhibitor AZD2014 further attenuates formation of MYD88/TLR9/BCR super-complex when compared to ibrutinib alone (115). CC-115, a dual mTOR/DNA-dependent protein kinase inhibitor, is now in a clinical trial and is able to revert CD40-mediated resistance to venetoclax and also inhibits BCR signaling in CLL patients with acquired idelalisib resistance (174). Inhibiting the PI3K/mTOR/Akt pathway has been shown to be successful also in *in vitro* drug screening in DLBCL, where PI3K inhibitors synergized with ibrutinib (129). The same study, confirmed by others, proved a synergy between

ibrutinib and inhibition of IRAK4, a mediator for TLR and NFκB activation whose targeting is studied not only in DLBCL but in CLL as well (129, 175, 176). Furthermore, the synergy with ibrutinib was also seen in combination with selinexor, an XPO1 inhibitor, although this seems to be regulated again *via* the PI3K/mTOR/Akt pathway as selinexor restores a nuclear abundance of FoxO3a and PTEN after ibrutinib treatment, resulting in the inhibition of PI3K/mTOR/Akt signaling activation (**Figure 1**) (121, 129). Absence of tumor suppressors FoxO3a and PTEN in the nucleus could be an explanation for ibrutinib resistance in 2p+ CLL patients who overexpress *XPO1* and why selinexor and next-generation XPO1 inhibitors seem to be efficient in preclinical CLL and MCL models where it reduces NFκB binding to DNA (92, 130–132). Another molecule that could be targeted together with BTK is the MP3K14 enzyme, a member of an alternative NFκB pathway constitutively activated in ibrutinib-resistant MCL patients due to mutations in *BIRC3*, *TRAF2*, or *TRAF3* (**Figure 1**) (84, 85).

An attractive strategy to increase the treatment efficacy is combining drugs that are already approved in therapy. Recently, ibrutinib has been shown to be efficient with venetoclax, a BH3 mimetic that inhibits anti-apoptotic molecule BCL2 and is approved for CLL treatment (125, 137, 138, 177). This combination is rational as ibrutinib induces BCL2 expression and on the other hand, decreases anti-apoptotic MCL1 levels, which can be a cause of venetoclax resistance (**Figure 1**) (30, 139–141). Higher BCL2 levels have also been found in DLBCL patients with poorer response to ibrutinib therapy (142). Furthermore, ibrutinib inhibits malignant cell proliferation while venetoclax targets preferentially resting subpopulations, potentially explaining the synergy of these two drugs in B cell malignancies (129, 143). Combining ibrutinib with fludarabine, a purine analog commonly used together with cyclophosphamide and anti-CD20 monoclonal antibody rituximab to treat CLL patients, has been shown to be synergic *ex vivo* (125). Ibrutinib with immune modulator lenalidomide and rituximab is under investigation in DLBCL and MCL but has not been successful in CLL (178–181). A profoundly studied possibility for therapy is combining "BCR inhibitors" with widely-used anti-CD20 monoclonal antibodies (28). However, adding rituximab to ibrutinib did not bring any clinical benefit and this is likely due to ibrutinib downregulating CD20 levels and/or interfering with effector cell functions (28, 30, 182, 183). Furthermore, ibrutinib has been shown to negatively regulate anti-CD20 induced apoptosis in MCL cell lines (184). A combination of ibrutinib with a more efficient anti-CD20 antibody, obinutuzumab, is now approved for CLL therapy. However, the real benefit of obinutuzumab still remains unclear as the control arm of the clinical trial was chlorambucil with obinutuzumab (185). In the ELEVATE clinical trial, acalabrutinib or acalabrutinib plus obinutuzumab were both superior to chlorambucil plus obinutuzumab (170). Even though it is not yet clear whether this combination reduces the occurrence of acquired BTK inhibition resistance, it is true that re-distributing malignant cells to the peripheral blood makes malignant cells more susceptible to monoclonal antibodies (145, 186). An interesting option is ibrutinib combined with anti-ROR1 monoclonal antibody in CLL, which is expected to have a great specificity for malignant B cells, and ROR1 levels are not reduced during ibrutinib therapy (187). Promising results have also been obtained from a fixed-duration treatment with venetoclax and obinutuzumab in CLL (188). Therapy nowadays aims for a time-limited treatment setting as it would lower the selection pressure, leave shorter time for cells to compensate for inhibition of targeted pathway, and reduce toxicities in patients.

It has been reported that patients who relapse or are intolerant to one kinase-inhibitor benefit from a change to different small-molecule inhibitors rather than chemotherapy (189). Novel therapeutic targets and drugs are therefore being investigated. Promising results have been obtained by targeting different players in BCR signaling such as PKCβ or MALT1 whose inhibition is effective in ABC-DLBCL, MCL, and naïve as well as ibrutinib-resistant CLL (190–193). Cerdulatinib, a dual SYK/JAK-STAT inhibitor, targets BCR signaling and is also able to overcome microenvironmental protection and blocks proliferation in ibrutinib-resistant primary CLL samples and BTK^{C481S} lymphoma cell lines (**Figure 1**) (133). Directly targeting microenvironmental interactions, migration and adhesion could also have potential use in therapy of ibrutinib-resistant patients *via* the use of natalizumab or CXCR4 inhibitor plerixafor (45, 145). Lastly, malignant cells of ibrutinib-resistant CLL and MCL patients show metabolic reprogramming, which has also been suggested as a possible therapeutic target (87, 144).

EFFECTS OF IDELALISIB AND MECHANISMS OF RESISTANCE

Another molecule in BCR signaling widely therapeutically targeted in B cell malignancies is PI3K. Activated PI3K/Akt axis in B cell malignancies and its signaling pathway's role in cell survival makes it an attractive therapeutic target (3, 151, 194, 195). PI3K exists in four catalytic isoforms: p110α, p110β, p110γ, and p110δ. p110α (PI3Kα) and p110δ (PI3Kδ) are both needed for "tonic" (antigen-independent) BCR signaling, while only p110δ is needed for antigen-induced BCR signaling (196). The PI3Kδ isoform is targeted by widely-used idelalisib, which has been approved for the treatment of CLL, FL, and non-Hodgkin lymphomas. Idelalisib not only thwarts the PI3K/Akt/mTOR pathway but also inhibits cell migration towards chemokines and adhesion to stromal cells, which, just like with ibrutinib, leads to an initial increase in the number of lymphocytes in the peripheral blood caused by lymphocyte migration out of the tissues in CLL. This is accompanied by a reduction in Akt phosphorylation and other downstream effectors as well as by apoptosis induction (8, 197–199). Unfortunately, due to serious adverse effects and infections, it has been suggested to primarily give idelalisib to CLL patients with progression on ibrutinib or indolent NHL patients with progression on two prior therapies (14, 200, 201). As already mentioned, promising results have recently been seen in a clinical trial of a combination of ibrutinib and a next-generation dual PI3Kδ/CK1ϵ inhibitor, umbralisib (127). Furthermore, the importance of the p110γ subunit is now

emerging in CLL. Its activation does not respond to BCR stimulation but increases in response to CD40L/IL4 and cells with overexpressed PI3Kγ show an enhanced cell migration towards CXCL12. A dual PI3Kδ/γ inhibitor, duvelisib, seems to have a bigger impact on cell migration than idelalisib alone and is now approved to treat CLL/SLL and FL (197). Another PI3K inhibitor, copanlisib, is approved for relapsed/refractory FL. It is a pan-class I PI3K inhibitor that inhibits all four PI3K isoforms with higher selectivity against PI3Kα and PI3Kδ (202). Its advantage lies not only in its molecular mechanism as inhibition of other PI3K isoforms can lower viability and migration of B cells, but it also seems to cause fewer adverse events in patients when compared to idelalisib (197, 203–205).

Despite idelalisib's and other PI3K inhibitors' ability to initially control the disease in some patients, a fraction of patients develop resistance. Unfortunately, unlike in ibrutinib treated patients, the mechanisms of resistance remain mostly unclear (8, 206). No recurrent mutations were found in patients progressing on idelalisib nor have they been found in a mouse model resistant to PI3Kδ inhibition (207, 208). Two studies have shown the role of MAPK signaling in resisting PI3Kδ inhibition. Firstly, Murali et al. confirmed activating mutations in MAPK pathway leading to Erk phosphorylation in patients resistant to PI3K inhibition and suggested that blocking Erk might sensitize patients to PI3K inhibitors (**Figure 1**) (209). Secondly, a non-genetic mechanism of resistance was found in PI3Kδ resistant mice, where the upregulation of insulin-like growth factor 1 receptor (IGF1R) led to MAPK signaling activation. IGF1R upregulation was caused by FoxO1 and GSK3β and the resistance was resolved by inhibiting the receptor with linsitinib (208). Initial experiments with copanlisib point to IL6 signaling as a main player in copanlisib resistance; levels of IL6 and phosphorylation of STAT5, Akt, p70S6K, and MAPK were increased in copanlisib-resistant B cell lymphoma cell lines and the resistance was reversible by JAK inhibitor (210).

Extensive research is needed in order to reveal the resistance mechanisms for PI3K inhibitors and it seems that they might be more complex than in BTK inhibition. Despite their adverse effects in patients and the emergence of BCL2 inhibitors and next-generation BTK inhibitors, the PI3K/mTOR/Akt signaling pathway plays an important role in B cell malignancy pathogenesis and its inhibition might find its therapeutic place.

DISCUSSION

Targeting BCR signaling is now a commonly used therapy strategy for B cell malignancies. Unfortunately, some patients are primarily resistant to "BCR inhibitors" or develop resistance during the course of treatment. Furthermore, the now required continuous treatment often leads to toxicities and forces a change of therapy. *BTK* and *PLCG2* mutations are the most common and best-described mechanisms of resistance to BTK inhibitor ibrutinib, although recurrent mutations in other genes or aberrations in larger chromosomal regions have been described as being responsible for the resistance across the B cell malignancies. Interestingly, malignant B cells are able to overcome BTK inhibition by non-genetic mechanisms as well. These include activation of compensatory survival pathway, such as PI3K/mTOR/Akt, NFkB, or MAPK signaling pathways. Compensatory survival is also provided by the upregulation of anti-apoptotic BCL2, MYC or adhesion involved integrins. Even though a lot is known about BTK inhibition, PI3K inhibitor resistance remains largely unclear. Several studies point to MAPK pathway activation as a compensatory mechanism to PI3K inhibition, but further research is needed in this area.

Resistance caused by *BTK* mutations can be solved by third-generation BTK inhibitors now in clinical trials or by BTK degradation by PROTAC-based compounds; mutations in *PLCG2* by combining BTK inhibition with RAC2 or SYK and LYN inhibition. Compensatory survival by upregulating other signaling pathways could be solved by combining several inhibitors as well. The synergy between blockage of BTK and the PI3K/mTOR/Akt pathway has been shown repeatedly in CLL, DLBCL and MCL. It remains largely unknown how the non-coding part of the genome influencing BCR signaling is affected by "BCR inhibitors" and if (de)regulation of these molecules could contribute to therapy resistance or be directly used as a therapeutic target (20, 31, 34, 35). Novel therapeutic targets and strategies are still being investigated and their inhibition is tested alone or in combination with "BCR inhibitors". These combinations should be supported by analyzing the responses of malignant cells to individual drugs and using multi-omics to identify possible compensatory signaling pathways to be co-targeted by small-molecule inhibitors. Furthermore, co-targeting two kinases in seemingly the same pathway can also have a synergistic effect, as observed by BTK and PI3K inhibition's synergy. Analyzing individual patient-to-patient response to "BCR inhibitors" could help to identify specific compensatory pathways and this could help to define a personalized combinatorial therapy. However, it is likely that there might be some universal responses to "BCR inhibitors" in each B cell malignancy and this could follow pre-existing mechanisms that allow B cells to survive for an extended time without antigen encounter. Single cell analysis of the response to BTK/PI3K inhibitors in multiple patients could help to understand if the response or adaptation to therapy follows a generally uniform course or if there are major intra- and inter-patient specific mechanisms. Besides transcriptomics and proteomics, research should focus on describing the cells' immunophenotypic profiles after "BCR inhibition" to identify surface molecules that could be targeted by monoclonal antibodies, since this can be of high specificity for cancer cells and low toxicity in general. The time dynamics of changes during therapy should also be studied in order to describe the mechanisms of early and long-term adaptation and potentially identify an optimal time point for adding a second drug in combination. Improving clinical efficacy of drug combinations containing "BCR inhibitors" should allow a time-limited treatment with a deep molecular response, decreased chance of resistance and limited toxicities associated with long-term therapy.

AUTHOR CONTRIBUTIONS

LO and MM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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CD200 and Chronic Lymphocytic Leukemia: Biological and Clinical Relevance

Giovanni D'Arena[1], Vincenzo De Feo[2], Giuseppe Pietrantuono[3], Elisa Seneca[3], Giovanna Mansueto[3], Oreste Villani[3], Francesco La Rocca[4], Fiorella D'Auria[5], Teodora Statuto[5], Luciana Valvano[5], Francesca Arruga[6], Silvia Deaglio[6], Dimitar G. Efremov[7], Alessandro Sgambato[8] and Luca Laurenti[9]*

[1] Hematology, "S. Luca" Hospital, ASL Salerno, Vallo della Lucania, Italy, [2] Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Salerno, Italy, [3] Hematology and Stem Cell Transplantation Unit, IRCCS Centro di Riferimento Oncologico della Basilicata, Rionero in Vulture, Italy, [4] Laboratory of Preclinical and Translational Diagnostics, IRCCS Centro di Riferimento Oncologico della Basilicata, Rionero in Vulture, Italy, [5] Laboratory of Clinical Research and Advanced Diagnostics, IRCCS Centro di Riferimento Oncologico della Basilicata, Rionero in Vulture, Italy, [6] Cancer Immunogenetics Unit, Department of Medical Sciences, Molecular Biotechnology Center, University of Turin, Turin, Italy, [7] Molecular Hematology, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy, [8] Scientific Direction, IRCCS Centro di Riferimento Oncologico della Basilicata, Rionero in Vulture, Italy, [9] Hematology Institute, IRCCS Fondazione Policlinico Universitario A. Gemelli, Rome, Italy

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Jérôme Paggetti, Luxembourg Institute of Health, Luxembourg

Reviewed by:
Jorge Castillo, Dana–Farber Cancer Institute, United States
Michael Mian, Ospedale di Bolzano, Italy

***Correspondence:**
Giovanni D'Arena
giovannidarena@libero.it

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CD200, a transmembrane type Ia glycoprotein belonging to the immunoglobulin protein superfamily, is broadly expressed on a wide variety of cell types, such as B lymphocytes, a subset of T lymphocytes, dendritic cells, endothelial and neuronal cells. It delivers immunosuppressive signals through its receptor CD200R, which is expressed on monocytes/myeloid cells and T lymphocytes. Moreover, interaction of CD200 with CD200R has also been reported to play a role in the regulation of tumor immunity. Overexpression of CD200 has been reported in chronic lymphocytic leukemia (CLL) and hairy cell leukemia but not in mantle cell lymphoma, thus helping to better discriminate between these different B cell malignancies with different prognosis. In this review, we focus on the role of CD200 expression in the differential diagnosis of mature B-cell neoplasms and on the prognostic significance of CD200 expression in CLL, where conflicting results have been published so far. Of interest, increasing evidences indicate that anti-CD200 treatment might be therapeutically beneficial for treating CD200-expressing malignancies, such as CLL.

Keywords: CD200, chronic lymphocytic leukemia, prognosis, diagnosis, flow cytometry

INTRODUCTION

Mature B-cell leukemias are heterogeneous in clinical and biological features. Despite the large body of studies published, difficulties to get a firm diagnosis still exist in some cases due to the lack of disease-specific markers and overlapping immunophenotypes. CD200 has recently emerged as a useful tool to better discriminate among several chronic leukemias. In addition to the usefulness of this marker in the diagnostic setting, it also has a prognostic role and may represent a potential therapeutic target in chronic lymphocytic leukemia (CLL).

In this review, we provide a summary of published data on the biological and clinical relevance of CD200 in CLL, identified through a literature search of the MEDLINE, Google Scholar, and Scopus databases, aiming at providing an update of the published literature on this topic. The search comprised the terms "CD200", "chronic lymphocytic leukemia", and "chronic B cell leukemias" without a date restriction. All articles and Meeting Abstracts we found were evaluated and included in this review.

CD200 ANTIGEN AND CD200 RECEPTOR

The surface membrane glycoprotein CD200, formerly termed OX-2, is encoded by the 29,744 bp long CD200 (OX-2) gene located on the long arm of chromosome 3 (3q13.2) (1).Three transcript variants of CD200 are known: variant 1, 2,226 bp long, containing 7 exons and encoding the 269 amino acids long isoform a; variant 2, 2,301 bp long, containing 7 exons and encoding the 294 amino acids long isoform b; and variant 3, 2,085 bp long, missing an exon and encoding the 153 amino acids long isoform c (2).

The CD200 glycoprotein is a single-pass, type I, highly conserved membrane protein, belonging to the immunoglobulin superfamily, spanning the membrane once, with the N-terminus on the extracellular side of the membrane (2). It is composed of two extracellular (one variable and one constant) immunoglobulin-like domains, a single transmembrane region, and a cytoplasmic tail (**Figure 1**) (3).

The receptor for CD200 (CD200R) also has two immunoglobulin-like domain. The phylogenetic analysis demonstrated that the receptor is closely related to CD200 and probably evolved by a gene duplication (2). As a matter of the fact, the genes for human CD200 and CD200R are closely linked on chromosome 3 (4). However, the CD200R has a longer cytoplasmic tail with signaling motifs different from CD200 (5).

CD200 is normally expressed on a variety of cell types, including thymocytes, B lymphocytes, a subset of T lymphocytes, neurons, endothelial cells, some dendritic cells, kidney glomeruli, and syncytiothrophoblasts (6). The expression of CD200R is more restricted and includes myeloid leucocytes, such as macrophages, dendritic cells, and mast-cells, as well as B lymphocytes and a subset of T lymphocytes (7, 8).

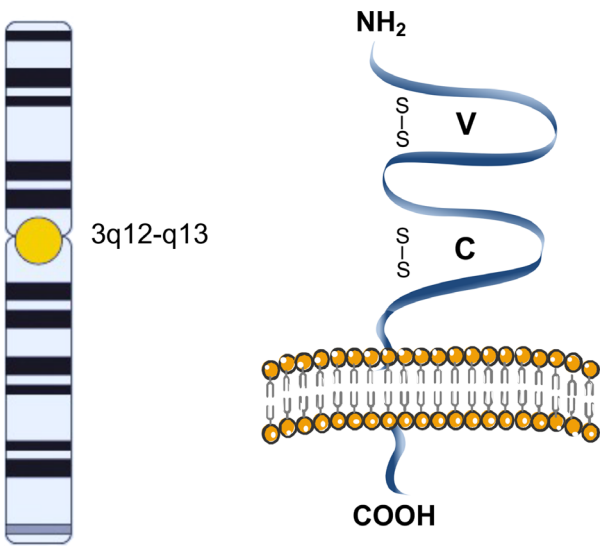

FIGURE 1 | Schematic representation of CD200. The CD200 glycoprotein has two extracellular like Immunoglobulin domains formed by disulfide bonds, one variable (V) and one constant (C), a single transmembrane region, and a cytoplasmic tail.

FUNCTION OF CD200 AND ITS ROLE IN CANCER

CD200, by means of the interaction with its receptor, induces the suppression of T-cell mediated responses, limiting inflammation in a wide range of inflammatory diseases (**Table 1**). The inhibition of macrophage function, induction of regulatory T-helper cell type (Th1) to Th2 cytokine profile switch, suppression of natural killer cell function, and inhibition of tumor-specific T-cell immunity have been all experimentally demonstrated (9–14, 15–18). Consistent with its immunosuppressive role, CD200-deficient mice are susceptible to tissue-specific autoimmunity (15). Gorczynski and co-workers demonstrated that the interaction of CD200 with CD200R is able to decrease the production of Th1-like cytokines, such as interleukin (IL)-2 and interferon (IF)-γ, and increase the release of Th2-like cytokines, such as IL-10 and IL-4 (16). In addition, the same group reported that the CD200/CD200R interaction induces the *in vitro* differentiation of T lymphocytes toward $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs) (16).

Moreaux et al demonstrated that CD200 mRNA is overexpressed on cells of several types of cancers compared to their normal counterparts, including chronic lymphocytic leukemia (CLL) (19). Kretz-Rommel et al elaborated a tumor model on the basis of the previous demonstration that CD200 is up-regulated in CLL and that the up-regulation in multiple myeloma (MM) and acute myeloid leukemia (AML) correlates with adverse prognosis (20–22). These authors firstly demonstrated that human peripheral blood mononuclear cells (hPBMCs) and Namalwa tumor cells (Burkitt's lymphoma cell line lacking CD200 expression) simultaneously injected in

TABLE 1 | CD200:CD200R interaction and negative control of immunity.

KEY FACTS
Reduced Th1 cytokine (IL-2, IFNγ) production (9)
Increased IL-10 and IL-4 production (9)
Induction of Tregs (10)
Inhibition of mast cell degranulation (11, 12)
Downregulation of basophilic function (13)
Suppression of natural killer cell function (14)

CD200R, receptor of CD200 antigen; Th, T helper; IL, interleukin; IFN, interferon; Tregs, regulatory T-cells.

NOD/SCID mice show reduced tumor growth with respect to that observed in mice in the absence of hPBMCs (23, 24). When the Namalwa tumor cells were engineered to express human CD200 on their surface and simultaneously injected with hPBMCs, CD200 expressed on tumor cells prevented hPBMCs from eradicating the cancer cells. This tumor model clearly demonstrated the immunosuppressive activity of CD200. Moreover, treatment with anti-CD200 monoclonal antibodies (Abs) was also highly effective in this model and inhibited the growth of Namalwa CD200 tumor cells in NOD/SCID hu-mice by >90%. The same study showed that CD200 is a marker of activated T cells and that the use of IgG1 anti-CD200 (a constant region variant that can mediate ADCC) resulted in efficient target cell killing of these lymphocytes by ADCC. Taken together, these data have relevant implications for the immunotherapy of cancer patients with anti-CD200.

RELEVANCE OF CD200 IN DIAGNOSIS OF CHRONIC LYMPHOPROLIFERATIVE DISORDERS

The neoplasms of mature B cells are heterogeneous diseases currently included in the "mature B-cell lymphoid neoplasms" of the WHO classification (25, 26). Immunophenotypic profile, cytogenetics, and molecular biology must be all taken into account as a multidisciplinary integrated approach to differentiate the disease entities belonging to this category. However, difficulties in defining some cases still exist. Diagnostic accuracy is crucial in diagnosing neoplasms that require different specific treatments.

Flow cytometric characterization of chronic lymphoproliferative disorders represents a cornerstone in the diagnostic approach to neoplasms of mature lymphocytes (27). In the early 90s, a British group in London proposed a scoring system (Matutes score), which was based on analysis of 5 membrane markers: CD5, CD22, CD23, FMC7, and surface immunoglobulin (SmIg) (28). A score of 1 was assigned for each of the following immunophenotypic features: CD5 positive, CD22 weak or negative, CD23 positive, FMC7 negative, SmIg weak. The total score, according to this scoring system, is usually 4 or 5 for typical CLL cases, and 3 or less for other mature B-cell lymphoid neoplasms (28). A few years later, the same group improved the diagnostic accuracy of the score (from 91.8% to 96.8%) simply replacing CD22 with CD79b (29).

The Matutes score is used worldwide as a diagnostic tool. However, some cases of chronic lymphoid neoplasms are still misdiagnosed, including some cases belonging to a more aggressive entity, such as mantle cell lymphoma (MCL). In this context, CD200 has been shown to have differential expression in B-cell neoplasms and to well discriminate CLL from MCL and hairy cell leukemia (HCL) and its variant form (v-HCL) (**Figure 2**) (30–32).

The reports on the relevance of CD200 expression to differentiate B cell chronic lymphoid neoplasms that have been published so far are listed in **Table 2**. Firstly, Palumbo et al in 2009 demonstrated that CLL can be differentiated from MCL according to the expression of CD200 (all patients with CLL expressed CD200, whereas all patients with MCL were negative) (31). At the same time Brunetti et al, analyzing only patients with HCL, showed that all patients with typical HCL expressed CD200 (30). Shortly after, Dorfman & Shahsafaei studied by means of flow cytometry bone marrow and lymph node aspirates from patients with different mature B cell lymphoid neoplasms, showing that CLL and HCL cases were all CD200 positive, prolymphocytic leukemia (PLL) were positive in 80% of cases, while MCL, splenic marginal zone lymphoma (SMZL), and follicular lymphoma (FL) were all negative (32). Since then, several other researchers analyzed samples from patients with mature B-cell lymphoid neoplasms (33–63). Overall, the published data confirmed the positivity in all patients with

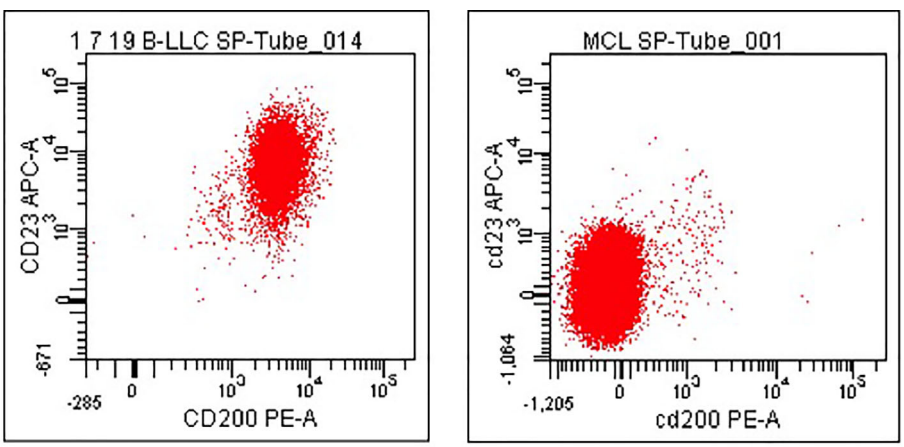

FIGURE 2 | CD200 expression in CLL and MCL. Dot plots showing a case of CLL in which both surface CD200 and CD23 were expressed (left panel) and a case of MCL in which both antigens were found negative (right panel). B-cells only have been gated for the analysis.

TABLE 2 | CD200 expression and differential diagnosis in chronic B-cell leukemias: published data at a glance.

Reference	No. Patients	Samples evaluated	CLL	MCL	HCL	HCL-v	MZL	FL	LPL	Other B-cell neoplasms
Palumbo et al. (31)	91	PB	79/79 (100%)	0/14 (0%)	–	–	–	–	–	–
Brunetti et al. (30)	10	PB; BM	–	–	10/10 (100%)	–	–	–	–	–
Dorfman and Shahsafaei (32)	73	BM, LN[#]	21/21 (100%)	0/10 (0%)	12/12 (100%)	–	0/10 (0%)	0/16 (0%)	8/10 (80%)	MALT: 0/4 (0%); B lymphoblastic leukemia/ lymphoma: 10/10 (100%); DLBCL: 0/12 (0%); mediastinal large B-cell lymphoma: 8/ 8 (100%); BL: 0/8 (0%); MM: 10/13 (77%); HL: 12/13 (92%); nodular lymphocyte predominant HL: 0/14 (0%)
Bhatnagar et al. (33)	100	PB; BM	78/78 (100%)	0/7 (0%)	–	–	–	–	–	–
El Desoukey et al. (34)	49	PB	31/31 (100%)	0/4 (0%)	2/2 (100%)	–	0/4 (0%)	0/8 (0%)	–	–
Cherian et al. (35)	66	Not reported	–	–	–	–	–	–	–	Not reported data as percentage expression of CD200 in 51 cases of CLL/ SLL and 15 cases of MCL. Most cases studied can be separated using a cut off of 1.10 for the CD200 MFI ratio
Kern et al. (36)	100	PB, BM	58/59 (98,3%)	2/14 (14,3%)	–	–	–	–	–	CLL/PL cases: 26/27 (96.3%)
Alapat et al. (37)	107	PB; BM; LN; body fluids	19/19 (100%)	0/4 (0%)	–	–	–	–	3/7 (43%)	B-ALL: 19/20 (95%); T-ALL: 0/5 (0%); MM: 37/52 (71%)
Pillai et al. (38)	180	BM, PB, LN; body fluids and other tissues	–	–	23/23 (100%)	1/1 0(10%)	–	–	–	Not specified the number of positive/ negative cases but only reported MFI (with range) for each disease category. CLL/SLL showed CD200 with MFI of 5,965 compared with MCL and FL which had MFIs of 397 and 521, respectively.
Dasgupta et al. (39)	56	Not reported	28/28 (100%)	2/10 (20 %)	3/3 (100%)	–	–	–	–	4/8 (50%) not otherwise specified
El-Sewefy et al. (40)	40	Not reported	30/30 (100%)	1/10 (10%)	–	–	–	–	–	
Sandes et al. (41)	159	PB; BM; LN; CSF	56/56 (100%)**	0/14 (0%)	13/13 (100%)	–	6/6 (100%)	8/11 (73%)	2/4 (50%)	PL: 2/7 (28%) SRPBL: 1/1 (100%) CD5-CD10- unclassifiable NHL: 21/31 (68%) CD5+ NHL*** 9/23 (39%)
Challagundla et al. (42)	364	PB; BM; FNA; LN	119/119 (100%)	58/61 (95%)*	7/7 (100%)	–	9/26 (35%)°	°°	3/3 (100%)°°	
Karban et al. (43)	200	PB	200/20 (100%)	4/46 (8,7%)	–	–	–	–	–	–

(Continued)

TABLE 2 | Continued

Reference	No. Patients	Samples evaluated	CLL	MCL	HCL	HCL-v	MZL	FL	LPL	Other B-cell neoplasms
Sorigue et al. (44)	248	PB; BM; LN; CE	106/106 (100%)	–	–	–	–	–	–	MBL-CLL like: 106/106 (100%); DLBCL: 7/32 (22%) of which 4/11 (36%) ABC; 3/20 (15%) GCB; 0/1 (0%) cell of origin not determined; 1/4 (25%) BL.
Lesesve et al. (45)	124	PB; BM	57/69 (83%)	0/10 (0%)	2/4 (50%)	–	1/16 (6%)	0/7 (0%)	–	MBL-CLL like: 8(13(61%); MBL-non-CLL like 1/3(33%); PL: 0/2 (0%)
Fan et al. (46)	374	PB; BM	268/271 (99 %)	1/31 (35%)	3/3 (100%)	–	75% strong	–	–	PL: 5/7 (%) weakly, 2/7 (%) strong
Naseem et al. (47)	77	Not reported	54/54 (100%)	1/6 (16%)	5/5 (100%)	–	–	1/2 (50%)	–	Other CLPD did not express CD200
Rahman et al. (48)	3	Not reported	–	–	–	3/3 (100%)	–	–	–	–
Rahman et al. (49)	160	PB; BM; FNA, ascites fluid	98/98 (100%)	0/24 (0%)	6/6 (100%)	0/1 (0%)	3/6 (50%)	2/4 (50%)	2/4 (50%)	DLBCL: 3/5 (60%); BL: 0/1 (0%); PBL: 0/1 (0%); CD5-CD10- undefined lymphomas: 4/10 (40%)
Ting et al. (50)	97	PB; BM; LN; pleural fluid	56/56 (100%)[§]	0/6 (0%)	2/2 (100%)	–	–	–	–	40 pts of which 22 diagnosed with lymphomas with subtype (not reported in the paper), and 18 unclassified (of which 10 diagnosed with lymphoma without subtype, and 8 non-CLL MBL)
Arlindo et al. (51)	124	PB; BM	61.1 (41.4/89.2)	3.5 (2.1/4.1)	220.3 (163.1/297.5)	36.1 (22.1/50.1)	8.3 (4.5/13.2)	2.6 (1.8/11.4)		Atypical CLL: 113.7 (70.4/122.2)
Mason et al. (52)	79	PB;BM;LN	–	–	34/34 (100%)	0/3 (0%)	1/22 (5%)	–	13/20 (65%)	
Starostka et al. (53)	188	PB; BM; LN; pleural fluid	158/161 (98.3%)	7.9%	–	–	63.6%	–	–	
Miao et al. (54)	653	PB; BM	355/365 (97%)	12/41 (29%)	1/1 (100%)	2/2 (66.7%)	8/20 (40%)	13/17 (76.5%)	30/35 (85.7%)	MALToma: 4/4 (100%); CD5+ and CD5- unclassified B-cell chronic lymphoproliferative disorders: 119/153 (78%)
Poongodi et al. (55)	77	PB; BM	54/54 (100%)	1/6 (16,7%)	5/5 (100%)	–	2/2 (100%)	1/2 (50%)	–	DLBCL: 1/3 (33.3%); unclassifiable lymphoma: 2/3 (66.7%); SLL: 1/1 (100%)
Favre et al. (56)	96	PB	84/84 (100%)	1/30 (3%)	7/7 (100%)	–	13/14 (93%)	–	–	SRPBL: 6/15 (40%)
Falay et al. (57)	339	PB; BM	295/306[♀] (95.8%)	2/33 (6.1% dimly)	–	–	–	–	–	
D'Arena et al. (58)	427	PB; BM	312/322 (97%)	4/21 (19%)	15/15 (100%)	–	27/53 (51%)	3/12 (25%)	0/4 (0%)	
Debord et al. (59)	135	PB, BM	–	3/63 (5%)	–	–	–	–	–	68/72 (94%) low grade B-cell lymphoma not otherwise specified
Mora et al. (60)	120	PB	64/64 (100%)	1/5 (20%)	–	–	13/19 (68.4%)	2/2 (100%)	–	MBL: 14/14 (100%); SLL: 3/3 (100%); other B-CLPD (not otherwise specified): 9/13 (69.2%)^
Myles et al. (61)	307	PB; BM; LNH, other body fluid or tissue	231/241 (96%)	62/66 (94%)	–	–	–	–	–	

(Continued)

TABLE 2 | Continued

Reference	No. Patients	Samples evaluated	CLL	MCL	HCL	HCL-v	MZL	FL	LPL	Other B-cell neoplasms
Soong et al. (62)	189	PB; BM	121/121 (100%)	1/10 (1%)	–	–	–	–	–	54/68 of B-chronic lymphoid leukemias, including MCL cases
El-Neanaey et al. (63)	50	PB	30/30 (100%)	–	5/5 (100%)	–	–	–	–	14/25 (56%) B-NHL not subclassified

CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; HCL, hairy cell leukemia; HCL-v, hairy cell leukemia variant; MZL, marginal zone lymphoma; FL, follicular lymphoma; LP, lymphoplasmacytic lymphoma; PL, prolymphocytic leukemia; SRPBL, splenic diffuse red pulp small B-cell lymphoma; NHL, non-Hodgkin's lymphoma; HL, Hodgkin lymphoma; SLL, small lymphocytic lymphoma; DLBL, diffuse large B-cell lymphoma; ABC, activated B cell-like; GCB, center B cell-like; MM, multiple myeloma; PBL, plasmablastic lymphoma; PB, peripheral blood; BM, bone marrow; LN, lymph node; CE, cavity exudate; FNA, fine needle aspiration; CSF, cerebrospinal fluid; IHC, immunohistochemistry; MFI, mean fluorescence intensity.

**Three cases of MCL moderate or bright CD200 expression (all three cases showed cyclin CD1 overexpression by IHC; two cases had t(11;14)(q12;q32) by FISH and conventional cytogenetics also; one case showed del(14q31q32) in three of 20 metaphases. Flow cytometry immunophenotyping in all three cases showed an atypical pattern of CD23 expression, positive on most lymphoma cells).*

***11 cases were atypical-CLL (CD23 absent or dimly expressed in six cases and the following antigens expressed with moderate/strong intensity: FMC-7 in eight cases; CD79b in five cases, and smIg in four cases).*

****CD5+ NHL (non-CLL, non-MCL).*

#CD200 expression was studied by means of immunohistochemistry (IHC).

ºmostly nodal or MALT types.

ºº90 FL cases were studied and a spectrum of CD200 expression ranging from negative to moderate was found (not reported the number of positive cases); only three LPL cases were studied with moderate expression of CD200.

§56 CLL of which 13 CLL-like MBL; 7 patients with CLL were atypical. Both typical and atypical were found all CD200 positive; MFland percentage of CD200 expression were found not different; no differences were found between typical and atypical CLL also.

§No difference between typical and atypical CLL. Multivariate analysis for MCL and atypical CLL discrimination, it was demonstrated that the most determinant molecule was CD200 (p<0.0001, 95% CI).

**MFI >1,000 was used to represent positive staining while MFI ≤1,000 to represent negative staining for CD200.*

^CD200, added to the canonical 5 markers, improved the diagnostic accuracy of Matutes score from 86.7% to 92.5% (p<.01).

CLL and HCL and the negativity in almost all patients with MCL (**Table 2**).

Focusing on the assessment of fluorescence intensity of CD200, Lesesve et al used the ratio of mean fluorescence intensity (MFI) of CD160/CD200 on leukemic cells/controls (45). CD160 is a glycosylphosphatidylinositol-anchored cell surface molecule belonging to the immunoglobulin superfamily that was found to be expressed in patients with CLL (64, 65). Only 60% of patients showed surface positivity for CD160. Both markers were positive in 55% of CLL but only in 2% of other B-cell neoplasms, and absence of both markers occurred in 12% of CLL and in 86% of other B-cell neoplasms.

Taken together, these data showed that CD200 has a high sensitivity for CLL diagnosis, being expressed in most of the CLL cases. However, it is expressed also in other B-cell malignancies, including MZL, HCL, and even some cases with indolent, non-nodal entity MCL, thus showing low specificity.

Some authors tried to implement the diagnostic ability of monoclonal antibodies-based scores using CD200 (58, 66–68). Recently, Köhnke et al proposed the so-called "CLLflow score" including CD200 in the Matutes score and showing an improvement in the specificity for the diagnosis of CLL (66). Briefly, the score is calculated by adding the percentage of CD200+ and CD23+/CD5+ B cells and then subtracting the percentages of CD79b+ as well as FMC7+ B cells. A score >0 is consistent with the diagnosis of CLL while a score ≤ 0 with the diagnosis of a non-CLL disease entity (67–69). In our hands a simplified score system for the diagnosis of CLL, in which only 4 markers are used (CD5, CD23, CD200, and SmIg), showed a higher sensitivity and specificity with respect to the Matutes score (58).

Interestingly, very recently Sorigue et al, using three immunophenotype-based diagnostic approaches (Matutes score, D'Arena score and CD43 expression) analyzed 597 patients with a chronic lymphoproliferative disorder (CLPD) and found that patients with concurring CLL-like or non-CLL like results according to the three diagnostic strategies were diagnosed with CLL (n = 441) and non-CLL (n =99), respectively (**Figure 3**) (69, 70). 'Discordant' patients (n = 57) were further re-evaluated taking into account individual cytometric markers and cytogenetics data and only 16 patients (2.7%) were not assigned to a reasonable diagnosis (**Figure 3**). The latter cases were considered "borderline lymphoproliferative disorders", a loosely-defined concept that would include any chronic lymphoid disorder in which the diagnosis of CLL cannot be either made or ruled out.

In another recent study, Sorigue et al conducted a systematic review of the use of CD200 in the differential diagnosis of CLPDs (71). They evaluated the positive predictive value of CD200 on the prevalence of the disorders evaluated for the differential diagnosis. Twenty-seven publications were included in this systematic review (accounting for 5,764 patients). The median positivity rate and percentage of CD200 positive cells in patients with CLL was 100% and 95%, respectively, whereas it was 4% and 8% in MCL, and 56% and 62% in other CLPDs. The authors concluded that CD200 is a suboptimal marker in discriminating

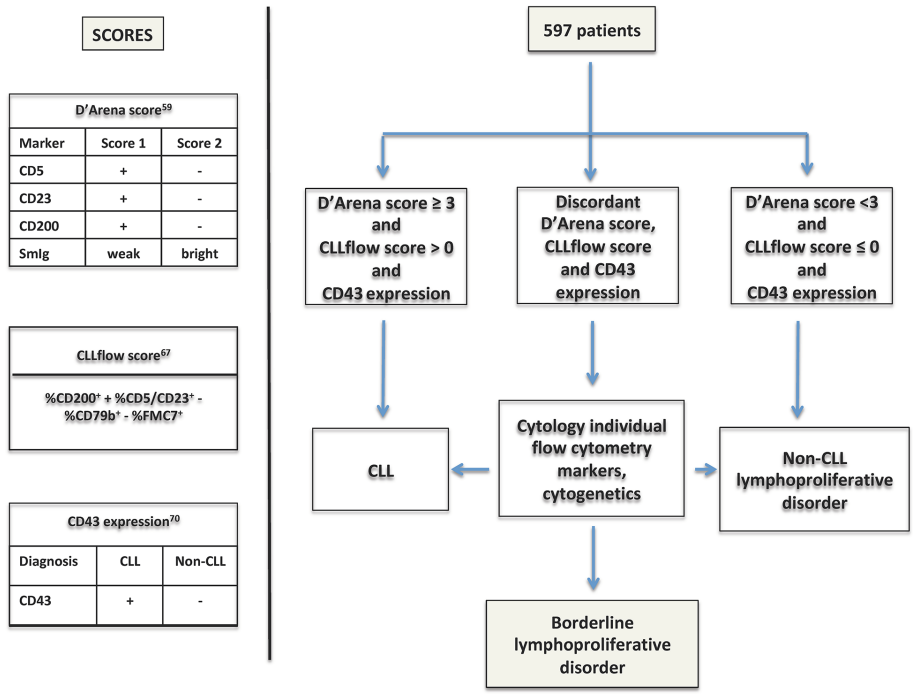

FIGURE 3 | Overview of the study by Sorigue et al. (70). The algorithm of the study using the three diagnostic scores to assign a diagnostic category is explained in the text (58, 66, 69).

CLL from CLPDs other than MCL. These findings suggest that assessment of CD200 within scores rather than as a single marker, as reported by some authors, would be more useful (66–68).

In addition, some authors investigated whether a different expression of CD200 between CLL and monoclonal B-cell lymphocytosis (MBL) exists. Sorigue et al demonstrated that all cases of both CLL and CLL-like MBL were CD200 positive. However, MBL cases showed a lower CD200 MFI than CLL. Moreover, both CLL with trisomy 12 and CLL-like MBL displayed lower CD200 MFI than CLL with other cytogenetic abnormalities. In contrast, Rawstron et al did not find any difference in expression of a large body of markers, including CD200, between CLL-type MBL and CLL (72). However, differences in the expression of other markers were observed, including lower expression of CD38, CD62L, and CD49d and higher expression of LAIR-1, CXCR5, and CCR6 on CLL-type MBL compared to CLL. In conclusion, despite CLL-type MBL being phenotypically identical to CLL for a large body of antigens, certain differences exist, particularly with respect to proteins involved in the homing to lymphoid tissue.

CD200 AND PROGNOSIS IN CLL

Little is known about the prognostic significance of CD200 expression in CLL. As a matter of fact, conflicting results have been reported so far. Few data have been published and both percentage of CD200 positivity and fluorescence intensity have been used to estimate CD200 expression on CLL B-lymphocytes. Firstly, Wang et al in 2014 identified two distinct groups of CLL patients according to the expression of CD200 on bone marrow B cells (CD200 low group: <50%; CD200 high group: ≥ 50%) (73). As reported in **Table 3**, correlations were found with some clinical-biological features of CLL patients with <50% CD200+ cells. In contrast, using the same methodological approach, El Din Fouad et al found other correlations in patients with a lower percentage of CD200+ CLL cells (<50%) (74). Moreover, while the former study did not report data on TTT, response to therapy or OS, the latter found no correlation with response to treatment or OS. In a large cohort of patients with CLL, Miao et al, evaluating the CD200 mean fluorescence intensity (MFI) instead of the percentage of positivity, found that patients with lower CD200 MFI had a significantly shorter TTT with respect to patients with higher CD200 MFI (75). However, no correlation was found between CD200 MFI and OS, and CD200 MFI did not maintain its predictive value on TTT in multivariate analysis. More recently, using a more standardized flow cytometric approach, we measured the CD200 MFI on CD19+ and CD19- lymphocyte subpopulations, calculating the relative fluorescence intensity (RFI) as a ratio of the CD200 MFI on CD19+ and CD19- cells (76). Lower and higher CD200 RFI values were found to be associated with del11q and del13q14, respectively. In addition,

TABLE 3 | CD200 expression and prognosis in CLL.

Reference	No. Patients evaluated	CD200 positivity cut-off	Correlations
Wang et al. (73)	40	50% of B-cells (<50%: CD200 low group; ≥50% CD200 high group)	<50% of CD200-positive B-cells positively correlated with younger age, female gender, lower WBC, lower lymphocyte absolute and percentage number, lower lymph node involvement, lower ZAP-70 positive cells, early disease stage. No data on TTT, response to therapy and OS.
El Din Fouad et al. (74)	43	50% of B-cells	>50% of CD200-positive B-cells positively correlated with older age, lymphocytosis, hepatomegaly, splenomegaly, higher Rai and Binet stage. No correlation with response to treatment and OS.
Miao et al. (75)	307	CD200 MFI cut-off: 189.5 (<lower group; ≥higher group)	Lower CD200 MFI positively correlated with shorter TTT.
D'Arena et al. (76)	105	CD200 RFI cut-off: 13	Lower CD200 RFI positively correlated with del11q and negatively correlated with del13q14. CD200 RFI greater than the mean value of the entire cohort positively correlated with longer OS.

WBC, white blood cell count; TTT, time to treatment; OS, overall survival; MFI, mean fluorescence intensity; RFI, relative fluorescence intensity (ratio of MFI of CD200 on CD19+ lymphocytes/MFI of CD200 on CD19- lymphocytes).

CD200 RFI greater than the mean cohort value was detected in patients with longer OS. However, these data have to be confirmed in patients with a longer follow-up.

In summary, conflicting results have been reported on the prognostic role of CD200 expression in CLL so far. Methodological and sampling differences, such as the analysis of percentage of positive cells versus MFI, bone marrow versus peripheral blood cells, and different flow cytometry instrument settings and/or gating strategy, could account for these differences. Further studies are needed to better understand this issue before reaching definitive conclusions.

CONCLUSIONS AND FUTURE DIRECTIONS

CD200 expression is useful in better classifying B-cell CLPDs. The addition of CD200 to flow cytometry marker panels addressing the diagnosis of this heterogenous group of B cell neoplasms may be particularly helpful in distinguishing some disease entities, in particular CLL and MCL, whose clinical behavior and prognosis are quite different. On the contrary, expression of CD200 does not appear to have a relevant role as a prognostic indicator in CLL according to data published so far, despite limited in number. However, this issue needs to be better addressed by studies on a larger cohort of patients and using standardized methodologies. Finally, the potential role of CD200 as a therapeutic target must be taken into account in particular for diseases that highly express CD200, such as CLL and HCL (24, 77). In fact, CD200 is known to act as an immunosuppressive molecule that is upregulated on primary CLL B-cells (18). Moreover, an elegant work by Kretz-Rommel and co-workers demonstrated that CD200 expression by tumor cells suppresses antitumor responses in an animal model (22). In a similar fashion, Gorczynski et al showed that by manipulating CD200:CD200R interactions it is possible to cure local tumors and distant metastases in a murine breast cancer model (78, 79). Very recently, the first-in-human study investigating the therapeutic use of the recombinant humanized monoclonal anti-CD200 antibody samalizumab in 23 patients with advanced CLL and 3 patients with MM was published (phase 1 study NCT00648738) (80). Treatment was associated with mild to moderate adverse events and resulted in a dose-dependent decrease in CD200 expression on CLL cells. Decreased tumor burden was also observed in 14 CLL patients, with one of them achieving a durable partial response, while 16 patients maintained a stable disease. These results, although preliminary, suggest that samalizumab could represent an immune checkpoint inhibitor with activity in CLL.

Finally, there is some evidence that serum levels of soluble CD200 may be related to disease progression and prognosis in patients with CLL (79, 81, 82). In particular, Wong et al showed that CD200 can be released from CD200+ neoplastic cells by ectodomain shedding (81). This event is regulated by ADAM28 (79, 82). Interestingly, both the membrane and the soluble form of CD200 is able to engage CD200R, which in turn can result in increased tumor growth (20, 83). This happens by means of reduction of immune reactivity and/or increment of induction/activation of regulatory T cells in the tumor microenvironment (83, 84). Taken together, more and more convincing data have been reported in the literature from which the relevant role of CD200 is emerging not only as a diagnostic and prognostic tool but also as a potential therapeutic target in various neoplasms including CLL.

AUTHOR CONTRIBUTIONS

GD'A, GP, VD, FA, SD, DE, AS, and LL designed the project, revised the scientific literature, and wrote the paper. GD'A, GP, ES, GM, and OV followed patients with chronic lymphocytic leukemia. FL, FD'A, TS, and LV performed flow cytometric studies. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

REVIEW
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Angiogenesis in Lymph Nodes Is a Critical Regulator of Immune Response and Lymphoma Growth

Lutz Menzel[1]*, *Uta E. Höpken*[2] *and Armin Rehm*[1]*

[1] Translational Tumor Immunology, Max Delbrück Center for Molecular Medicine, Berlin, Germany, [2] Microenvironmental Regulation in Autoimmunity and Cancer, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany

Tumor-induced remodeling of the microenvironment in lymph nodes (LNs) includes the formation of blood vessels, which goes beyond the regulation of metabolism, and shaping a survival niche for tumor cells. In contrast to solid tumors, which primarily rely on neo-angiogenesis, hematopoietic malignancies usually grow within pre-vascularized autochthonous niches in secondary lymphatic organs or the bone marrow. The mechanisms of vascular remodeling in expanding LNs during infection-induced responses have been studied in more detail; in contrast, insights into the conditions of lymphoma growth and lodging remain enigmatic. Based on previous murine studies and clinical trials in human, we conclude that there is not a universal LN-specific angiogenic program applicable. Instead, signaling pathways that are tightly connected to autochthonous and infiltrating cell types contribute variably to LN vascular expansion. Inflammation related angiogenesis within LNs relies on dendritic cell derived pro-inflammatory cytokines stimulating vascular endothelial growth factor-A (VEGF-A) expression in fibroblastic reticular cells, which in turn triggers vessel growth. In high-grade B cell lymphoma, angiogenesis correlates with poor prognosis. Lymphoma cells immigrate and grow in LNs and provide pro-angiogenic growth factors themselves. In contrast to infectious stimuli that impact on LN vasculature, they do not trigger the typical inflammatory and hypoxia-related stroma-remodeling cascade. Blood vessels in LNs are unique in selective recruitment of lymphocytes via high endothelial venules (HEVs). The dissemination routes of neoplastic lymphocytes are usually disease stage dependent. Early seeding via the blood stream requires the expression of the homeostatic chemokine receptor CCR7 and of L-selectin, both cooperate to facilitate transmigration of tumor and also of protective tumor-reactive lymphocytes via HEV structures. In this view, the HEV route is not only relevant for lymphoma cell homing, but also for a continuous immunosurveillance. We envision that HEV functional and structural alterations during lymphomagenesis are not only key to vascular remodeling, but also impact on tumor cell accessibility when targeted by T cell–mediated immunotherapies.

Keywords: lymphoma, B cell malignancy, angiogenesis, lymph node, tumor microenvironment, reactive endothelium, lymphocyte trafficking, high endothelial venule

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Reviewed by:
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Patricia Perez Galan, Institut de Recerca Biomèdica August Pi i Sunyer (IDIBAPS), Spain

***Correspondence:**
Lutz Menzel
lutz.menzel@mdc-berlin.de
Armin Rehm
arehm@mdc-berlin.de

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INTRODUCTION

Lymph nodes (LNs) are strategically positioned hubs of the immune system, connecting the lymphatic system with the blood circulation, filtering antigens and organizing the encounter of lymphocytes with antigen presenting cells (APCs). The LN parenchyma is tightly packed with numerous types of immune cells and susceptible for their immigration and release during conditions of homeostasis, inflammation and tumor transformation. The complex reciprocal interactions of stromal cells and immune cells in LNs shape an adapted microenvironment that supports angiogenesis and increased LN vascularization (1). Although numerous studies reported vascular remodeling and expansion in LNs upon pathogen or tumor cell encounter, the detailed mechanisms and the participating cells of these angiogenic processes are not yet identified. In this review, we delineate the current state of knowledge and propose probable cellular interactions that participate in vascular growth in LNs. In particular, we will focus on the intricate relationship between immune cells and vascular cells as a major pillar of the tumor microenvironment (TME).

B cell non-Hodgkin lymphoma (B-NHL) is a heterogenous group of hematological malignancies that arise from B lymphocytes at various stages of differentiation. Lymphomas grow in the bone marrow and in the secondary lymphatic organs (SLOs), with a predominance of LNs and spleen, but they can also manifest in non-lymphoid tissues (2). The genetic and epigenetic alterations and the intracellular pathway dysregulations responsible for the pathogenesis and progression of lymphomas have been extensively studied and led to tremendous advancements in therapeutic intervention strategies (3). The idea of tumor dependency on cells in the surrounding of a a priori benign environment and on adapted organ properties goes back to Rudolph Virchow in the 19th century (4). The crucial influence of the cellular context in which lymphoma cells arise and lodge attracts growing interest, and the investigation of the TME became an increasingly appreciated field in cancer research (5, 6). The TME constitutes about half of the tumor mass in indolent follicular lymphoma (FL) and marginal zone lymphoma (MCL), whereas the proportion in aggressive diffuse large B cell lymphoma (DLBCL) is generally lower and scarce in Burkitt's lymphoma (BL) (7). On the extreme, in classical Hodgkin lymphoma (cHL) only about 2%–3% of the cells comprise the malignant Hodgkin-Reed-Sternberg cells (8). Hence, the composition and the dependency of the different B-NHL and cHL on the TME differ substantially between the entities (7). What distinguishes solid tumors and their metastasis most from lymphoma is that within SLOs, transformed B cells encounter a TME infrastructure that genuinely supports survival of benign B cells. These tissues undergo refinement to the needs of the tumor cells induced by a continuous reciprocal crosstalk of tumor, immune and mesenchymal stromal cells (9).

The complex interactions of transformed B cells and the TME lead to extensive changes of the vasculature within the affected organs, which is considered to have a substantial prognostic impact on the patients' disease outcome (7, 10). The stromal compartment, mainly comprised of blood vessels, lymphatic sinuses and the fibroblastic reticular network is tightly interconnected and regulated. In some respect, it can be considered to represent a joint structural compartment in which its distinct subcompartments grow and remodel in a synchronized manner (11, 12). While the crucial role of lymphatic vessels during lymphoma growth and dissemination is undisputed (13, 14), here we will highlight the influence of blood endothelial cells (BECs) and the blood vasculature, which comprise the main provider of nutrition for proliferating and differentiating immune and tumor cells. In addition, the blood vasculature shapes a major dissemination route for benign immune and transformed cells (15, 16).

EXPANSION OF BLOOD VASCULATURE IN LNs DURING DEVELOPMENT, INFLAMMATION, AND CANCER

Tumors often recapitulate developmental traits of tissues in which they arise. The stem cell-like phenotype of many tumors is characterized by gene expression signatures that are associated with embryonic stem cell identity and underlines the close transcriptional relationship between neoplastic and developmental tissue (17, 18). Similar to rapidly developing and growing organs, tumors require blood vessels to access oxygen and nutrients. The initiation of blood vessel expansion, referred to as angiogenic switch, occurs at different stages during tumorigenesis, depending on the tumor type and the respective TME. The onset of neo-vascularization and vascular remodeling is a multifactorial processes orchestrated by activating and inhibiting factors whose balance determines whether BECs stay quiescent or get activated (19).

Therefore, it is useful to recapitulate the essential steps during development to understand the basal mechanisms of the microenvironmental remodeling in LNs. Blood vessels in LNs reside within the stromal scaffold and are crucial for the delivery of oxygen, nutrients, and cells. The critical delivery function was demonstrated by the rapid occurrence of necrosis in LNs upon ablation of the arterial feeding vessel in rats (20). During development (**Figure 1**), LNs evolve from budding lymphatic veins that form a primordial lymph sac, also known as LN anlagen. Studies with transgenic mice lacking lymphatic vessels due to the deficiency for the transcription factor (TF) Prox1 or appropriate lymphangiogenesis factors, e.g., vascular endothelial growth factor-c (Vegfc$^{+/-}$), revealed a compromised LN development (21, 22). LN anlagen recruit hematopoietic lymphoid tissue-inducer (LTi) cells, which in turn stimulate local mesenchymal cell differentiation into lymphoid tissue-organizer (LTo) cells. The accumulation and interaction of lymphotoxin (LT) $\alpha_1\beta_2$ on LTi cells and LTβ receptor (LTβR) expressing LTo cells results in a self-amplifying loop of LTi recruitment and LTo differentiation that drives the LN development (23). The lymphoid organogenesis is accompanied by the maturation of blood vasculature driven by locally generated retinoic acid (RA) (24). RA is presumably provided by neurons localized adjacent to the developing LN. It directly regulates the proliferation of endothelial cells, but also induces CXCL13 expression in LTo cells via binding to the RA receptor-related orphan receptor (RORγt). CXCL13 in cooperation with its receptor CXCR5 is the exclusive inducer of the initial clustering of LTi cells in LN anlagen independently of LT-LTβR signaling (25). A ubiquitous expression of the mucosal addressin cell adhesion molecule-1 (MadCAM-1) on

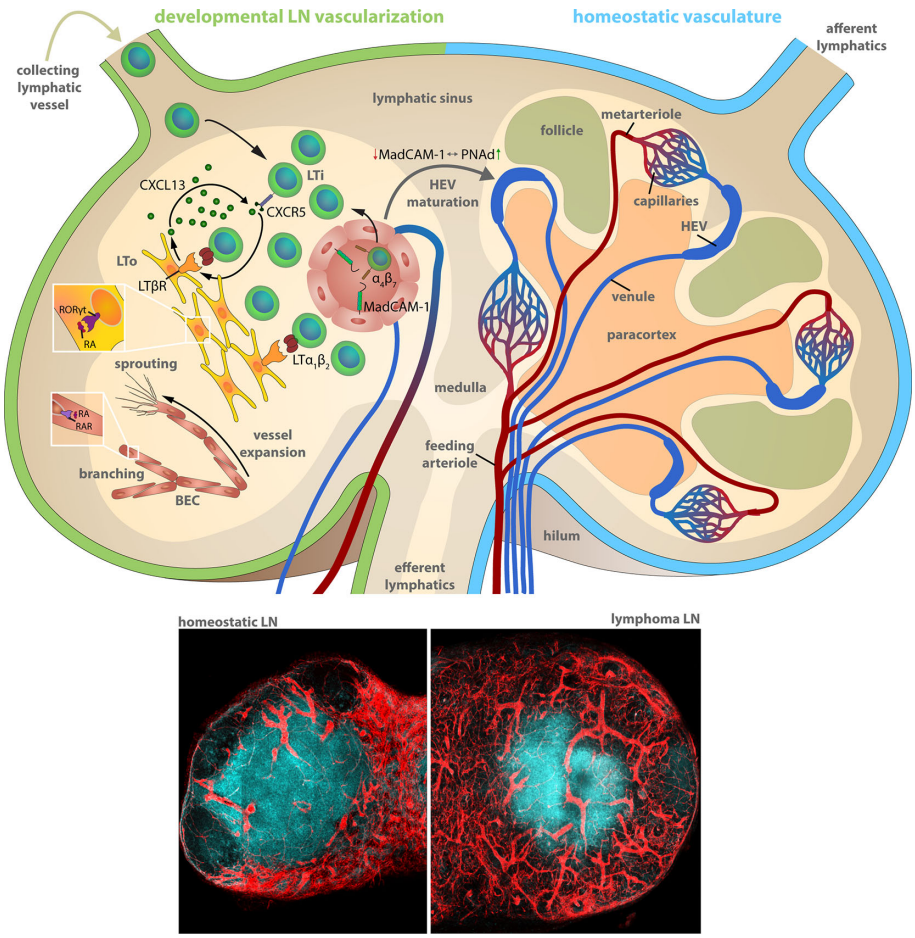

FIGURE 1 | Lymph node vascularization in development and under homeostatic conditions. The LN compartments during LN development (left) and homeostatic conditions (right). Left: Lymphoid organogenesis is driven by recruitment of Lymphoid tissue-inducer (LTi) cells that stimulate lymphoid-organizer (LTo) cells via lymphotoxin (LT) $\alpha_1\beta_2$ - LT β receptor signaling, which secrete LTi-recruiting CXCL13 in turn. LTi recruitment from the blood circulation and the afferent lymphatics accumulates LTi cells within the LN anlagen resulting in a self-amplifying process of LN development. $\alpha_4\beta_7$ integrin-expressing LTi recruitment and extravasation utilizes the mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1) on the luminal surface of blood vessels. MadCAM-1 switches to peripheral node addressin (PNAd) expression during differentiation of mature high endothelial venules (HEVs) within peripheral LNs. The formation of the blood vessel network comprises sprouting and branching of expanding blood vessels driven by retinoic acid (RA) stimulation of the RA receptor (RAR) on blood endothelial cells (BECs). Right: The blood circulation enters the LN during homeostatic conditions via the feeding arteriole at the LN hilum, proceeds along the medullary cord and branches into metarterioles that feed the capillary networks around the medulla and at the subcapsular sinus. HEVs are post-capillary venules with a characteristically enlarged vessel diameter. The venous backflow leaves the LN in a bundle of venules at the hilum. Bottom: Representative histochemistry sections (vessels: *Cadherin5*$^{fluoresent\_reporter}$, red) of murine LNs during homeostasis and during progression of a murine high-grade B cell lymphoma.

developing venous blood vessels in the LN mediates the directed immigration of the α4β7 integrin expressing LTi cells (26–28). Notably, the expression of MadCAM-1 in peripheral LNs of newborns switches during the formation and maturation of high endothelial venules (HEVs) into the expression of the peripheral node addressin (PNAd). PNAd expression marks the completion of the maturation of the postcappillary vessels to highly differentiated HEVs that provide all prerequisites for the functional transmigration of blood-borne lymphocytes into the developing and homeostatic LN (**Figure 1**) (26).

The vascular system of LNs in adult mammals is composed of arteries, capillaries, post-capillary venules and veins (29). Arteries are characteristically located at the periphery of the LN. The feeding arteriole enters the LN at the hilum and exhibits a gradual decrease in diameter at its few branching points alongside the medullary cords until it reaches the subcapsular capillary network. Capillaries form a dense network under the subcapsular sinus and around the medullary cords, whereas they are markedly less dense in cortex regions and sparse within the paracortex under homeostatic conditions. The vessel diameter abruptly increases at the capillary to post-capillary transitions. These post-capillary venules, referred to as HEVs, are primarily located within the cortex in the interfollicular space. HEVs form loop-like structures following a centripetal course that ends in

transitions to veins at the corticomedullary junctions. Finally, a bundle of larger main veins leave the LN though the hilum (**Figure 1**) (29, 30).

Tumor growth is often accompanied by the ingrowth of blood vessels and the formation of a vascular network, consistent with the need for malignant cells to have access to the circulation system. Tumor vascularization occurs either through co-option of the pre-existing vasculature, or by induction of neovascularization. Vessel co-option is a non-angiogenic process in which tumor cells utilize pre-existing blood vessels of surrounding tissue to support tumor growth, survival and metastasis (31). In contrast, neovascularization involves a series of complex and sequential events: (I) activation of microvascular endothelial cells, (II) enzymatic degradation of the vascular basal membrane, (III) gradual degradation of other extracellular matrix (ECM) components, (IV) endothelial cell migration and proliferation, (V) lumen formation within neo-sprouts, (VI) branching of the neo-vessel, and (VII) formation of a functional vessel network by fusion with neighboring vessels to initiate blood flow (32, 33). Located at the leading edge of the vascular sprout, tip cells form cellular protrusions or filopodia to guide migration toward a source of angiogenic growth factors. Simultaneously, they signal to adjacent endothelial cells via Delta-like ligand (DLL)-Notch interactions not to adapt the tip cell phenotype, but to maintain the proliferative stalk cell phenotype and to form a vascular lumen (34, 35). The vascular endothelial growth factors (VEGFs) are the major contributors to angiogenesis. The local secretion of VEGF-A and its gradient forming deposition on the ECM triggers endothelial tip cell formation via binding to VEGFR2, resulting in endothelial cell proliferation and migration and eventually, formation of tube structures resembling new capillaries (35–38). VEGF-B, VEGF-C, and VEGF-D are other members of the VEGF family of which VEGF-C plays a critical role upon LN remodeling because it is the most potent inducer of lymphangiogenesis as a ligand of VEGFR3. VEGFR3 is known for its involvement in physiological and tumor-associated lymphangiogenesis and lymphatic metastasis (39, 40). Apart from lymphatics, VEGFR3 is highly expressed at the leading-edge of BECs that undergo sprouting (41) and was recently shown to coregulate the expansion of the blood vessel network in LNs in a *Myc*-driven high-grade B cell lymphoma mouse model (42). Fibroblast growth factors (FGFs) stimulate endothelial cell migration and proliferation in a very potent manner, which in *in vitro* experiments even exceeds the stimulation capacity of VEGF-A (43, 44). FGF-1 stimulates proliferation and differentiation of all cell types necessary for the formation of arterial vessels, including endothelial and smooth muscle cells. The angiogenic potency of FGFs extends to prompt fibroblastic cells (e.g., pericytes, smooth muscle cells, and mural cells) and recruits them for vessel formation and maturation during tumorigenesis (45). FGF-2, the second most abundant growth factor of the FGF family, promotes endothelial cell proliferation and the physical organization of the endothelial cell tube-like formation during developmental vessel assembly (46, 47).

The integral investigation of the highly complex vascular network and the unique features of its parts in context of the compartmentalized architecture of the LN has long been a challenge for microscopic image analysis. Because higher order anatomical data sets were obtained from such advanced optical imaging approaches, algorithms for data handling were also demanding to generate. Over the last couple of years, novel tissue preparation methods (48, 49), imaging systems and computational rendering strategies evolved, which enable contextual and organ-wide topological analyses in three-dimensional spaces and over time. In particular, optical projection tomography (OPT) and light sheet microscopy have been established to study anatomical and functional features of LN, e.g., to quantify capillary and HEV structures and their contextual relationship to B cell follicles and dendritic cells (DCs) throughout the organ (50–52). A combination of microscopic imaging and computational modulation of the hydrodynamic properties of vessels in LNs revealed a tight connection of the hydraulic conductivity between lymphatic and blood vessels and the respective hydrodynamic conditions within the LN. These biophysical conditions are vital for inter- and intra-LN transport mechanisms and immunological functions, and most likely for lymphoma B cell dissemination and immunosurveillance as well (53, 54). Up to date, these dynamic conditions are not easy to mimic in organoid models. However, in an early 3D organoid model mimicking a LN exposed to tissue injury or inflammation, the interstitial flow affected the fibroblastic reticular cells (FRCs) that enwrap conduits transporting fluid from the subcapsular sinus to HEVs. Blocking this flow led to CCL21 downregulation, indicating that increased lymph flow as a hydrodynamic factor acts on the paracortex and thus, affects the remodeling and functionality of conduits and FRCs (55). In line, mechanosensing of conduit flow deprivation by FRCs in Peyer's patches resulted in dysfunctional HEVs and disturbed mucosal immune responses (56). Similar processes are also conceivable during lymphoma growth within LNs, where a gradual loss of HEVs in numerous B-NHL was described many years ago (57). A comprehensive and continuous blood vessel network of LNs under homeostatic conditions has been revealed (54, 58) and brought up an analysis pipeline for detailed and whole-organ investigations of the LN vasculature upon perturbations through inflammation, lymphoma homing and LN solid tumor metastasis. Recently, utilization of single cell transcriptome analysis methods revealed a broad overview of the heterogeneity of ECs throughout several different murine organs, including the spleen and LN as representatives for SLOs (59, 60).

THE BLOOD VASCULATURE IS PART OF THE REACTIVE STROMAL INFRASTRUCTURE DURING INFLAMMATION AND CANCER DEVELOPMENT

Inflammation, vessel reorganization and angiogenesis are intimately connected processes. In adults, angiogenesis usually occurs during pathological settings such as infection, wound

healing and cancer. Notably, hematopoietic cells and endothelial precursors share common $CD34^+$ stem and progenitor cells (61).

Growth of solid tumors is typically associated with inflammation that triggers tissue-protective and pro-tumorigenic mechanisms. Inflammatory responses in normal tissue and cancer are initiated and maintained by local tissue or cancer associated macrophages (TAMs) and DCs. Sustained inflammation further leads to recruitment of bone marrow–derived monocytes, neutrophilic granulocytes, myeloid-derived suppressor cells (MDSC), and tissue or tumor infiltration of lymphocytes from the SLOs. Especially cytokines and chemokines, transcriptionally regulated downstream of NF-κB signaling pathways in immune cells, promote cell survival and proliferation, recruit more immune cells and re-shape the TME. Pro-inflammatory cytokines like IL-6, TNFα and IL-17, increase the proliferation rate of other inflammatory immune cells and prime the tumor to overcome suboptimal microenvironmental conditions including lack of nutrients, growth factors and hypoxia (62). Inflamed tissue and solid tumors are often characterized by insufficient oxygen supply that triggers angiogenesis. Hypoxia, which is the major driver of vascular alterations in solid tumors, stabilizes the TF HIF-1α, the master regulator of pro-angiogenic factor expression such as VEGFs, CXCL12, and COX-2 (63–65). The presence of a constant pro-angiogenic milieu in solid tumors often causes a disturbed maturation and pruning of blood vessels. The division in arterioles, capillaries and venules can be deficient and results in an aberrant distribution of vessel caliber, influencing the blood flow. Morphologically, a poorly organized, malformed vessel network develops under these conditions (66, 67). The endothelial junctions in such malformed networks are often defective and lead to enhanced permeability and elevated interstitial fluid pressure (68). Pericytes can be partially detached and newly build blood vessels often fail to recruit sufficient pericyte coverage, causing an unevenly distributed basement membrane, vessel fragility, and risk of hemorrhage (69, 70). Besides the structural and functional defects, the specific transcriptional response of tumor vasculature is not only related to angiogenesis and vessel integrity, but affects endothelial activation and recruitment of leukocytes as well. Pro-angiogenic signaling leads to endothelial anergy, reduced response to pro-inflammatory signaling and decreased expression of adhesion molecules and chemokines necessary for capture and trans-endothelial migration of leukocytes (71, 72).

In LNs, which are the authochthonous environment for most B-NHL, the pre-existing vasculature takes part in the massive remodeling process during immune responses, best studied for strong inflammatory stimuli in mice (50, 73, 74). LNs are plastic organs able to expand to a multiple of their normal size within days including an extensive remodeling of the vascular-stromal compartment. The rapid expansion of the LN size and cellularity includes early events of remodeling of the feeding artery, causing an increased blood flow and LN hypertension accompanied by an increase of the vascular permeability (75, 76) and increased interstitial pressure. The capillary network within the cortex and medulla expands toward the paracortex, and post-capillary venules are reorganized (30). Skin allograft-draining LNs in rats exhibited a progressive elongation and branching of HEVs resulting from focal proliferation of endothelial cells in the transition zone from high to low endothelium (77). Several years later, Bajénoff and colleagues revisited these observations and investigated the BEC proliferation applying a multicolor fluorescence fate-mapping mouse model. They found similar proliferation foci in post-capillary venules as proposed by Anderson and Anderson. In addition, an extensive expansion of the LN vasculature relying on the sequential assembly of endothelial cell proliferative units upon inflammation was observed. Clonally proliferating HEV cells (73) and capillary resident precursors (60) comprised local progenitors for HEV elongation and capillary neo-vessels during BEC turnover and vessel sprouting. Interestingly, recruitment of bone marrow–derived endothelial cell progenitors did not contribute to the local LN vascular alterations in this model. LN expansion stimulated by several immunization strategies in mouse experiments, e.g., bone marrow–derived DCs (78), ovalbumin/complete Freund's adjuvant (OVA/CFA) (79), OVA/alum (80), oxazalone (11), and lymphocytic choriomeningitis virus (LCMV) infection (50) indicated similar courses of vessel expansion, starting with early proliferation events that last for up to 5–8 days. The remodeling eventually ends with a gradual re-establishment of the vascular endothelial cell quiescence, a normalization of the vascular bed and restoration of the normal LN size (30, 73, 78).

IMMUNE CELLS ARE MEDIATORS OF ANGIOGENESIS

Both innate and adaptive immune cells have an intricate relationship with angiogenesis. They are involved in regulation of BEC proliferation, migration and activation and they provide a large spectrum of pro-angiogenic mediators apart from their genuine immunological function. Hence, immune cells induce, support or antagonize angiogenic processes during inflammation and tumor growth (**Figure 2** and **Table 1**) (124, 125). Angiogenesis is also important for the progression of B cell lymphoma, however the role of angiogenic factors and the composition of pro-angiogenic immune cells within LNs varies between different entities.

A leading immune cell source for growth factors and chemokines to promote angiogenesis under inflammatory and tumorous conditions are myeloid cells (126). **Macrophages** are phagocytic immune cells and important regulators of tissue homeostasis, morphogenesis and repair. In LNs, macrophages are an abundant immune cell population that is divided into subcapsular sinus macrophages (SSM), medullary sinus macrophages (MSMs), and medullary cord macrophages (MCMs) (127). Monocytes from the blood stream and macrophages from LN remote tissues (e.g., bone marrow) infiltrate the LN attracted by a variety of chemotactic factors, among others CCL2, CXCL12, and the macrophage migration inhibitory factor (MIF) (128–130). Tumor-associated macrophages (TAMs) play a prominent role during progression of chronic lymphocytic leukemia (CLL) by supporting tumor cell survival (131) and regulation of the TME (132). The presence and

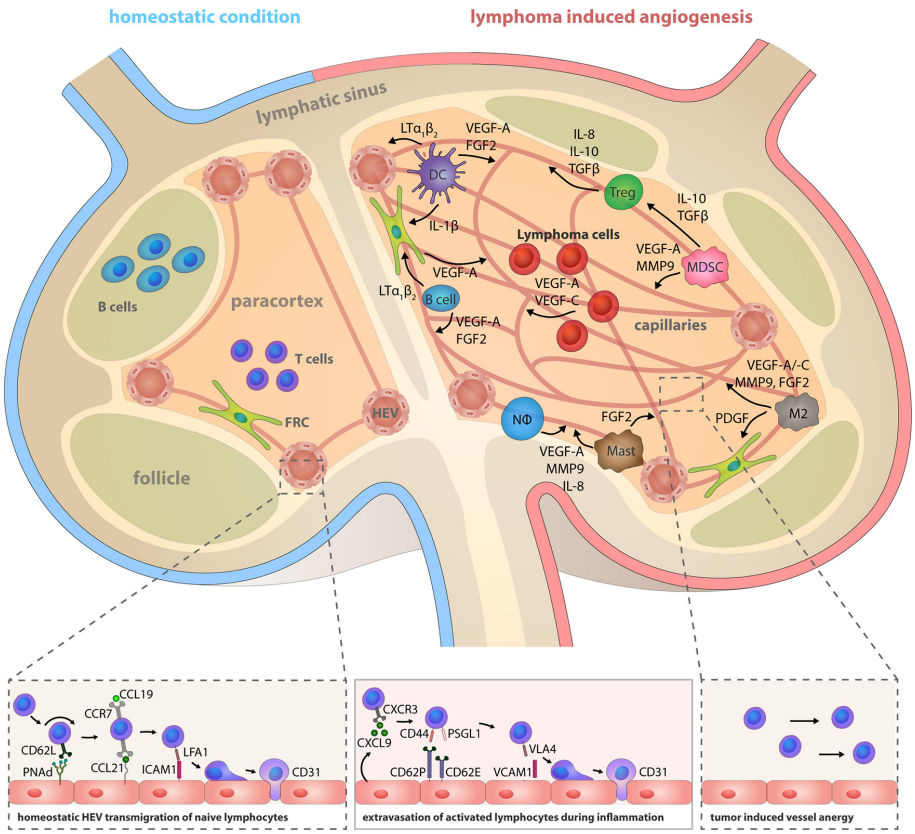

FIGURE 2 | Lymphoma induced angiogenesis in LNs and participating immune cells. Top: The LN compartments represented under homeostatic conditions (left) and lymphoma-activated angiogenesis (right). Lymphoma growth is characterized by a strong LN volume expansion and blood vasculature growth. Remodeling of the stromal infrastructure involves an increase of the microvessel density (MVD), as effectuated by direct angiogenic stimulation through lymphoma B cells cells, but concomitantly also through reciprocal crosstalk of cells in the TME and recruited immune cells. Notably, the initiation of the angiogenic switch in lymphoma is independent from hypoxia-induced HIF1α pathway activation. Tumor polarized DCs (CEBP/β^{high}) control the HEV differentiation status via $LT\alpha_1\beta_2$ and LIGHT presentation; they release IL-1β and hereby take part in the blood vessel growth by inducing VEGF-A expression in FRCs. They also secrete the angiogenic factors VEGF-A and FGF2. B cells express $LT\alpha_1\beta_2$, which exerts minor effects on HEVs, but a predominating stimulatory effect on FRCs. Expression of the chemokines CCL2, CXCL12, and MIF recruits additional immune cells into the LN. Regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), M2-polarized macrophages, neutrophils and mast cells are capable of producing the pro-angiogenic factors VEGF-A, VEGF-B, VEGF-C, MMP9, IL-8, IL-10, TGFβ, and FGF1/2. Bottom left: HEVs express PNAd, CCL21, and ICAM1 and thereby constitute the transmigration routes for lymphocytes under homeostatic conditions. Interaction of CD62L, CCR7, and LFA-1 on naïve lymphocytes with these HEV-associated surface receptors and chemokines initiates lymphocyte rolling, HEV wall adhesion and eventually, transmigration into the LN parenchyma. Bottom middle: Inflammatory vessels in reactive LNs recruit activated lymphocytes by CXCL9 secretion and replace the homeostatic receptors on endothelial cells with CD62P, CD63E, and VCAM1 that are interaction partners of leukocyte-expressed CD44, PSGL1, and VLA4. Bottom right: The lymphoma induced expansion of the blood vessel network favors the assembly of smaller anergic endothelium that is insufficiently equipped for lymphocyte extravasation.

polarization of macrophages during CLL is critical for the tumor progression, as indicated by a CLL-associated skewing of T cells toward antigen-experienced phenotypes and T cell exhaustion, which could be reversed by monocyte and macrophage depletion. Thus, interference with macrophage polarization in CLL turned out to be a promising target for immunotherapy (133). Similar to TAMs in leukemia, macrophages likely support angiogenesis in lymphoma as well, both in cHL and B-NHL. M2-polarized macrophages induce an immunosuppressive milieu in cHL, comprising the majority of the PD-L1 expressing cells, located in close proximity to the Hodgkin Reed Sternberg (HRS) cells (134). In this disease-defining tumor cell population, high frequencies of alterations on chromosome 9p24.1, involving copy number gain and amplifications, have been shown to increase the abundance of the PD-1 ligands, PD-L1 and PD-L2 (135). Furthermore, Epstein-Barr virus (EBV) infection can increase expression of PD-1 ligands in cHL as well (136). The TAM derived PD-L1 in conjunction with the HRS-cell derived PD-1 ligands PD-L1 and PD-L2 may neutralize the anticancer activity of PD-1+ T cells and natural killer cells, a process that can be reversed by utilizing PD-1 blocking antibodies (137). TAMs were also frequently found in FL and DLBCL, among them often polarized and pro-angiogenic M2-like macrophages, which secrete angiogenic factors and re-arrange the ECM by matrix metalloproteinase (MMP) release for vascular expansion (138, 139).

TABLE 1 | Immune cells derived pro-angiogenic factors.

Cells	Condition	Angiogenic Factors	Reference
MΦ	Inflammation (mouse, LPS, LTA/MDP)	VEGF-A/C/D	(81)
	Development (zebra fish)	VEGF-A	(82)
	Inflammation (mouse, OVA/CFA)	IL-1β via FRCs	(83)
	Hypoxia (in vitro)	VEGF, bFGF, CXCL8, COX2, HGF, MMP12	(84)
	Mouse/chicken angiogenesis model	MMP2, MMP9	(85)
	Human Monocytes (*in vitro*)	VEGF-A	(86)
	Mouse Matrigel Assay (*in vivo*)	IL-1β	(87)
	Mouse Matrigel Assay (*in vivo*)	FGF2, PlGF	(88)
	Human atherosclerotic plaques	VEGF-A	(89)
	Mouse solid tumors	PDGFβ	(90)
	Squamous carcinoma	VEGF-C	(91)
	human cell lines *in vitro*	TP	(92)
	Ovarian carcinoma	MMP9	(93)
	Breast carcinoma	VEGF-A	(94)
DC	OVA/CFA inflammation (mouse)	VEGF-A	(95, 96)
	Inflammation (mouse, LPS)	PGE_2	(97)
	LPS, PGE_2 *in vitro* (mouse)	FGF2	(95)
	development and homeostasis (mouse)	$LT\alpha_1\beta_2$	(98, 99, 100)
	Inflammation (mouse, OVA/CFA)	IL-1β via FRCs	(83)
	co-culture with NK cells (in vitro)	VEGF-C	(101)
	Il-10 stimulation (in vitro)	Osteopontin	(102)
NΦ	Mouse Matrigel Assay (*in vivo*)	VEGF-A, MMP9	(103)
	Human cells, angiogenesis assay (in vitro)	VEGF-A, IL-8	(104)
	Mouse wound healing assay	VEGF-A	(105)
MC	human skin	VEGF-A, IL-8, MCP-1	(106) (107)
	Human lung mast cells (in vitro)	MMP9, VEGF-A/B/C/D	(108)
	Thyroid cancer	IL-8	(109)
MDSC	Mouse tumor models	VEGF-A, G-CSF, MMP9	(110)
	Mouse melanoma model	VEGF-A	(111)
	Mouse ovarian cancer model	VEGF-A	(112)
	Multiple myeloma mouse model	MMP9	(113)
	Colorectal cancer mouse model	MMP9	(114)
T cells	Inflammation (mouse, OVA/Montanide)	$LT\alpha_1\beta_2$ via FRCs	(115)
	HUVECs (in vitro)	GM-CSF, IL-8	(116)
	Ischemia mouse model and *in vitro*	IL-10, amphiregulin	(117, 118)
	Type 2 Diabetes	Amphiregulin, IL-10	(119)
	Systemic sclerosis	IL-8, MMP9, VEGF-A induce EPCs diff.	(120, 121)
	Hypoxia (*in vitro*, ovarian cancer)	VEGF-A	(118)
B cells	Inflammation (mouse, LPS)	VEGF-A	(122)
	Inflammation (mouse, OVA/Montanide)	$LT\alpha_1\beta_2$ via FRCs	(115)
	Virus infection (mouse, LCMV)	$LT\alpha_1\beta_2$	(50)
	In vitro tube formation	VEGF-A, FGF2, PDGFA	(123)

MΦ (macrophages), DC (dendritic cells), NΦ (neutrophils), MC (mast cells), MDSC (myeloid-derived suppressor cells).

Regarding angiogenesis, macrophages play a crucial role at each step of the angiogenic cascade, starting from blood vessel sprouting to vessel maturation and remodeling of the vascular network. Pro-inflammatory conditions polarize classical activated macrophages (M1), whereas anti-inflammatory conditions give rise to alternatively activated macrophages (M2) including angiogenesis associated macrophages (140–142). For example, in cHL HRS-cell derived TGF-β, IL-13 and M-CSF educate monocytes or TAMs to become immunosuppressive M2-polarized TAMs (Aldinucci D, Casagrande N, 2016, Cancer Letters; Skinnidder and Tak Mak, 2002, Blood).

Macrophage-derived MMP2 and MMP9 proteases cleave the ECM to break open matrix remodeling to pave the way for endothelial sprout migration (85). Pro-angiogenic growth factors, such as VEGF-A, MMPs, IL-1β, FGF2, and transforming growth factor beta 1 (TGFβ1), are part of the repertoire secreted by macrophages in a pro-angiogenic milieu (85–89). Upon hypoxic conditions that lead to HIF1α activation, macrophages are able to establish capillary-like networks in which they line a vessel micro-tunnel and express lineage aberrant endothelial markers such as CD31, von Willebrand factor and Cadherin-5, leading to the assumption that macrophages may transdifferentiate into ECs under specific conditions (143, 144). Macrophages also function as cellular chaperones during anastomosis of vascular sprouts by guiding endothelial tip cells to undergo sprout fusion (82). Such macrophages expressed the markers tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE2) and neuropilin-1 (Nrp1), indicating that they are M2 polarized cells

with properties similar to TAMs (82, 145). New blood vessels need to undergo maturation to become functionally stable. A crucial step in this process is the integration of new blood vessels into the established stromal environment and the recruitment of pericytes to strengthen vascular junctions. Macrophages are highly abundant around new blood vessels and help to recruit pericytes by secretion of the platelet-derived growth factor β (PDGFβ) (90, 146).

Dendritic cells (DCs) are sentinel cells that connect the innate and adaptive branches of the immune system wherein they have important roles in host defense against pathogens and in generating anti-tumor immune responses. The classical DC compartment of the spleen is comprised of lymphoid tissue-resident DCs, whereas LNs also include non-lymphoid tissue-migratory DCs (147). Especially $CD11c^{medium}MHCII^{high}$ DCs are associated with the initiation of vascular expansion after bone marrow–derived DC (BMDC) transfer, whereas $CD11c^{high}MHCII^{medium}$ DCs accumulate later in the process and promote the re-establishment of vascular quiescence (78). Apart from their predominant immunologic role as professional APC population, DCs carry a wide range of angiogenic mediators to modulate vascularization. They do so by engaging cognate signaling receptors, such as VEGFR2 on endothelial cells or by recruiting and stimulating adjacent cells or cells of the TME (96). LN-resident DCs are closely associated with FRCs and sample conduit-conveyed antigens within the paracortical and interfollicular zone, where they are located in the proximity of HEVs (148). This spatial proximity suggests that DCs are likely to be a link between immune cells, vasculature and mesenchymal stromal cells. The development, maturation and lineage commitment of DC subsets is differentially regulated by acomplex TF network, depending on homeostatic, inflammatory, and tumorous conditions. We recently demonstrated that the TF C/EBPβ plays a crucial role in murine DC maturation and immunogenic functionality under homeostatic and lymphoma-transformed conditions (149). In the presence of lymphoma cells, enhanced expression of C/EBPβ in DCs was observed which transformed them into an immature, tolerogenic and pro-tumorigenic subtype (150). Such aberrant maturation stages may potentially affect the angiogenic capacities of the DCs as well. The crucial role of DCs for the LN vasculature was elaborated in several studies (12, 78, 79, 96) and revealed the DC-coordinated remodeling mechanisms of blood- and lymph-vasculature, and the FRC network as well. The DC associated increase of VEGF-A in reactive LNs further includes stimulation of a pro-angiogenic program in FRCs and the recruitment of blood-borne cells that participate in the angiogenic process. IL-1β expression by recruited $CCR7^{+}$ $CD11c^{+}$ DCs is associated with the enrichment of VEGF-A expressing FRCs at the border of the LN paracortex (83). The angiogenic role of DCs in lymphoma LNs has not been investigated yet; however, in reactive LNs, resident classic DCs produce biologically active VEGF-A downstream of the inflammation-associated TFs HIF-1α, STAT3, and CREB. HIF-1α and STAT3 are generally related to hypoxic conditions, whereas CREB phosphorylation is the consequence of autocrine and paracrine prostaglandin E2 (PGE_2) signaling (151). The PGE_2 production is directly connected to pathogen induced toll like receptor-4 (TLR4) signaling and therefore, delineates the connection of infection induced LN reactivity with angiogenic vessel formation (152). DCs also release other classical angiogenic growth factors like FGF2, endothelin-1 (ET-1), CXCL12, and COX-2. FGF2 activates endothelial cells and induces VEGF-A expression in mesenchymal cells, but also recruits and activates macrophages and mast cells that in turn exhibit angiogenic properties (152, 153). DCs further have the capacity to modulate angiogenesis in an indirect manner through secretion of the monocyte and granulocyte attracting chemokines CXCL8, CXCL1, CXCL2, CXCL3, and CXCL5 (154). The recruited myeloid cells can be triggered to secrete the pro-angiogenic IL-1β by a signaling pathway that includes classical DC-derived osteopontin (155). DCs are not only associated with vascular expansion, but also with the re-establishment of vascular quiescence and stability in the process of reinstallation of the LN homeostasis (78).

Neutrophilic granulocytes are the most abundant type of leukocytes throughout the body, representing the pioneering cells that are recruited to injuries and thus, they are frontline defenders against pathogens. Neutrophils infiltrate LNs guided by inflammatory cytokines like IL-1β and TNFα, the complement factors C3a and C5a, along the CXCR4-CXCL12 axis, and eventually they are also attracted by a plethora of inflammatory chemokines (156, 157) (Capucetti, Albano, Bonecchi, Frontiers in Immunology, 2020). Neutrophils are a source of soluble mediators that exert important angiogenic functions. VEGF-A, IL-8, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), and MMP9 are the most important activators of angiogenesis produced by these cells (103, 158). Interestingly, neutrophils are able to release VEGF-A-enriched granules upon TNFα stimulation and thus, promote vessel growth during inflammation (105). This can become an self-amplifying process since neutrophil-derived VEGF stimulates neutrophil migration via an autocrine amplification mechanism, a process that likely contributes to pathological angiogenesis during inflammation and cancer (159). Human polymorphonuclear granulocytes have been demonstrated to directly induce the sprouting of capillary-like structures in an in vitro angiogenesis assay, mediated by secretion of both pre-formed VEGF from cell stores and de novo synthesized IL-8 (104). In the murine Eμ-*Tcl1* model, mimicking CLL, a tumor-associated neutrophil (TAN) population with a B cell helper-like polarization was identified. Selective depletion of these TANs retarded leukemia progression in SLOs substantially (160).

Mast cells (MC) are hematopoietic tissue resident immune cells that are classically recognized as the main effector cell type of IgE-mediated immediate allergic reactions, however they are also frequently associated with tumorigenesis (161–163). According to their protease expression, mast cells are divided in two phenotypical populations: the $trypase^{+}$ $chymase^{-}$ (MC_T) and the $trypase^{+}$ and $chymase^{+}$ (MC_{TC}) cells (164, 165). MC produce several proangiogenic factors, among them VEGF-A, VEGF-B, MMP9, and FGF-2. In addition, mast cells chemotactically respond to VEGF-A and FGF2, indicating that a connection between mast cell accumulation at tumor sites, angiogenesis and tumor growth exists (166, 167).

Myeloid derived suppressor cells (MDSCs) contribute to the induction of an immune suppressive and tumor permissive microenvironment. They are frequently found in SLOs like spleen, but they are rare in LNs (168). However, they are able to modulate the L-selectin expression of naïve T and B cells, preventing efficient HEV adhesion, transmigration, and

subsequent antigen encounter within LN parenchyma (169, 170). MDSCs promote the formation of T regulatory cells (Tregs), the secretion of immunosuppressive IL-10 and TGF-β, and inhibit the activity of cytotoxic CD8 T cells via expression of arginase-1 (Arg1) and inducible nitric oxidase (iNOS) within the TME of several tumor entities including B cell lymphoma (171, 172). Moreover, MDSCs directly influence the tumor stroma by inducing differentiation of cancer-associated fibroblasts (CAFs) (173, 174). Pro-angiogenic properties of MDSCs during tumor progression have been reviewed recently (175). MDSCs and their progenitors, immature myeloid cells are usually not present in LNs during steady state conditions. However, inflammatory conditions and tumor-derived factors (e.g., CXCL12, GM-CSF, and CCL2) induce the activation and accumulation of MDSC in SLOs (176–178). MDSCs exhibit numerous immunomodulatory properties that have considerable potential to influence angiogenic processes in LNs, either through direct triggering of ECs, or by stimulating leukocyte and stromal cells to establish an angiogenic milieu (175). MDSCs are able to promote tumor angiogenesis through releasing VEGF-A and MMP9. Mouse models suggest that MDSCs integrate into the line of vessel-decorating endothelial cells (179). In mouse melanoma, MDSC contribute to A2B adenosine receptor-induced VEGF-A production (111, 180). VEGF-A in turn stimulates MDSC recruitment from the bone marrow, creating a self-enhancing feedback loop that promotes immunosuppression and vessel growth (112). One of the reasons why several angiogenic tumors occur to be insensitive to VEGF-A-targeted therapy is the presence and recruitment of MDSCs. These cells secrete high amounts of VEGF-A which might lead to neutralization of the VEGF-inhibition and additionally, they establish pro-angiogenic signaling pathways involving several other cells of the TME (110, 181). Moreover, MDSCs limit T cell adhesion and extravasation by VEGF-A stimulated suppression of endothelial ICAM-1 and VCAM-1 expression during tumor angiogenesis (71, 182).

Lymphocytes, the major regulatory and executive cell subset of the adaptive immune response are also able to influence angiogenesis during inflammation and cancer, although their specific implications are still enigmatic.

T cells comprise different subsets involved in lymphomagenesis, including naive T cells, memory T cells, and Treg cells (183). Several negative regulators of T cell activation act as checkpoints to fine-tune the immune response and regulate hyperactivation. Cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) are the most potent examples of T cell immune checkpoint molecules (ICB) (184). Cancer patients often display dysfunctional antitumor T cell responses because of the signaling pathways downstream of these receptors. PD-1 and CTLA-4 inhibition are subject of extended clinical studies and led already to impressive response rates in some tumor entities, among them melanoma, non–small cell lung cancer and for hematopoietic tumors, (184–186), in cHL as well (187, 188). By targeting abnormal formation of tumor vessels, anti-angiogenic agents potentially result in an enhanced infiltration of anti-tumor effector cells, making the combination of immune checkpoint inhibitors and anti-angiogenic agents a promising and complementary approach in cancer adoptive T cell therapy (189). On the other hand, as a result of IFNγ and IL-12 stimulation, microvascular endothelial cells express checkpoint molecules like PD-L1 (190, 191). In line, arterial vessels express PD-L1 and PD-L2 after toll like receptor (TLR)-3 activation upon bacterial infection (192). The regulatory and angiogenic effects of $CD4^+$ T helper cells (Th cells) are strictly associated with their differentiation. Cytotoxic $CD8^+$ T cells and $CD4^+$ Th1 cells produce IFNγ that restrains endothelial cell proliferation and induces expression of the angiostatic chemokines CXCL9/10/11 in TAMs (126, 193). *In vitro* studies revealed that Th2- and Th17 cell-conditioned medium triggered endothelial sprouting, whereas medium of Th1 cultures induced vascular regression. Conditioned medium from Tregs had a minor or no effect (116). In vivo, $CD4^+$ T cells display opposing effects on vascularization depending on their subset differentiation. Th1 cell-derived IFNγ impairs angiogenesis in ischemic tissue, an effect that is counteracted by regulatory $CD4^+$ T cells (Tregs) that antagonize the immunologic Th1 cell response by secreting anti-inflammatory IL-10 and TGFβ. Thus, Tregs display rather indirect pro-angiogenic properties, most likely by paracrine effects on other potentially pro-angiogenic immune cells (e.g., macrophages, DCs, mast cells) (119, 194). T cell recruitment, survival and functionality are highly dependent on tumor-polarized myeloid cells and tumor-derived factors. The typical immuno-suppressive milieu of the TME is characterized by polarizing factors, shifting CD4+ T cell differentiation toward $CD4^+CD25^+FOXP3^+$ Tregs. In the aggressive Myc-driven murine lymphoma model, this polarization process is promoted by DCs expressing increased amounts of the TF C/EBPβ (144).

In ovarian cancer, Tregs were selectively recruited into the tumor tissue via CCL22 and CCL28 production by the tumor cells and subsequently, Treg-induced secretion of high amounts of VEGF-A to promote endothelial cell proliferation (118, 195). A striking example for Treg recruitment represents cHL; here, Tregs are attracted via the Hodgkin-Reed-Sternberg cell-secreted chemokines CCL17 and CCL22, which engage the Treg-expressed chemokine receptor CCR4 (196), or by the chemokine CCL20 that binds to CCR6 (Baumforth, Birgersdotter, Machado, Am J Pathol, 2008). Th cells and cytotoxic T cells are required to mediate the anti-angiogenic effect of IL-12. IL-12–activated lymphocytes effectuate inhibition of tumor growth and function as anti-vascular agents that release higher amounts of IFNγ while they down-regulate VEGF in neighboring cells (197, 198). Noteworthy, the presence of IFNγ comes at the expense of an induction of PD-L1 on numerous stromal cell types, among them endothelial cells (199, 200); this process is likely to counteract the beneficial effects of IFNγ-secreting effector T cells which may be rendered dysfunctional (201). The infiltration of tumor sites by cytotoxic $CD8^+$ T cells is usually correlated with a favorable clinical prognosis, however immunosuppressive conditions can polarize these cells to $CD8^+FOXP3^+$ regulatory cells with similar immunomodulatory and angiogenic properties as $CD4^+$ Tregs (202–205). Studies of coronary artery disease and systemic sclerosis found T cells with angiogenic potential in blood samples of patients and demonstrated that these $CD3^+CD31^+CXCR4^+$ cells (referred to as angiogenic T cells) play a vital role for the colony formation

and differentiation of endothelial progenitor cells (EPCs) in the bone marrow (110, 206). Such EPCs have been detected in the circulation and in LN samples from patients with B-NHL as well, although their influence on lymphoma-induced vessel growth is still elusive (207, 208). However, inflammation models argue against a significant functional role of BM-recruited EPCs in LN vascularization (73).

B cells are frequently found to be part of the TME (209); however, their role in tumor progression and vascularization is still unclear. They can directly promote angiogenesis by secreting pro-angiogenic factors such as VEGF-A, FGF2, and MMP9 (210), or indirectly by polarizing macrophages to the M2 pro-angiogenic phenotype (211). Transgenic mice ($CD19^{Cre}$/$hVEGF\text{-}A^{fl}$) overexpressing human VEGF-A in murine B cells exhibited a VEGF-A induced lymphangiogenesis and an expansion of HEVs in LNs. The authors of the study speculated that the unphysiologically high levels of human VEGF-A might not directly influence the LN lymph- and blood vasculature, but may rather cause an accumulation of pro-angiogenic macrophages (122). In a mouse model of LCMV infection, B cells were shown to be required for LN tissue remodeling and vessel expansion. Surprisingly, the latter was independent of VEGF-A signaling pathways, but required $LT\alpha_1\beta_2$-expressing B cells (50). A recent study emphasized the angiogenic capacity of a B cell subset during eosinophilic esophagitis and in patients with melanoma. These pro-angiogenic B cells were identified by the surface markers $IgG4^+CD49b^+CD73^+$ and shown to promote vascular tube formation *in vitro* through VEGF-A, FGF2, and PDGFA expression (123). Taken together, although B cells express VEGF-A and $LT\alpha_1\beta_2$ during certain conditions, their role in LN angiogenesis is not well understood. Potentially, B cells may exert pro-angiogenic effects themselves, but also through stimulation of other stromal cell types, such as FRCs and macrophages (**Figure 2**).

B CELL LYMPHOMA-INDUCED VASCULAR CHANGES ARE DEPENDENT ON THE ENTITY AND STATE OF LYMPHOMA PROGRESSION

The clinical importance of angiogenic processes and mechanisms for the growth of solid tumors is well recognized (212, 213). Therapeutic concepts from solid tumors targeting the VEGF-A/VEGFR1/2 axis have been adopted for combinatorial therapies of B-NHL, resulting in rather disappointing clinical outcomes (214, 215). We recently showed that angiogenic processes in LNs in a mouse model of high-grade B cell lymphoma are induced by signaling pathways distinct from solid tumors. In sharp contrast to most solid tumors, lymphoma growth in LNs was not associated with hypoxic conditions or inflammation. Instead, lymphoma affected vessel expansion via the VEGF-C/VEGFR3 and $LT\alpha_1\beta_2$/LTβR signaling axes (42). In patients, the growth of tumor cells in low-grade B-NHLs is usually exponential for a few months and remains in a steady state as indolently growing tumor mass for years. This indolent lymphoma is considered to be avascular with dormant endothelial cells within the TME. In contrast, high-grade B-NHL progression is often accompanied by a so called "vascular phase", which represents extensive vascularization of LNs (216, 217). Such intermediate- and high-grade B-NHLs grow exponentially without intermission phase until they reach a mass critical for a patient's survival. As a clinical indicator of the vascularization, B-NHLs are usually quantified by terms of the microvessel density (MVD). Immunohistology using anti-CD31 antibody staining is still considered the "gold standard" of blood vessel detection, even though there is substantial variation between different studies due to the heterogeneity of the lymphoma stroma and different scoring methodologies. In some cases, the marker CD34 is used to detect the blood vasculature. Notably, lymphatic vasculature also expresses CD31, but at much lower levels (42, 218, 219).

Non-invasive assessment of tumor vascularization *in vivo* is possible by using Doppler sonography, contrast-enhanced dynamic magnetic resonance imaging (dMRI) and positron emission tomography-computer tomography (PET-CT). These techniques do not allow a direct quantification of the blood vessel density but provide information on the functional status of the blood vessels, e.g., vessel integrity, permeability, perfusion and metabolism (220). Another diagnostic approach to detect ongoing angiogenesis *in vivo* is the serological quantification of growth factors. VEGF-A levels in the serum of patients with progressive NHL were significantly elevated in comparison to patients in complete remission (221, 222). Elevated VEGF-A levels have been found in aggressive B cell lymphoma subtypes including MCL, DLBCL, but also in indolent lymphoma, such as CLL and small lymphocytic lymphoma (SLL), respectively (223–225). A variety of commonly used B-NHL cell lines secrete measurable VEGF amounts under serum starvation conditions, whereas other angiogenic factors like the placental growth factor (PlGF) and FGF-2 are not expressed (226). However, the detection of angiogenic factors in clinical serum samples gives no information on the cellular source of these molecules and is not a reliable indicator of angiogenesis in the compartment of interest. Previously, a group of angiogenesis experts published consensus guidelines for the use and interpretation of angiogenesis assays, which involve in vivo, ex vivo explantation, and in vitro bioassays. They explicitly highlighted critical aspects that are relevant for the execution of angiogenesis detection and proper interpretation (227).

Mantle cell lymphoma (MCL) is an aggressive B cell neoplasm that comprises 6% of all NHL cases (228, 229). It is susceptible to paracrine signaling from the microenvironment and in turn shapes the microenvironment by secreting soluble factors (230). MCL is genetically characterized by overexpression of the *CCND1*gene, encoding for cyclin D1 (231). Recent studies identified a subgroup of MCL that has a more indolent behavior with a clinical presentation as leukemic disease, exhibiting minimal LN distribution and a frequent splenomegaly. These tumors also overexpress cyclin D1 but lack expression of the sex determining region-Y-box11 (SOX11), a TF specifically expressed in conventional MCL and associated with an aggressive and angiogenic phenotype (232). These results have

been confirmed in MCL patient samples by using immunohistochemistry, demonstrating a correlation between an increased MVD and high levels of SOX11 expression (233). Experiments with MCL tumor xenotransplants in mice, in cell lines, and in primary MCL samples revealed that SOX11 actively modulates angiogenesis by up-regulation of the platelet-derived growth factor α (PDGFα), which is a competent inducer of an FRC-associated pro-angiogenic program (234, 235). Moreover, SOX11 overexpression promotes B cell receptor signaling represses Bcl6 transcription and upregulates PAX5 to avoid B cell differentiation into memory B cells or plasma cells. PAX5 supports tumor cell homing and invasion via up-regulation of CXCR4 and the focal adhesion kinase (FAK) (236–238). The absolute monocyte count in MCL correlated with the prognosis and supports the hypothesis that the TME is relevant for MCL tumor progression (239). $CD68^+$ and $CD163^+$ macrophages were found in MCL LNs without exception. Substantial numbers of VEGF-C expressing macrophages were found in a mouse xenotransplantation model as well (240). Treatment with the immunomodulator lenalidomide depleted monocytes and VEGF-C expressing macrophages, resulted in impaired functional lymphangiogenesis. However, a relevant impact on lymphoma-associated blood vessel growth in MCL was not investigated in this study. Of note, in human MCL anti-inflammatory and pro-angiogenic $CD163^+$ cells (M2-like) outnumbered the more inflammatory $CD68^+CD163^-$ macrophages (233), indicating a propensity to stimulate angiogenesis. This M2-like polarization of macrophages is actively driven by MCL derived CSF-1 and IL-10 (241). MCL cells exhibit increased expression of the T cell, B cell, and monocyte recruiting chemokines CCL4 and CCL5 compared to normal B cells (242). T cell infiltration has been considered as a prognostic marker in MCL in which $CD8^+$, and particularly $CD4^+$ T cell frequencies are higher in indolent MCL and decrease with more aggressive histological and clinical presentation (243). In contrast, a recent study reported an expanded vascularization of MCL associated with a high infiltration of $CD4^+$ and $CD8^+$ T lymphocytes (233). The differences might be explained by a weak comparability of data that were either correlated with the clinical outcome, or with the SOX11 expression level in MCL, two hallmarks that are not always correlated. A more detailed T cell characterization of CD4/CD8 T cell subsets is required for a more reliable assessment of the T cell–related influence on angiogenesis and the clinical outcome in MCL. Interestingly, MCL cells itself express the VEGFR-1, providing a strong rationale to target VEGF in order to interfere with angiogenic processes and concomitantly, with autocrine survival signals (230, 244).

Angiogenesis is likely a part of MCL progression, driven by MCL derived PDGFα. Therapeutical interference with PDGFR-β signaling, the receptor for PDGFα, can be achieved with receptor tyrosine kinase (RTK) inhibitors. Some PDGFR-β targeted drugs have been tested in clinical trials for B-NHL but failed to bring significant benefit (217). In contrast, immunomodulating drugs (IMiD) like thalidomide and lenalidomide have anti-angiogenic properties and showed great potential in combination with rituximab for the treatment of untreated or relapsed MCL patients (245, 246).

Follicular lymphoma (FL) is the second most common B-NHL, accounting for 20% of all B-NHL cases (247). The disease affects LNs, spleen and frequently also the bone marrow. Neoplastic follicles in FL have a lower proliferative index in comparison to reactive germinal centers. However, the proliferative capacity of FL cells increases gradually with the FL grade. FL progression requires the supporting infrastructure of the follicular TME to maintain survival, a requirement that gets progressively lost in the process of transformation to aggressive DLBCL (248, 249). Follicular dendritic cells (FDCs) are one branch of this supporting infrastructure. They are of mesenchymal origin and represent a crucial stromal cell population supporting the germinal center reaction and maintenance of the B cell follicle in LN and spleen (250, 251). FDC secreted B cell survival factors such as Indian hedgehog (HH), the B cell activating factor (BAFF), and IL-15 are potentially pro-tumorigenic (252, 253). CXCR5-controlled access to FDCs conferred survival and proliferation stimuli to CLL B cells in the murine Eμ-*Tcl1* model, which mimics some aspects of indolent tumor growth (253). Similar to reactive LN follicles, neoplastic follicles in FL preserve the organized FDC network structure at least in early stages of the disease progression (254). FL-FDC cross-talk induces a pro-angiogenic expression pattern in FL cells, including secretion of VEGF-A and VEGF-C (255). This cross-talk is crucially dependent on the phosphoinositide-3-kinase δ (PI3Kδ), providing therapeutic intervention options with PI3K specific inhibitors like idelalisib, which is approved for the treatment of FL, CLL, and SLL (256). The second branch of the supportive infrastructure in follicles are the $CD4^+CXCR5^+PD1^+$ T follicular helper (Tfh) cells, which provide vital survival signals for FL cells by secreting IL2, IL4, IFNγ, and by CD40L presentation (9, 257). FL cells are further dependent on proliferation and survival signals of the B cell receptor (BCR) in interaction with FDCs and TAMs (258). Elevated numbers of M2-like TAMs are found in the immediate microenvironment of FL cells and neo-vascular sprouts within the follicle (138). However, the prognostic value of $CD163^+$ TAMs remains controversial and is highly dependent on the prior course of treatment (259). In sum, FL appeared to be less prone to induce relevant vascular changes, whereas LNs of high-grade B-NHLs exhibited a dense and aberrantly distributed vasculature within the paracortical zone. In contrast to most other B-NHL malignancies in which high levels of pro-angiogenic factors and an increased MVD is associated with an adverse prognosis, high level FL vascularization correlates with a beneficial disease course (260–262). The improved clinical outcome apparently correlated with the increased vascularization, but was surprisingly independent of follicular VEGF-A expression (223, 263). Some studies stated a minor vascular remodeling in FL compared to reactive LN or follicular hyperplasia, or even vascular regression constraining the growth of reactive and neoplastic follicles (260, 264). Therefore, the clinical significance of angiogenesis in FL remains uncertain. In one clinical trial, addition of the anti-VEGF bevacizumab during

rituximab treatment of relapsed FL significantly improved the progression-free survival (265). The potential of angiogenesis inhibition upon treatment of FL requires further evaluation in larger clinical trials.

Collectively, according to the data currently available it seems that angiogenesis is important for high-grade lymphoma, but has less impact on indolent FL growth.

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoid tumors worldwide accounting for 30% of all diagnosed NHL and characterized by the large size of neoplastic B cells and usually a very aggressive clinical presentation (266). Lenz et al. identified gene expression profiles in LN from patients pre-treated with the combination therapy anti-CD20 antibody, cyclophosphamide, doxorubicin, vincristin, prednisolone (R-CHOP), dividing DLBCL in two distinct subgroups that are predictive of the clinical outcome (267). The "stromal-1" signature includes expression of extracellular matrix (ECM) elements, ECM remodeling factors (*MMP2*, *MMP9*, *M1-MMP*, *PLAU*, *TIMP2*) and is associated with a favorable prognosis. The "stromal-2" signature" was found in tumors with an increased MVD and is characterized by markers of endothelial cells (*Pecam1*, *Vwf*, *Kdr*, *Tek*). The latter signature is associated with a poor clinical outcome, emphasizing the critical impact of angiogenic processes on aggressive B-NHL progression. Several studies investigated the clinical consequences of the "stromal-2" signature and confirmed the correlation of a high MVD with an adverse outcome and a shorter overall survival rate (268–271). The relationship between MVD and DLBCL behavior was the object of many studies and was found to be associated with poor prognostic parameters such as splenic involvement, high mitotic rate, and capsular invasion (268–272). Gomez-Gelvez et al. reported contradictory results, showing that high MVD is associated with rather better progression-free survival (PFS) and event-free survival (EFS) (273). Several other studies also failed to draw a connection between the MVD, tumor grade and prognostic outcome (274–277). A DLBCL mouse xenotransplantation model demonstrated that the inhibition of the paracrine VEGFR-2 pathway reduced growth of an established lymphoma and correlated with decreased tumor angiogenesis (226). DLBCL cells often overexpress the phosphodiesterase 4B (PDE4B), which intracellularly catalyzes the hydrolysis of cyclic-AMP (cAMP). The cAMP-PDE4B axis modulates signaling of PI3K and AKT and therefore acts upstream of VEGF-A expression. Experiments with genetically or pharmacologically inhibited PDE4B resulted in decreased VEGF-A expression in lymphoma cells and reduced angiogenesis in the Eμ-*Myc* high-grade lymphoma mouse model (278).

In a gene expression study on relapsed or refractory DLBCL, patients with the ABC-like DLBCL subtype that had low $VEGF_{121}$ isoform expression, exhibited a significantly better overal survival than those with high $VEGF_{121}$ gene expression levels (279). Interestingly, $VEGF_{121}$ low transcript levels were associated to a gene signature reflecting immune response and T cell activation.

DCs are likely a major source of VEGF-A in LNs with DLBCL. Functionally, DCs could be involved in lymphoma TME remodeling, but their number in DLBCL LNs is significantly lower than in reactive LNs. Lower expression levels of the LN homing receptors CD62L and CCR7 in DCs in LNs of DLBCL patients were thought to result in reduced DC immigration. However, it remains elusive if the DCs lose the receptor expression upon arrival in the LN, or whether these cells are recruited via alternative routes (280). In an aggressive *Myc*-driven lymphoma model in mice a tumor-specific DC differentiation occurs that promotes tumor cell survival and favors the maturation of monocytic-derived DCs ($MHCII^{medium}$) (149, 150). Alongside tumor repressing M1 macrophages, "alternatively" activated M2 macrophages exhibit angiogenic capacities, they are frequently found in DLBCL and often correlate with a poor prognosis (281–283). Although numerous studies reported an association between TAMs and MVD in DLBCL, others could not find a correlation between $CD68^+$ macrophages and an increased MVD (270). Such controversies can probably be best explained by variabilities in the methodological approaches. The macrophage marker CD68 represents M1 and M2 macrophages and therefore, produces inaccuracies in the interpretation of studies concerning the macrophage-MVD correlation. The addition of the marker CD163, which rather recognizes M2 activated macrophages, including angiogenic macrophages, provides a more reliable view on the role of macrophages in DLBCL (284). Elevated numbers of macrophages have been correlated with poor prognosis in DLBCL (282). However, in therapeutic setting macrophages are required to confer treatment effects when patients were treated with anti-CD20 antibody (e.g., Rituximab). Here, macrophages mediate tumor cell depletion via the macrophage Fc-gamma receptor (FcγR) expression (215). Another abundant immune cell population in LNs of DLBCL patients is mast cells with a predominance of MC_T-type (tryptase-positive) cells. MC_{TC}-type (tryptase-positive and chymase-positive) and $CD4^+$ Th_2 were shown to express IL-4 in DLBCL and therefore, they may actively promote survival of the tumor cells (285). Hedström and coworkers examined 154 DLBCL cases and suggested that the infiltration of mast cells reflects the inflammatory immune response of the endogenous anti-tumor defense and is therefore related to a favorable outcome (286). The gradual increase of the MVD was correlated with an increasing number of mast cells. Although mast cells are considered to be bystanders in tumor immunology, additional pro-angiogenic effects of these cells are likely as they secrete relevant amounts of different VEGFs, FGF-2, trypase, and granzyme B. The latter has a pro-angiogenic effect via the enzymatic mobilization of ECM-bound FGF-1 (287, 288). The wide range of physiological conditions and tumor entities that include mast cell-supported angiogenesis and the respective recruitment and signaling pathways were excellently reviewed by Ribatti et al. (289).

Apart from the direct effect on immune and tumor cells, surprisingly, the application of the VEGF-A inhibiting antibody bevacizumab to R-CHOP therapy increased adverse cardiac events, yet without increasing the therapeutic efficacy in DLBCL patients (214, 215). From these studies it can be inferred that the increased MVD in DLBCL patients may be simply a correlation with minor importance for the disease course, or that other non-VEGF-A

angiogenic pathways prevail and cause enhanced vascular assembly instead. In a study of Pazgal et al., VEGF-C, VEGF-D, and VEGFR3 were expressed in both lymphoma cells and endothelial cells of the blood and lymphatic vasculature. They reported a significant correlation of the VEGF-C expression and the presence of blood vessels. VEGF-D expression correlated with the patient International Prognostic Index (IPI) Score and the patients' overall survival (14). These results may indicate that apart from its role as primary signaling pathway for lymphatic vessels, the VEGF-C–VEGFR3 axis also has implications on angiogenic processes of LN blood vessels. A study in breast cancer demonstrated that VEGFR3 is significantly upregulated in the endothelium of new blood vessels. The results also suggested that VEGF-C secreted by the intraductal carcinoma cells acts predominantly as an angiogenic growth factor for blood vessels, although other immune or stromal cells might be involved in this paracrine signaling network as well (290). An experimental study using a *Myc*-driven aggressive lymphoma mouse model, which resembles important aspects of aggressive B-NHL, supported this hypothesis, showing that the MVD expansion was triggered by lymphoma-provided VEGF-C, in a synergistic activity with $LT\alpha_1\beta_2$ (42).

Representing a high-grade and angiogenesis-associated lymphoma type, multiple clinical trials with anti-angiogenic agents for the treatment of DLBCL have been conducted. Most of the treatment approaches using single agent angiogenesis inhibitors failed to prove a beneficial effect. However, combinatorial treatment strategies such as R2-CHOP (lenalidomide, R-CHOP) (291, 292), brought encouraging results. Such oberservations emphasize that anti-angiogenesis therapies might not be effective when applied alone, even in highly vascularized lymphoma, but are valuable components in combination with other drugs.

Burkitt's lymphoma (BL) represents around half of all malignant non-Hodgkin lymphoma in children and around 2% in adults (293). The BL pathogenicity is usually associated with the infection of B cells with the Epstein-Barr virus (EBV). EBV gene products induce BL cell-derived soluble factors that result in inhibition of neo-vascularization and eventually tumor necrosis and regression (294). However, in *in vivo* experiments, EBV-positive cells induced massive recruitment of leukocytes at the tumor border and the development of granulation tissue with large numbers of blood and lymphatic vessels (295). Surprisingly, aggressive BL displayed the highest MVD in comparison to intermediate DLBCL and indolent B-NHL (42, 262, 287). In support of this observation, BL showed increased vascularization relative to benign lymphadenopathies and can produce several angiogenic factors, although it is not yet known whether this is due to *Myc* gene overexpression or the EBV transformation (296–298). BL were found to be closely associated with VEGF-producing $CD68^+VEGFR1^+$ myeloid cells located around the neo-vasculature. The newly formed blood vessels were identified by the absence of pericyte coverage as result of the rapid vessel growth (299, 300). Genetic depletion of this subpopulation of CD68+VEGFR1+ myeloid cells was sufficient to inhibit angiogenesis in experimental lymphoma (301). To our knowledge, to date there are no clinical data or published treatment strategies of BL that target angiogenesis specifically.

Classical Hodgkin Lymphoma (cHL) is characterized by mono-nucleated Hodgkin and multi-nucleated Reed-Sternberg (HRS) cells, which comprises tumors with mixed cellularity, nodular sclerosis and lymphocyte-rich or lymphocyte-depleted subtypes. Different from other lymphoma, HRS cells are the minority of cells within the affected LN. Most of the cells in cHL tumors are cells of the TME, indicating a prominent role of benign immune cells and the LN stroma (302). A crucial role of angiogenesis and increased MVD have been reported for cHL and correlate with a poor prognosis (303). Similar to observations in highly vascularized LNs in an aggressive B-NHL mouse model (42) and in immunohistochemically characterized B-NHL patient specimen (277), in cHL HIF-1α was only moderately expressed (304), suggesting that angiogenesis in cHL is not hypoxia-driven and may utilize other angiogenic pathways instead. In childhood cHL, HRS cells express VEGF, MMP-2 and MMP-9. However, the expression of these factors did not correlate with the MVD and neovascularization level (305, 306). On the other hand, VEGF-D, a ligand for VEGFR3 and usually associated with lymphangiogenesis, is expressed in HRS cells at high abundance and correlated with high numbers of microvessels (307). Moreover, in vitro HRS cell-derived TGF-β, FGF-2, and VEGF supported HUVEC tubulogenesis (308, 309). Secretion of Ltα by HRS cells activated endothelial cells, which enhances adhesion molecule expression and consequently, recruitment of T cells. This mechanism amplifies the inflammatory milieu in the cHL TME through conditioning of the blood vasculature (310).

Commonly attributed to the occurrence of angiogenic M2 macrophages, TAMs are linked to poor outcome in HL. Interestingly, lack of macrophages, but also high numbers of macrophages is associated with a poorer disease-free survival and overall survival, whereas intermediate numbers are associated with a better outcome. This macrophage paradox suggests that a lack of TAMs is beneficial for HL growth, while TAMs have an inhibitory effect with increasing numbers (311). The inhibitory effect seems to be displaced by an adverse effect of TAM-induced angiogenesis, supposedly predominated by $CD163^+$ M2-like TAMs (312). High numbers of $CD163^+$ TAMs correlate with elevated VEGF-A levels and an increased MVD, indicating that CD163 is an independent prognostic marker in cHL (313). Interestingly, although the particular signaling pathways within TAMs remain elusive, pre-clinical experiments with PI3K-Akt pathway inhibition suggested a connection to macrophage M2-polarization (314, 315), which could be a promising anti-angiogenic intervention clue by prevention of pro-angiogenic activity of M2-like TAMs.

ANTI-ANGIOGENIC THERAPIES IN COMBINATION WITH CHEMOTHERAPIES

Cancer therapy earlier than the 1970s was solely focused on targeting the actual cancer cells. Judah Folkman's discovery that tumor growth is angiogenesis-dependent led to a profound paradigm shift in cancer therapy (316, 317). Sprouting angiogenesis plays an essential role in tumor growth, invasion,

progression, and metastasis, targeting this process is a promising strategy to inhibit growth and spread of solid tumors. Clinical trials and treatment strategies of anti-angiogenesis therapy in B-NHL were recently reviewed (217). Angiogenesis inhibitors are classified into direct and indirect agents. Direct inhibitors target vascular ECs and include endostatin, arrestin, and tumstatin. Indirect angiogenesis inhibitors target tumor cells or cells of the TME to prevent the expression of pro-angiogenic factors or block their activity (318). The anti-VEGF monoclonal antibody Bevacizumab was the first anti-angiogenesis drug approved by the FDA for the treatment of metastatic colon, ovarian, renal, non-squamous cell lung cancer, and glioblastoma multiforme. Unfortunately, clinical significance was only reached in glioblastoma multiforme treatment (319, 320), a result that could not be confirmed in other studies (321). In contrast to Bevacizumab, treatment with tyrosine kinase inhibitors (e.g., Sorafenib) that interfere with the signal transmission of VEGFRs resulted in remarkable effects throughout several cancer entities. Combination of tyrosine kinase inhibitors and conventional chemotherapy have not been beneficial (322). Conventional chemotherapy can cause direct cytotoxicity of endothelial cells, but this effect is non-selective and only observed upon the maximal tolerated dose (MTD). Insufficient tumor and vascular bed destruction can effectuate a strong hypoxic condition, which results in release of chemoattractant CXCL12. Accordingly, MTD chemotherapy potentially increases systemic CXCL12, which recruits bone marrow–derived EPCs. These cells can cause recurring angiogenesis in mouse models of solid tumors (323, 324). Therefore, an anti-angiogenesis therapy that is complementary to chemo- or immunotherapy is aimed at restricting pro-angiogenic bystander effects of the tumor treatment. In addition, instead of aiming for a complete vascular eradication, the paradigm in anti-angiogenic therapies shifted to vascular normalization (325, 326).

Rituximab has become an essential part of first-line treatment of several B cell lymphoma entities, foremost of DLBCL. However, ongoing research aims to improve the therapeutic efficiency and the reduction of the relapse rate of drug-resistant lymphoma cells. Tumor anti-angiogenesis therapy approaches are one branch of such research, in which Bevacizumab and Endostatin were the most promising representatives for lymphoma treatment (327, 328). VEGF-A has a crucial role in promoting vessel growth, but is also considered to be an immunosuppressive factor that modulates the migration and function of several immune cells, e.g., DCs and mast cells. The potential pharmaceutical targeting of the VEGF/VEGFR axis to modulate anti-tumor immunity has been reviewed recently (329).

An important challenge of anti-angiogenic therapy in solid tumors as well as in lymphoma is the identification of the particular angioactive receptors throughout different tumor entities and individual patients. The inhibition of intracellular signaling hubs is a strategy to overcome the targeting of distinct angiogenic tyrosine-kinase receptors. Class I PI3Ks are involved in the signal transduction of many pro-angiogenic signals and control cell growth, survival, motility, and metabolism (330). PI3Kδ inhibition in lymphoma potentially also interferes with tonic signaling in tumor cells, e.g., via the BCR signaling pathway (331), or breaks the Treg-mediated immune tolerance (332). Interestingly, PI3K activity is essential for macrophage M2 polarization (333) and therefore, a potential target to hamper M2-like angiogenic macrophages. Inhibition of PI3K signaling represents a valuable therapeutic strategy to target different indolent B cell lymphoma entities, among them FL, CLL, SLL, and more recently, they showed promise in T cell lymphomas as well (334, 335). The combinatorial treatment of the first generation PI3K inhibitor idelalisib with rituximab or bendamustine revealed favorable response rates in FL patients (334), but serious adverse effects due to bacterial and viral infections were observed. Additionally, immune-mediated and hematologic adverse events occurred. Beyond that first generation PI3K inhibitor, newer PI3K inhibitors such as copanlisib and duvelisib were introduced for patients with relapsed and progressive FL, CLL, SLL, respectively. These inhibitors differ in their preference for PI3K isoforms which are expressed differentially in various tissues (336). Despite relevant side effects of PI3K inhibitors, they have been judged clinically manageable and thus, prompted an FDA approval for relapsed and refractory indolent B-NHL (335, 337). Published reports on anti-angiogenic therapies in B-NHL allow the conclusion that the complex mechanisms of angiogenesis in lymphoma are incompletely understood and require further pre-clinical and translational research to develop reliable and effective anti-angiogenic treatment strategies. Moreover, new anti-angiogenic treatment regimens need to be validated regarding an actual reduction of tumor growth, since sole targeting of angiogenic factors often fail to cause substantial tumor regression (**Figure 3**) (340).

OUTLOOK

Vascular remodeling and angiogenesis have been increasingly recognized as crucial factors in the pathophysiology of B-NHLs. We here present an integrated concept that includes angiogenic processes of the LN TME beyond the proliferation and survival of endothelial cells stimulated by the VEGF-VEGFR axis. In human, the angiogenic properties of LN resident and recruited immune cells are still insufficiently understood. Studies to address such functional states are limited because tissues from appropriate human patients are rarely available. Notably, most of the human data available so far are observations on clinically progressed and even terminal stage lymphoma LNs. Flow cytometry analysis of blood samples is usually limited to a few entities (e.g., FL, CLL, and MCL), common markers and cannot readily be correlated with pathohistological observations due to the lack of tissue specimen. Availability of LN tissue from progressed disease stages is often limited to scarce material from fine-needle biopsies. As a useful surrogate, mouse models of reactive LNs and LNs with lymphoma growth demonstrated that the angiogenic processes are regulated by a timely complex interplay of immune, tumor, and stromal cells (42).

In the future, modern methods like single-cell RNA sequencing alone or with spatial resolution, or single-cell analysis in combination with proteomics will help to resolve the complexity of participating cells and their heterogenous differentiation status.

This technique requires much less input material for a high resolution analysis at the genome, protein, or epigenome level (341–343). Even patient-derived specimen from fine needle biopsies seem amenable to such analysis, allowing then a kinetic description of LN remodeling in the course of diagnosis, treatment response, and eventually relapse. Single-cell RNA sequencing will further enable the discrimination of different endothelial cell subtypes and their differentiation traits (344). The compartment of BECs is comprised of several functionally distinguishable subpopulations that further differentiate during angiogenesis. To date, we know little about the role, differentiation conditions and distribution of these subpopulations in LNs. Such transcriptional observations need to be correlated with the topology of the single cells and cell networks within the complex LN (345). Modern imaging methods, e.g., light sheet microscopy and intravital 2-photon microscopy enable the complex spatial integration and the investigation of dynamic processes *in situ*, which have long been restricted to snapshot observations. Very recently, a new generation of flow cytometry devices became available that, based on a spectrum wide detection of fluorophores, allow a simultaneous detection of a multi-fold higher number of cell markers. The possibility to determine extended marker panels with small sample sizes will not only improve basic knowledge in the pre-clinical context, but will also provide innovative approaches for clinical diagnostics (346, 347).

Improving insight into angiogenesis is also of considerable relevance for the emerging immunotherapies using chimeric antigen receptor (CAR)- and T cell receptor (TCR)-transgenic T cells and NK cells. It is reasonable to suggest that tumor blood vessels have a leading role in granting effector T cell access to the LN and the tumor niche formed therein. For example, solid tumors condition an endothelial activation status that can be considered immunologically "silent" (348). However, reactivation of such vessel-lining endothelial layers is a prerequisite for the adhesion and transmigration cascade of naive and therapeutic T cell populations. We envision that this endothelial tuning is not only applicable to solid tumors, but also to LN-localized lymphatic neoplasm. Except for cHL, immune checkpoint blockade (ICB) targeting PD-1 or CTLA-4 has not shown relevant benefit in other B cell neoplasm. Because ICB efficacy depends on the presence of a repertoire of antigen-specific T cells, a rational sequence of immunotherapeutic interference in B-NHL would start with a vessel induction toward a more activated or even inflammatory state. It seems not even necessary to overactivate local endothelial cells, as shown by the application of a modified TNFα cytokine that upregulates adhesion molecules, but then even eradicates solid tumors through rapid destruction of the tumor neovasculature (349). Enhanced adhesion, e.g., involving ICAM-1 and VCAM-1 up-regulation, may be sufficient to allow T cells to get access to the primary lymphoma site in the deep parenchyma. Finally, in a time window to be defined, application of ICB might then unleash the activity of effector T cells that already invaded the tumor site.

Collectively, efforts to target tumor cells only or single lymphoma-promoting cellular stromal elements in the TME are unlikely to confer long lasting remissions. For example, although anti-CD19 CAR T-cell therapies have proven remarkable efficacy in B cell malignancies, they become ineffective due to CD19 antigen loss or downregulation (350, 351). Other contributing factors to

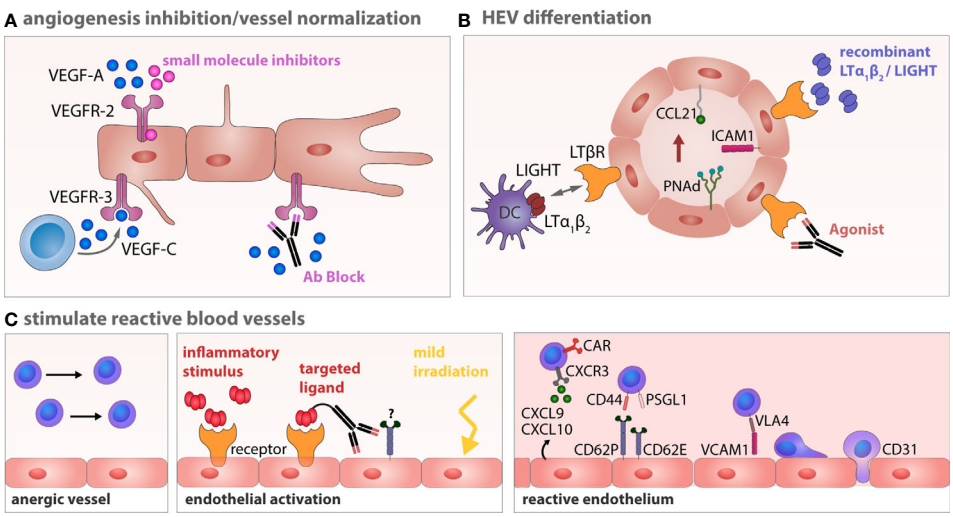

FIGURE 3 | Therapeutic strategies to induce vessel normalization and revert endothelial anergy in B-NHL. **(A)** Anti-angiogenesis therapy tageted at VEGFR-2 or VEGFR-3 can restore a normalized vessel network. **(B)** Targeting of the LTβR with $LT\alpha_1\beta_2$ and LIGHT expressing DCs, agonistic antibodies or recombinant factors potentially circumvents impaired lymphocyte homing by establishing or stabilizing HEV integrity within the lymphoma TME (338). **(C)** Vessel anergy can be changed by a targeted conversion of the endothelium toward a reactive endothelium using inflammatory cytokines, which might be site directed to avoid unintended systemic effects. Normalization of aberrant vessels and activation of the endothelium can also be achieved by locally applied low-dose gamma irradiation (339). Reactive endothelium within LNs is a prerequisite for an effective infiltration of effector T cells during cellular immunotherapy.

substantial rates of treatment failure might be the nodal immunosuppressive microenvironments in B-NHL (9, 352).What is needed is an integrative concept that blocks vicious feedback cycles in lymphoma. We suggest that combinatorial targeting of aberrantly polarized myeloid cell populations, blood endothelial activation, angiogenesis, and effector T cell dysfunction is a rational stepwise strategy (**Figure 3**). Advanced CAR T cell technologies try to integrate a few of these demands, for example by deleting the functionality of PD-1 (353, 354) and secretion of immune-stimulatory cytokines such as IL-12, IL-21, or IL-18 (355–357). We envision that the vasculature is important for control of lymphoma relapse. In this process, mutual stimulation of residual tumor cells, mesenchymal and hematopoietic stromal cells, and endothelial cells might favor neo-angiogenesis and eventually, re-shaping a growth supporting niche for lymphoma B cells.

AUTHOR CONTRIBUTIONS

LM: conceived the general idea, wrote the manuscript, and created the figures and tables. AR: conceived the general idea, co-wrote the manuscript, and edited figures and tables. UH: provided expert opinion/knowledge input and edited manuscript, figures, and tables. All authors contributed to the article and approved the submitted version.

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The Effect of *SF3B1* Mutation on the DNA Damage Response and Nonsense-Mediated mRNA Decay in Cancer

*Alexander C. Leeksma[1,2,3], Ingrid A. M. Derks[2,3], M. Haidar Kasem[4], Emine Kilic[5], Annelies de Klein[5], Martine J. Jager[6], Arjan A. van de Loosdrecht[7], Joop H. Jansen[8], Veronika Navrkalova[9], Laura M. Faber[10], Nadja Zaborsky[11,12,13], Alexander Egle[11,12,13], Thorsten Zenz[14], Sarka Pospisilova[9], Omar Abdel-Wahab[15], Arnon P. Kater[1,2] and Eric Eldering[2,3]**

[1] Department of Hematology, Amsterdam University Medical Centers, Location AMC, University of Amsterdam, Amsterdam, Netherlands, [2] Department of Experimental Immunology, Amsterdam University Medical Centers, Location AMC, University of Amsterdam, Amsterdam, Netherlands, [3] Lymphoma and Myeloma Center Amsterdam (LYMMCARE), Cancer Center Amsterdam (CCA) and Amsterdam Infection and Immunity Institute (AIII), Amsterdam, Netherlands, [4] Translational Functional Cancer Genomics, National Center for Tumor Diseases (NCT) and German Cancer Research Center (DKFZ), Heidelberg, Germany, [5] Department of Ophthalmology and Clinical Genetics Erasmus MC, Rotterdam, Netherlands, [6] Department of Ophthalmology, LUMC, Leiden, Netherlands, [7] Department of Hematology, Amsterdam University Medical Centers, Location VUMC, Amsterdam, Netherlands, [8] Laboratory of Hematology, Department Laboratory Medicine, Radboud University Medical Center, Nijmegen, Netherlands, [9] Center of Molecular Biology and Gene Therapy, Department of Internal Medicine-Hematology and Oncology, University Hospital Brno and Center of Molecular Medicine, CEITEC, Masaryk University, Brno, Czechia, [10] Department of Internal Medicine, Rode Kruis Ziekenhuis, Beverwijk, Netherlands, [11] Department of Internal Medicine III with Haematology, Medical Oncology, Haemostaseology, Infectiology and Rheumatology, Oncologic Center, Paracelsus Medical University, Salzburg, Austria, [12] Department of Internal Medicine III with Haematology, Salzburg Cancer Research Institute—Laboratory for Immunological and Molecular Cancer Research (SCRI-LIMCR), Salzburg, Austria, [13] Department of Internal Medicine III with Haematology, Cancer Cluster Salzburg, Salzburg, Austria, [14] Department of Oncology and Haematology, University Hospital and University of Zurich, Zurich, Switzerland, [15] Human Oncology and Pathogenesis Program and Leukemia Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, United States

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Martina Seiffert, German Cancer Research Center (DKFZ), Germany

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***Correspondence:**
Eric Eldering
e.eldering@amc.uva.nl

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Recurrent mutations in splicing factor 3B subunit 1 (*SF3B1*) have been identified in several malignancies and are associated with an increased expression of 3' cryptic transcripts as a result of alternative branchpoint recognition. A large fraction of cryptic transcripts associated with *SF3B1* mutations is expected to be sensitive for RNA degradation *via* nonsense-mediated mRNA decay (NMD). Several studies indicated alterations in various signaling pathways in SF3B1-mutated cells, including an impaired DNA damage response (DDR) in chronic lymphocytic leukemia (CLL). In this study, we investigated isogenic cell lines and treatment naïve primary CLL samples without any *TP53* and/or *ATM* defect, and found no significant effects of *SF3B1* mutations on the ATM/p53 response, phosphorylation of H2AX and sensitivity to fludarabine. Cryptic transcripts associated with *SF3B1* mutation status were observed at relatively low levels compared to the canonical transcripts and were validated as target for mRNA degradation *via* NMD. Expression of cryptic transcripts increased after NMD inhibition. In conclusion, our results confirm involvement of NMD in the biological effects of *SF3B1* mutations. Further studies may elucidate whether *SF3B1*-mutant patients could benefit from NMD modulatory agents.

Keywords: SF3B1, DNA damage response, splicing, nonsense-mediated mRNA decay, apoptosis

INTRODUCTION

Splicing factor 3B subunit 1 (*SF3B1*) is frequently mutated in different malignancies. In chronic lymphocytic leukemia (CLL), different studies reported a lower incidence (5–11%) of *SF3B1* mutations at diagnosis, which increased with therapy resistance to 15–20% (1, 2), and an association with poor prognosis (3, 4). In various other cancers, notably myelodysplastic syndrome (MDS; 25–30%) (5–7) and uveal melanoma (UM; 10–21%) (8–10), heterozygous *SF3B1* mutations are also highly prevalent. *SF3B1* mutations cause altered splice branchpoint recognition which results in increased 3' cryptic splicing, and concomitant frameshifts (11, 12). Alternative transcripts with a premature termination codon (PTC) ≥50–55 nucleotides before the last exon-exon junction are normally targeted for degradation *via* nonsense-mediated mRNA decay (NMD) (11, 13). Consequently, a substantial fraction of the *SF3B1*-associated cryptic transcripts is expected to be NMD-sensitive. The pathobiology of *SF3B1* mutations is of interest because the (defective) splicing machinery might be a therapeutic target (14). Homozygous splicing factor mutations are not observed and mutations in splicing factor genes show mutual exclusivity (15). The enhanced sensitivity of *SF3B1*-mutated cells to the splicing inhibitor H3B-8800 which is currently tested in phase I clinical trials (16) is in agreement with a therapeutic window of splicing factor inhibitors. Various studies have described the effects of *SF3B1* mutations on alternative branchpoint recognition and indicated alterations in several signaling pathways including the DNA damage response (17–19), telomere maintenance (18), NF-kB (15, 20), NOTCH1 (18) and MYC signaling (21), but there is no clear view or consensus on the resulting pathological mechanism(s).

Here, we expand on our earlier observation that *SF3B1* mutations in CLL associate with an altered response to DNA damage (DDR), with certain aspects resembling an ATM defect (17). Outcomes of this previous study included effects of *SF3B1* mutations on the ATM/p53 response after irradiation, higher phosphorylation of variant histone H2AX on Ser139 [γH2AX; a marker for DNA double stranded breaks (22)] at baseline and in response to irradiation, and a decreased sensitivity to fludarabine (17). To gain more insight into the underlying pathobiological mechanism of *SF3B1* mutations, we now investigated isogenic cell lines and an additional cohort of treatment-naïve primary CLL samples without a confounding *TP53* and/or *ATM* defect. Secondly, we analyzed the effect of NMD on *SF3B1*-associated cryptic transcripts in various primary cancer cells and isogenic cell lines.

METHODS

Cell Culture

NALM-6 isogenic knock-in cell lines including different hotspot mutations in the HEAT domain of SF3B1 (parental, K700E, K666N, and H662Q) were from a previous study (11) and mutations were confirmed by Sanger sequencing. UM cell lines 92.1 (SF3B1wt) and Mel202 (SF3B1mut) were acquired from Martine de Jager (department of ophthalmology LUMC, The Netherlands). Pancreas carcinoma (PDA) cell line panc1 (SF3B1wt) was obtained from the LEXOR group (Amsterdam UMC, The Netherlands) and panc05.04 (SF3B1mut) was directly bought from ATCC and CLL cell lines PGA (SF3B1wt) and CII (SF3B1mut) were a kind gift from Tanja Stankovic (Bournemouth, UK). Cell lines were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) with HEPES and L-glutamine (92.1, Mel202, PGA, CII, panc05.04, and NALM-6 cell lines) or IMDM (Lonza, Basel, Switzerland) with HEPES, L-glutamine (panc1), and supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (Invitrogen) and incubated in 5% CO_2 at 37 °C. Panc05.04 was cultured in the presence of 1% Insulin-Transferrin-Selenium (ITS -G) (Thermo Fisher Scientific, Waltham, MA, USA). Primary CLL cells were thawed and cultured in IMDM (Lonza, Basel, Switzerland) with HEPES, L-glutamine, 10% FCS, and Penicillin-Streptomycin (Invitrogen) for functional experiments and incubated in 5% CO_2 at 37 °C.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using the GeneEluteTM Mammalian Total RNA Miniprep kit (Sigma-Aldrich #RTN70) and cDNA was transcribed by RevertAid (Fermentas Inc., Hannover, Md #EP0451) using Random Hexamer Primers (Promega, Madison, USA #C1181) according to manufacturer's instructions. Primers used for detection of 3' cryptic transcripts associated with *SF3B1* mutation were designed based on results from transcriptomic analyses (12, 23) of *SF3B1* mutated cancer cells and are listed in **Supplemental Table 1**. Expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and qPCRs were performed using SYBR Green master mix (Applied Biosystems #4385617). Linear regression (LinReg) software was used for data processing. Relative expression was calculated by the comparative ΔCt method (24).

Sequencing of *SF3B1*

Cell lines and primary cells were sequenced with Sanger or next-generation DNA sequencing at the SF3B1locus. Primary MDS, CLL and UM cells were considered as SF3B1 mutated when a mutation was detected in the HEAT domain of SF3B1 with a variant allele frequency (VAF) ≥20%. Only treatment-naïve primary CLL cells negative for *ATM* (no del11q/and or *ATM* mutation) and *TP53* defects (no del17p and/or *TP53* mutation), at date of sampling were included for functional analysis of the DDR in *SF3B1* mutated samples. Patients characteristics and results of mutation analyses of samples used in this study are listed in **Supplemental Tables 2–5**.

Reverse Transcriptase Multiplex Ligation Dependent Probe Amplification

For RT-MLPA analysis cells were treated with or without irradiation (1Gy or 5Gy) and cultured for 16 h. RT-MLPA (MRC-Holland) was performed as described before, using an earlier validated RT-MLPA probe set, which includes several p53 and ATM target genes (*CD95*, *BAX*, *PUMA*, *p21*, *FDXR*, *PCNA*, *NME1*, *ACSM3*) (17). Expression was normalized to a panel of housekeeping genes.

Western Blot Analysis

Cells were lysed in Laemmli sample buffer and western blotting was performed using standard conditions. The following antibodies were used: PUMA (Sigma-Aldrich #PRS3043), p53 (Calbiochem #OP43), serine 15 phosphorylated-p53 (Cell Signaling #9284S), MDM2 (Santa Cruz #sc-965), KAP (Cell Signaling #5868), serine 824 phosphorylated-KAP (Cell Signaling #4127), p21 (Cell Signaling #2947), and β-actin (Santa Cruz #sc-1616). IRDye 800CW Goat anti-Rabbit IgG (LI-COR #926-32211), IRDye 800CW Donkey anti-Goat (LI-COR #926-32214), IRDye 680LT Donkey anti-Goat (LI-COR #926-32224), IRDye 680LT Goat anti-Mouse IgG (LI-COR #926-68020). Protein expression was quantified with Odyssey software (Li-Cor Biosciences) and corrected for the expression of β-actin.

Apoptosis Induction by Fludarabine or Doxorubicin

Cells were cultured in the presence of fludarabine (Sigma-Aldrich #F2773) for 48 h, and doxorubicin (Selleckhem #S1208) for 24 h at indicated concentrations. Apoptosis was measured by flow cytometry. Cells were stained with 0.01 μM of the viability dye Dihexyloxacarbocyanine Iodide (DiOC6, Molecular Probes #D-273) for 20 min at 37 °C and prior to analysis, TO-PRO-3 (Thermofisher Scientific #T3605) was added as a marker for cell death. Signals were measured on a FACS Calibur (BD). Specific cell death was calculated as [(% apoptosis treated cells - % apoptosis untreated cells)/% viable untreated cells]*100. Flow cytometry data were analyzed using FlowJo software (Treestar, Ashland, OR, USA).

γH2AX and CD95 Expression

Expression of γH2AX was measured using flow cytometry. Cells were irradiated (1Gy or 5Gy) and at indicated times, cells were permeabilized (Foxp3 staining kit; eBioscience) and stained using the following antibodies: isotype-AF488 (BD Biosciences #557782) or γH2AX-AF488 (phosphorylated-H2AX-ser-139; Cell Signaling #9719S). CD95 expression on NALM-6 cells was determined by flow cytometry using anti-CD95-FITC (BD biosciences #555673) following irradiation (1Gy or 5Gy) and 16 h culturing. Data were normalized for isotype control (isotype-AF488).

Statistical Analysis

Analyses were performed using Graphpad Prism software version 8. (Graphpad, La Jolla, CA, USA). Kruskal–Wallis test with Dunn's multiple comparison *post hoc* analysis was used for analysis of RT-MLPA data. A two-sided Mann–Whitney U test was used to identify differences between two groups. For apoptotic responses with >2 groups, one-way ANOVA with Dunnet's *post hoc* test was used. P-values <0.05 were considered statistically significant.

RESULTS

First, we studied the ATM/p53 response in isogenic NALM-6 cells with heterozygous *SF3B1* mutations (11). Confirmation of altered SF3B1 function in three *SF3B1*-mutated NALM-6 cell lines against their parental cell line was obtained through increased expression of the *SF3B1*-associated cryptic transcripts of *ATM*, *FOXP1* and *TTI1* (**Supplemental Figure S1A**).These transcripts were previously reported to be increased in SF3B1 mutated cells (12, 23, 25), and were considered as signature genes that might also be linked with pathobiological consequences. Various aspects of DDR functionality (17) were investigated: 1) irradiation (IR) followed by quantification of ATM/p53 target genes by RT-MLPA and analysis of proteins by western blot, 2) ATM functionality *via* KAP phosphorylation on Ser824, 3) γH2AX following IR, and 4) treatment with DNA damaging agents fludarabine and doxorubicin, followed by assessment of the apoptotic response by flow cytometry. In all of these aspects, the three *SF3B1*-mutated NALM-6 cell lines behaved identical to the parental cells (**Supplemental Figures S1B-C, S2-4**). Influence of cell cycle status was investigated with the cyclin-dependent kinase inhibitor palbociclib. Palbociclib induced growth arrest without cell death induction, but did not influence the ATM/p53 response following IR (**Supplemental Figure S5**).

Since our earlier studied CLL cohort contained a mix of untreated and chemotherapy-treated CLL patients, we next analyzed treatment-naïve primary CLL cells harboring *SF3B1* mutations (median VAF of 41.8%; **Supplemental Table S2**) for potential effects on the DDR using the same set of assays as applied previously (17). RT-MLPA revealed a significantly increased p21 mRNA in non-irradiated *SF3B1*-mutated CLL cells ($p < 0.001$; **Figure 1A**). This is in accordance with a recently identified link of *SF3B1* mutations with senescence and increased p21 protein levels (19). Non-irradiated *SF3B1*-mutated CLL cells showed a trend towards a higher expression of *ACSM3* with large variation between patients, which could not be linked with VAF of the mutation. Also, the response of ATM target genes *ACSM3* and *NME1* to IR was not affected by *SF3B1* mutation status (**Figure 1A**) (17). Identical effects of wild type (WT) and *SF3B1*-mutated samples in response to IR were observed in this cohort (**Figure 1A**). This was unlike the previous data on the mixed treatment cohort, where differences between WT, and *SF3B1*-mutated samples were apparent (17). Levels of Ser15 phosphorylated p53 and p53 (**Figure 1B**), γH2AX baseline/formation (**Figure 1C**) and sensitivity to fludarabine (**Figure 1D**) appeared unaffected in treatment-naïve *SF3B1*-mutated CLL cases. In summary, we could not detect mechanistic clues relating to a potential link between *SF3B1* mutation and altered DDR responses, using isogenic cell lines and a treatment-naïve CLL cohort.

Another possible pathological mechanism is increased expression of *SF3B1* associated 3' cryptic transcripts at the expense of the canonical mRNA and protein, as reported earlier (11). In addition, *SRSF2* mutations have been shown to affect both splicing and NMD, suggesting a role for NMD in the pathogenic effect of splicing factor mutations (26). Five cryptically spliced genes (*ANKHD1*, *ATM*, *FOXP1*, *MAP3K7*, and *TTI1*; **Figure 2A**), identified in previous transcriptomic analyses in *SF3B1*-mutated patients (12, 15, 18), were analyzed

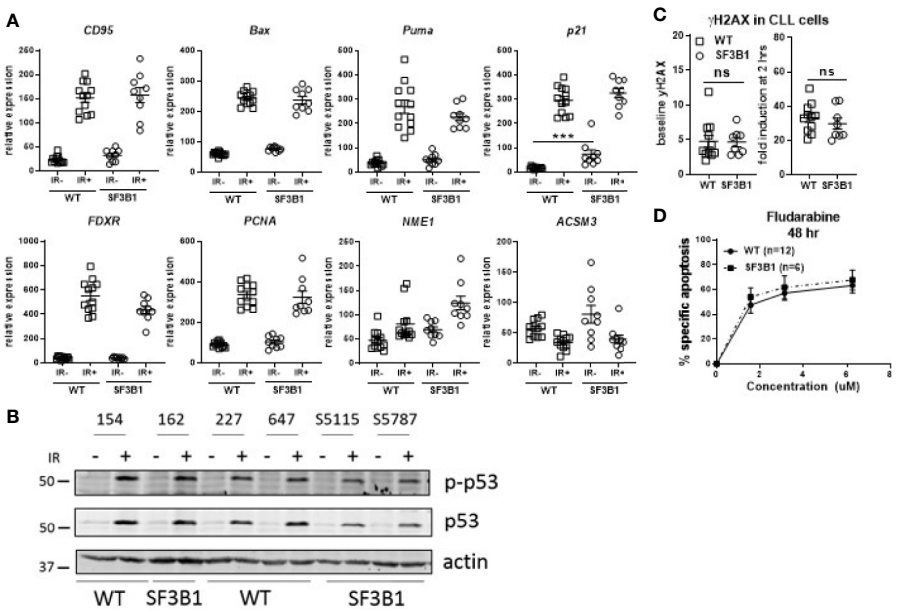

FIGURE 1 | Analysis of DDR response in treatment-naïve *SF3B1*-mutated CLL cells. **(A)** Results of RT-MLPA with specific probes for the detection of ATM/p53 target genes. Relative expression is shown of treatment-naïve *SF3B1*wt (WT, n = 12) and *SF3B1*mut (SF3B1, n = 9) primary CLL cells -/+ IR (5Gy) and 16 h culturing. Data is represented as mean ± SEM. Significance was determined by Kruskal-Wallis test with Dunn's multiple comparison *post hoc* analysis, ***p < 0.001. **(B)** Effects of IR (5Gy) followed by 16 h culturing on p-p53, p53, and β-actin measured by western blotting. **(C)** Formation of γH2AX measured by flow cytometry at baseline (left) and after irradiation (5Gy) and 2 h incubation (right). Data were normalized for isotype control and represented as mean ± SEM. ns, not significant. **(D)** CLL cells of *SF3B1*wt (n = 12) and *SF3B1*mut (n = 8) patients were treated with different concentrations of fludarabine as indicated. Cell death was assessed by $DIOC_6$/TO-PRO-3 staining and calculated as described in material and methods. Error bars are ± SEM. No significant differences were observed (Mann-Whitney).

in different cancer types. Percentages of cryptic transcripts versus canonical transcripts were quantified in primary material from genotyped CLL (**Supplemental Table S3**), MDS (**Supplemental Table S4**) and UM (**Supplemental Table S5**), and in cancer cell lines from different origin -/+ *SF3B1* mutation (**Figures 2B, C**, respectively). Increased expression of *SF3B1*-associated transcripts was indeed observed in all *SF3B1*-mutated cells compared to *SF3B1* WT cells in primary cancer cells and cancer cell lines. Ratios of cryptic versus canonical transcripts were gene-specific and differed between the investigated cancer cells. Cryptic transcripts were mostly present at 10–1,000-fold lower levels than the canonical transcripts, only for MAP3K7 it reached appreciable, though still modest levels.

NMD and its potential altered function/contribution in cancer has recently become of interest as a therapeutic target (13). To explore the effect of NMD on the expression of *SF3B1*-associated cryptic transcripts, we inhibited NMD in different cell lines -/+ *SF3B1* mutation. Cells were treated with the translation inhibitor cycloheximide (CHX), known for its ability to inhibit NMD (11), or pyrimidine related compound 1 (PRC1), a specific inhibitor of the PI3K related kinase SMG1 which regulates NMD activity *via* phosphorylation of UPF1 (27). NMD inhibition with CHX was confirmed by analysis of an established NMD transcript of the splicing factor *SRSF3* (**Figure 2D**) (26). As expected, a rapidly increasing expression of the NMD-sensitive *SRSF3* transcript was observed after CHX treatment in *SF3B1*-mutated cells originating from various cancer types (CLL, UM and pancreatic cancer). Expression of the *SF3B1*-associated cryptic transcript of *TTI1* also increased after NMD inhibition (**Figure 2D**). SMG1 inhibition with PRC1 also resulted in an increased expression of *SRSF3* and *TTI1* transcripts in *SF3B1*-mutated NALM-6 cells (**Figure 2E**). These results suggest that *SF3B1*-associated cryptic transcripts are degraded *via* NMD and suggest a link between NMD and the pathogenic effects of *SF3B1* mutations.

DISCUSSION

Various clinical trials have reported a negative effect of *SF3B1* mutations on survival in chemotherapy-treated CLL patients. Mutations in SF3B1 were associated with decreased survival after chlorambucil and fludarabine with and without cyclophosphamide in the UK CLL4 trial (4) and fludarabine, cyclophosphamide plus rituximab in the German CLL8 trial (3). To expand our previous study, we therefore selected treatment-naïve samples with a high VAF of *SF3B1* mutation. Our results demonstrate that *SF3B1* mutations do not directly affect the ATM/p53 response, at least not in uncompromised, untreated patients. The seeming differences between earlier reported data on a mixed cohort of patients might be explained by effects of chemotherapy; most likely this resulted in the outgrowth of cells with defects in genes other than *ATM* and/or *TP53*, while still causing a slightly defective DDR response. Therefore, we should consider that *SF3B1* mutations can affect genomic stability *via* other pathways (28),

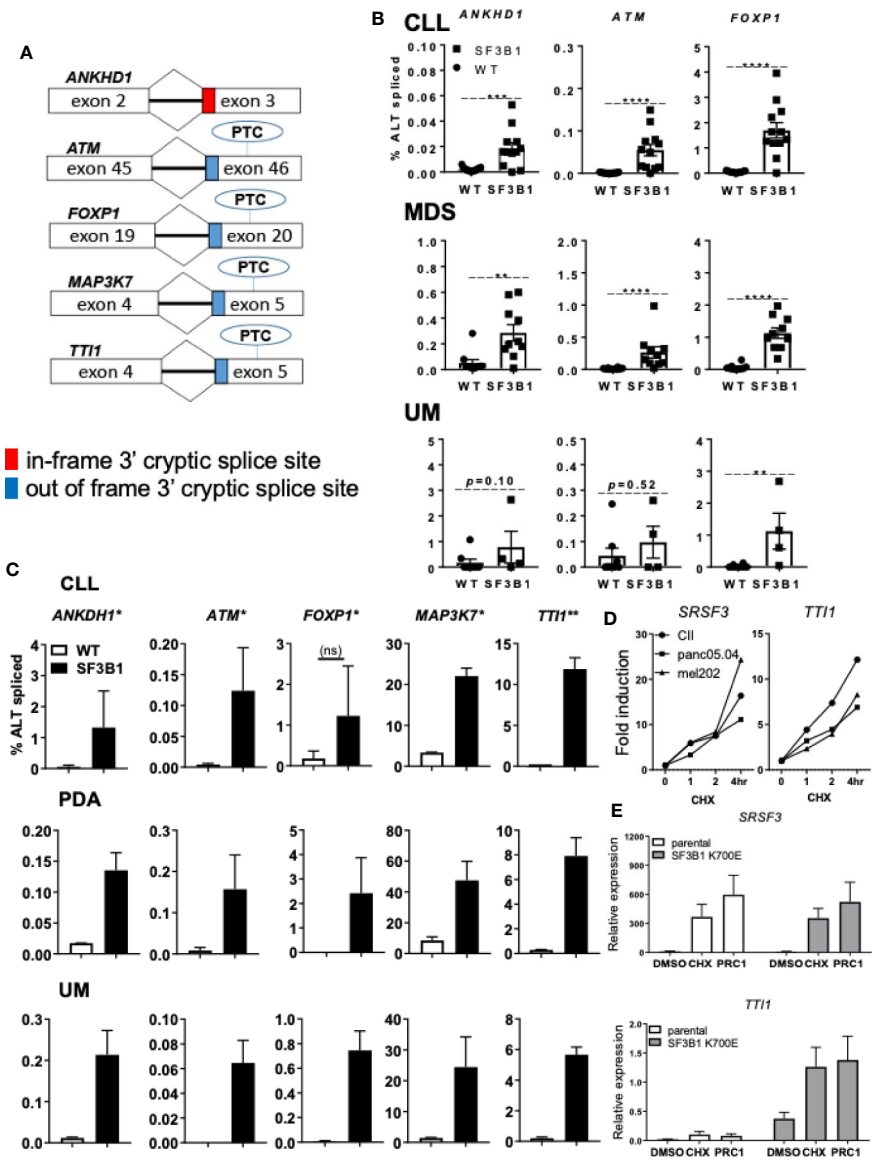

FIGURE 2 | Levels of *SF3B1*-associated 3' cryptic transcripts are often low and sensitive nonsense-mediated mRNA decay. **(A)** Schematic overview of five 3' cryptic transcripts associated with *SF3B1* mutation and the effect of alternative splicing on the introduction of a premature termination codon (PTC) in these transcripts. **(B)** Percentage ALT spliced (percentage cryptically spliced compared to the canonical transcript; % ALT spliced; y-axis) for qPCR analysis of *ANKHD1*, *ATM*, and *FOXP1* in primary CLL cells divided in *SF3B1*wt (n = 15) and *SF3B1*mut (n = 12), myelodysplastic syndrome (MDS) divided in *SF3B1*wt (n = 10) and *SF3B1*mut (n = 10) and uveal melanoma (UM) patients divided in *SF3B1*wt (n = 8) and *SF3B1*mut (n = 4). Primers specific for the 3' cryptic transcript and the canonical transcript were used. Bars represent mean ± SEM. Significant differences are presented as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Mann-Whitney U test). **(C)** Percentage ALT spliced was calculated for *ANKHD1*, *ATM* and *FOXP1*, *MAP3K7* and *TTI1* in the CLL cell lines PGA (*SF3B1*wt) and CII (*SF3B1*mut), pancreas carcinoma (PDA) cell lines panc1 (*SF3B1*wt) and panc05.04 (*SF3B1*mut) and UM cell lines 92.1 (*SF3B1*wt) and mel202 (*SF3B1*mut). Bars represent mean ± SEM of at least two independent experiments, three for *ANKHD1* in mel202 and six for *FOXP1* in mel202). **(D)** Fold induction of NMD-sensitive transcript of *SRSF3* (left) and cryptic transcript of *TTI1* (right) 1, 2, and 4 h after cycloheximide (CHX; 100 μg/ml) treatment in CLL, PDA and UM cell lines -/+ *SF3B1* mutation. **(E)** Relative expression of NMD-sensitive transcript of *SRSF3* (above) and cryptic transcript of *TTI1* (below) 6 h after DMSO, 100 μg/ml CHX or SMG1 inhibition by 1 μM pyrimidine related compound 1 (PRC1) in isogenic NALM-6 cells -/+ *SF3B1* K700E mutation. Bars represent mean ± SEM of three independent experiments.

or that other factors are associated with progression of *SF3B1*-mutant CLL patients. For example, mutations in splicing factors in MDS were linked with augmented R-loops and alternative transcripts were observed in genes involved in the suppression of R-loop formation (29). In addition, various altered transcripts resulting from *SF3B1* mutation were linked with diverse signaling pathways; decreased MAP3K7 expression leading to increased NF-kB activity (15), decreased expression of the uveal melanoma tumor suppressor gene BRD9 resulting in disruption of the non-canonical BAF chromatin-remodeling complex (30),

decreased phosphatase 2A subunit PPP2R5A leading to MYC stability (21), and an alternative transcript of *DVL2* was linked to overexpression of NOTCH1 in CLL (18). As hundreds of genes are associated with increased levels of cryptically spliced transcripts in *SF3B1* mutants (11, 12, 15, 18), it is to be expected that *SF3B1* mutations may have widespread effects on cancer cells. Indeed, differences in numerous pathways have been identified in a recent transcriptomic analysis (18). We observed a distinct increase in expression of cryptic transcripts associated with *SF3B1* mutations. Cryptic transcripts were mostly at 10–1,000-fold lower levels than the canonical transcripts, which may in most cases decrease the likelihood this would reach pathological levels. Yet, such transcripts could be augmented upon NMD inhibition, a finding that warrants further study. Hypothetically, the increased expression of cryptic transcripts observed in *SF3B1* mutants could be used therapeutically as NMD inhibition might result in the presentation of tumor specific neoantigens (31).

In conclusion, our results suggest a role for NMD in the biological effects of *SF3B1* mutations and indicate that *SF3B1* mutant patients could potentially benefit from NMD modulatory agents.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The study "B cell maligniteiten Biobank" was approved by the ethical committee Biobank Toetsing Comissie at AMC under the number METC 2013/159. Participants have local approval by their hospital RvB and local ethical committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AL, AK, and EE were responsible for the conception and design of the study. AL, ID, HK, EK, MJ, AdK, AL, JJ, VN, RK, NZ, AE, TZ, SP, OA-W, AK, and EE were involved in the analysis and interpretation of the data. AL, ID, and HK perfomed statistical analyses, made the figures, and performed the experiments. LF contributed clinical CLL samples. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.609409/full#supplementary-material

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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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Circulating Myeloid Regulatory Cells: Promising Biomarkers in B-Cell Lymphomas

Juliette Ferrant[1], *Faustine Lhomme*[2], *Simon Le Gallou*[1,3], *Jonathan M. Irish*[4,5] *and Mikael Roussel*[1,3*]

[1] UMR_S_1236, Univ Rennes, Inserm, Rennes, France, [2] Centre Hospitalier Universitaire de Rennes, Service Hématologie, Rennes, France, [3] Centre Hospitalier Universitaire de Rennes, Laboratoire Hématologie, Pôle Biologie, Rennes, France, [4] Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN, United States, [5] Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, United States

The monocyte/macrophage lineage has been shown to be involved in the promotion of a protumoral tumor microenvironment and resistance to treatment in B cell lymphomas. However, it is still poorly described at the single cell level, and tissue samples are not easily accessible. Thus, a detailed analysis of the circulating myeloid cell compartment in the different B lymphomas is needed to better understand the mechanisms of resistance to treatment and identify at risk patients. In this Perspective, we review current knowledge on the phenotypic and functional description of the circulating monocytic lineage in B cell lymphomas and provide first insights into the heterogeneity of these cell populations in health and lymphoma, using mass cytometry. Indeed, the monocytic compartment is a continuum more than distinct subpopulations, as demonstrated by our high-resolution approach, explaining the sometimes confusing and contradictory conclusions on the prognostic impact of the different populations, including monocytes and monocytic myeloid derived suppressor cells (M-MDSC). By identifying S100A9^{high} monocytic cells as a potential biomarker in diffuse large B cell lymphoma (DLBCL) in this proof-of-concept preliminary study including a limited number of samples, we underline the potential of circulating myeloid regulatory cells as diagnostic and prognostic biomarkers in B-cell lymphomas.

Keywords: monocyte, myeloid-derived suppressor cells, lymphoma, biomarker, mass cytometry

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***Correspondence:**
Mikael Roussel
mikael.roussel@chu-rennes.fr

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INTRODUCTION

Lymphomas are malignancies that arise from the lymphoid system with an involvement of the B lineage in 90% of cases (1). The lymphoma nomenclature, based on clinical, pathological, genetic, and molecular description (2), is constantly evolving. Different subtypes are described, depending on the stage of maturation from which the tumoral B cell derives. Beside the first historically described Hodgkin lymphoma (HL), non-Hodgkin lymphomas (NHL) include essentially mantle cell lymphoma (MCL), follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma, and chronic lymphoid leukemia (CLL). As in many cancers, the importance of the tumor microenvironment (TME) in the development of the disease and response to treatment has

been emphasized in B-cell lymphoma, in particular, lymphomas can be defined by their dependence to the TME (1, 3–6). The TME is in particular composed of immune cells including T cell subsets (follicular helper T cells [Tfh], regulatory T cells [Treg]), stromal cells, dendritic cells, and myeloid cells (monocytes, macrophages, myeloid derived suppressor cells [MDSC]) and is affected by the tumor localization (e.g. lymph node and bone marrow in FL or blood in CLL). The myeloid cell compartment has lately gained great interest and is extensively studied in several cancer types, including B lymphoma, due to its numerous key and ambivalent roles in pro-tumoral immune suppression, anti-tumor immunity, immunotherapy efficacy through antibody-dependent cellular phagocytosis (ADCP), or as a therapeutic target in immune-checkpoint blockade immunotherapy (1, 7). The monocyte/macrophage lineage has been shown to interact with tumoral and non-tumor B cells in lymphoma, and to be involved in the promotion of protumoral TME and resistance to treatment (8). However, the TME in lymphoma is heterogenous and still poorly described at the single cell level (9, 10). Moreover, there is currently an overall lack of biological material due to the popularity of the fine-needle aspiration for routine diagnosis. Thus, peripheral blood remains of interest for the analysis of immune cells and a fine knowledge of the circulating myeloid cell compartment in the different B lymphomas is needed to better understand the mechanisms of resistance to treatment and identify at risk patients. Herein, we propose to review current advances in the high-resolution description and functional role understanding of circulating monocytic lineage in B cell lymphomas and to discuss future perspectives.

Clinical Relevance of Circulating Myeloid Compartment in B Lymphoma Patients

Myeloid-cell related prognostic signatures have been demonstrated *in situ* in secondary lymphoid organs of DLBCL, FL, and HL by gene expression profiling studies (4–6), and several studies have shown an association between macrophage infiltration in lymphoma tissues and prognosis (5, 11–15), but the prognostic impact of myeloid cells in the blood is less clear in these pathologies. Nevertheless, soluble prognostic factors related to the biology of myeloid cells have been proposed to be of prognostic interest in these pathologies, including soluble PD-L1 (16, 17), soluble CD163 (18), CXCL10 (19), and IL-10 (19).

More recently, the circulating myeloid compartment, including in particular monocytes and MDSC, has been evaluated as prognostic biomarkers in B lymphoma. An increased monocyte count has been associated with poor prognosis in DLBCL (20–22), HL (23), MCL (24–26), and FL (27). A slightly different approach, with the lymphocyte/monocyte ratio, gives the same results in HL (23, 28, 29) and DLBCL (30). This could be linked to an increase in monocytic myeloid derived suppressor cells (M-MDSC), as it has been suggested in DLBCL (20, 31) and CLL, in which M-MDSC levels correlate with response to treatment (32–35). Concerning the granulocytic lineage, polymorphonuclear MDSC (PMN-MDSC) have been proposed as a marker of poor prognosis in DLBCL (36), but other works did not find any association between PMN-MDSC and DLBCL prognosis (31, 37). In HL, the presence of a $CD34^{pos}$ MDSC subtype in the blood has been associated with worsened prognosis (38).

Overall, there is ample evidence of the clinical relevance of the monocytic lineage, and in particular of circulating suppressor myeloid cells, in the prognosis of patients diagnosed with B lymphomas. However, the existence of conflicting data, largely due to inconsistent phenotype, highlights the need for an in-depth phenotypic study of these populations in order to better characterize them and evaluate their role in these pathologies.

Deciphering the Circulating Myeloid Regulatory Cell Phenotypes in B-cell Lymphomas

To fully understand the role of the monocytic lineage in the physiopathology of B-cell lymphomas, it is first necessary to clearly define these cell subsets. Unfortunately, this raises a pitfall in the different data available on the circulating myeloid compartment in B-cell lymphomas, indeed there is no consensus on the definition of M-MDSC and PMN-MDSC, and monocyte subsets are defined with a continuum of markers (CD14 and CD16). Nevertheless, some low-resolution phenotypical investigations have been performed on these cell subsets in B-cell lymphomas, using various and sometimes overlapping phenotypical definitions.

MDSC is a heterogeneous population of myeloid regulatory cells derived from polymorphonuclear cells (PMN-MDSC) and monocytes (M-MDSC) and defined by their immunosuppressive functions (39, 40). Their existence in inflammatory and cancerous diseases could reshape the TME or more distant sites. Since these cells were first described in mice and defined by their immunosuppressive functions, their identification and study in humans are challenging and discussed. In blood, PMN-MDSC (historically referred to as granulocytic MDSC [G-MDSC]) are classically identified as $CD11b^{pos}$ $CD14^{neg}$ $CD15^{pos}$ or $CD11b^{pos}$ $CD14^{neg}$ $CD66b^{pos}$, and M-MDSCs as $CD11b^{pos}$ $CD14^{pos}$ HLA-$DR^{neg/low}$ $CD15^{neg}$. It should be noted that these phenotypical definitions do not discriminate PMN-MDSC from normal neutrophils, as a consequence it is recommended to evaluate PMN-MDSC in the low density fraction after ficoll (39). Lin^{neg} HLA-DR^{neg} $CD33^{pos}$ $CD123^{neg}$ cells contain more immature progenitors named early-stage MDSC (e-MDSC) (39–41). Additional markers, including CD116, CD124, VEGF-R, CD11c, CD11b, PD-L1 are commonly used, and various phenotypes have been described in tumors (39, 40, 42).

In human B-cell lymphoma, various PMN-MDSC phenotypes were described. In particular, circulating $Lin^{neg/low}$ HLA-DR^{neg} $CD11b^{pos}$ $CD33^{pos}$ cells were increased in HL, FL, DLBCL, and MCL, compared to healthy donors blood (31, 37, 38), whereas $CD66b^{pos}$ $CD33^{dim}$ HLA-DR^{neg} were more abundant in the blood of HL, and indolent or aggressive NHL B-cell lymphomas (36). Finally, immature MDSC defined as $CD11b^{pos}$ $CD33^{pos}$ $CD14^{neg}$ $CD34^{pos}$ HLA-DR^{neg} were more

abundant in HL compared to blood from healthy donors (38). M-MDSC phenotype is more consistent and CD14^{pos} HLA-DR$^{low/neg}$ cells are accumulated in peripheral blood from FL, DLBCL, MCL, CLL, and HL when compared to healthy samples (20, 31–33, 43, 44). However, HLA-DR is expressed as a continuum and thus the M-MDSC identification remains subjective and will benefit from additional markers and high-resolution analysis.

Overall, comparing all these data is challenging due to the use of different and few phenotypic criteria to assess the myeloid compartment. To overcome these difficulties, high resolution tools, such as mass cytometry, could be of use to fully decipher at the phenotypic level these complex populations in tissue and in blood (45). Deep phenotyping of the circulating myeloid compartment in human blood in both healthy and B-cell lymphoma context is needed. Such data already exist for healthy donor blood, but have not been compared with blood samples obtained from lymphoma patients (9), and a clear landscape of blood mononuclear phagocytes in human health and B-cell lymphoma would allow relevant and reproducible functional experiments.

We propose here such analysis on blood monocytic cells from FL (n=3), DLBCL (n=5), and CLL patients (n=3), compared to healthy donors (n=3) (**Table 1**), using a previously described mass cytometry (CyTOF) panel dedicated to regulatory myeloid cell exploration (9, 46) (**Figure 1**). With one exception, all samples were obtained at diagnosis, before any treatment. Our analysis was performed on cryopreserved peripheral blood mononuclear cells (PBMC), thus allowing the exploration of monocytes and M-MDSC, but not PMN-MDSC. Using an unsupervised approach, we realized a dimension reduction followed by clustering, defining 8 monocytic clusters (**Figure 1A**). The hSNE representation shows all the CD14^{pos} myeloid cells compartment as a continuum, underlining the difficulty to clearly and consistently distinguish different subsets. Nevertheless, based on canonical markers expression, we can identify non-classical CD14^{dim} CD16^{pos} monocytes in cluster 1, classical CD14^{pos} CD16^{neg} HLA-DR^{pos} monocytes in clusters 4, 6, and 8, and putative CD14^{pos} HLA-DR^{low} M-MDSC in clusters 2, 3, 5, and 7 (**Figures 1A, B**). Interestingly, the hierarchical clustering of all 8 clusters do not delineate these 3 clusters groups, emphasizing the importance of other less classical markers (**Figure 1B**). In particular, clusters 2, 3, and 4 display a strong expression of S100A9, a protein which is involved in MDSC metabolism (49). These three clusters are mainly found in DLBCL, the most aggressive disease of our panel, in which they represent up to more than 90% of the monocytic population (**Figure 1B**). Of note, the cells in cluster 4 highly express HLA-DR, and would usually be included in the classical monocytes subset, even though their phenotype and presence in DLBCL patients may indicate a regulatory M-MDSC-like function. If clusters 1, 5, and 6 are found in all sample types, half of the cells present in cluster 5 come from FL patients. This cluster exhibits an interesting CD14^{pos} HLA-DR^{low} CD36^{pos} S100A9^{high} M-MDSC like phenotype (**Figure 1B**). Surprisingly, the cells in cluster 6 display heterogenous and partly low levels of HLA-DR, even though they highly originate from healthy donors. This intriguing result emphasizes the need to not reduce M-MDSC evaluation to HLA-DR expression. Finally, an unsupervised clustering can hide some residual heterogeneity inside the clusters, and thus mask disparities between sample types in a given cluster. For example, the cluster 1 gathers all non-classical monocytes, but looking more precisely at their phenotype across the different sample types, we can see that the cells from DLBCL patients express less HLA-DR and CD36, or that FL cells express more CD32 (**Figure 1C**). Of course, this preliminary study requires further investigations with a larger number of samples to overcome the limitations due to inter-sample variability, as underlined by the peculiar profiles displayed by one of the HD and one of the CLL samples. It is also difficult to conclude from these findings what impact these phenotypic differences have on cell function, since, for example, the CD32 subtype, which could be an activator or inhibitor, is not known. Linking these phenotypes to cell functionality and pathogeny would therefore require further functional studies.

Altogether, these results suggest that circulating monocytic cells display diverse and sometimes specific phenotypes depending on the physiological or lymphomatous context, and that these phenotypes may be related to cell functionality and/or severity of the disease.

TABLE 1 | Patients' characteristics.

Disease/ sample	Patient ID	Age at diagnosis (years)	Gender (Female/ Male)	Cell of origin (DLBCL) or grade (FL)
DLBCL	#1	76	F	ABC
	#2	66	M	GC
	#3	58	M	–
	#4	57	F	ABC
	#5	57	F	ABC
FL	#1	65	M	3a
	#2	80	F	1–2
	#3	76	F	1–2
CLL	#1	64	M	–
	#2	84	M	–
	#3	57	F	–
HD	#1	50	M	–
	#2	28	M	–
	#3	56	F	–

DLBCL, diffuse large B cell lymphoma; ABC, activated B-cell; GC, germinal center; cHL, classical Hodgkin lymphoma; FL, follicular lymphoma; CLL, chronic lymphocytic leukemia; HD, healthy donor.

Assessing the Myeloid Cells Functional Roles in B-Cell Lymphomas

Even if their definition remains quite elusive, particularly at the phenotypic level, it is now clear that MDSC are important players in B-cell lymphoma pathogenesis. Since the characterization of MDSC, although challenging, is classically based on functional assays (39), we have data on their functional role in tumors, including lymphoma.

In cancer, induction and expansion of MDSC can be induced by factors produced by the tumour or the TME cells (T-cells, macrophages, stromal cells), such as VEGF, GM-CSF, M-CSF, S100A8/9, IL-4, IL-6, or IL-10 (49). MDSC could derive from

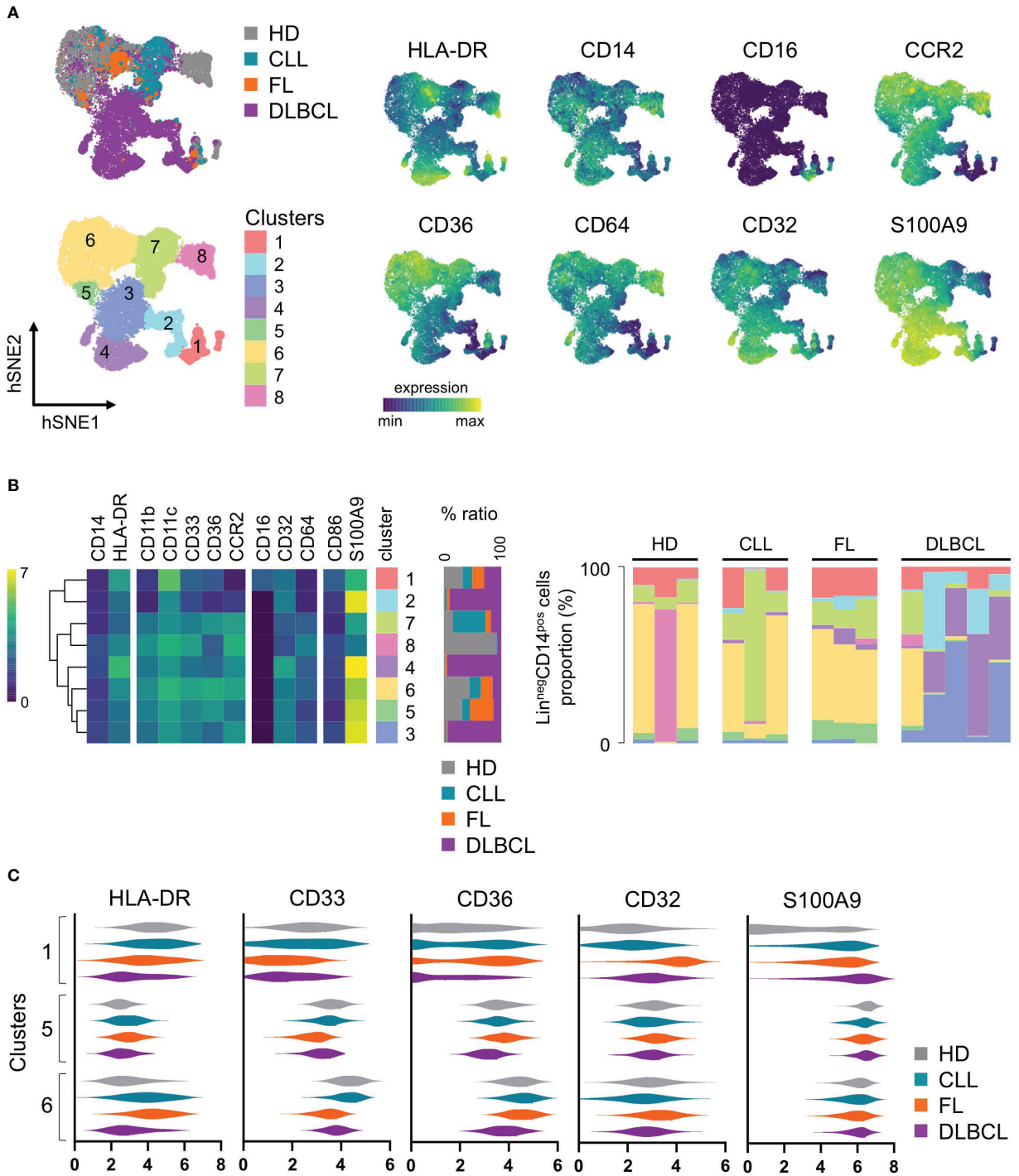

FIGURE 1 | Peripheral blood mononuclear cells (PBMC) from healthy donors (HD, n = 3), follicular lymphoma (FL, n = 3), diffuse large B-cell lymphoma (DLBCL, n = 5) and chronic lymphocytic leukemia (CLL, n = 3) patients were stained with a CyTOF panel already published and dedicated to regulatory myeloid cells assessment (9, 46). $Lin^{neg}CD14^{pos}$ viable cells were selected by manual gating on hSNE for each sample, using all channels and the default settings on Cytosplore (47, 48). Then, a hSNE algorithm followed by clustering was performed on all pooled selected cells (n=79,760) with Cytosplore (47, 48) using the following channels: CD11b, CD64, CD36, CCR2, CD45RA, CD123, CD86, CD33, CD11c, CD14, CD32, HLA-DR, CD16, and S100A9 and the default settings. Eight clusters were identified. **(A)** hSNE plot of $CD14^{pos}$ myeloid cells, colored by sample type, clusters, and selected marker expression. **(B)** Heatmaps of selected markers in each cluster (left), relative distribution of the cells contained in each cluster per sample type (middle), and relative proportion of each cluster in each sample (right). **(C)** Violin plots of selected markers on clusters 1, 5, and 6 for each sample type. Expression values are arc-sinh transformed. Hierarchical clustering, heatmaps and violin plots were generated with R v3.6.3, using Rstudio v1.2.5033 and the pheatmap package, and Graphpad Prism 8.4.3.

myeloid progenitors from the bone marrow or the spleen, or arise from monocytes (M-MDSC) or activated mature or immature PMN (PMN-MDSC) (50), after the activation of genes implicated in myeloid differentiation blockade or immune regulation. The main signaling mechanism involved seems to be the phosphorylation of transcription factors known as signal transducer and activator of transcription (STAT) protein family members like STAT3, STAT6, STAT1, STAT5 (40, 51). The fate of these circulating myeloid cells is not completely elucidated. MDSC can differentiate into macrophages and DC (52, 53), especially under hypoxia (54). It has been demonstrated in murine models of solid tumors that MDSC can differentiate into TAM at the tumor site (51, 55–57). MDSC are defined by their immunosuppressive capabilities. They can impair effector T- and NK-cell functions and polarize macrophages into « M2 » phenotype *via* the expansion of regulatory T cells (Treg), amino acids depletion through arginase-1 (Arg1) and Indoleamine 2,3-dioxygenase (IDO), ROS production through NOX2, expressions of immunoregulatory proteins PDL1, TGFβ, IL-10, and S100A12 (42, 58, 59). The crosstalk between MDSC, macrophages, and DC could enhance the tumor-induced immune suppression (60). MDSC could induce a systemic immune suppression by affecting trafficking of T- and B-cells and reducing antigen response outside tumor sites (61).

Some of these immunosuppressive mechanisms have been demonstrated in B-cell lymphomas. The role of arginine metabolism has been demonstrated in lymphoma mice models (54, 62), where an upregulation of inducible nitric oxide synthase (iNOS) and Arg1 is mediated in MDSC by hypoxia-inducible factor-1a (HIF1a) (54). Another mechanism identified in mice in the upregulation of Arg1 in both PMN-MDSCs and M-MDSC is an increase in the microRNA miR-30a expression, which leads to a decrease in SOCS3 mRNA and the activation of the Janus kinase/STAT3 pathway (63). It has also been demonstrated that MDSC arising from the bone marrow are recruited to the tumor through blood circulation, and that tumor infiltrating M-MDSC mediate the recruitment of Treg in tumor-bearing mice *via* CCL4 and CCL5 production (64). High levels of ROS were also reported in PMN-MDSCs from the blood of this lymphoma murine model (64). The role of MDSC in IL-10 production is suggested by results in B-cell NHL patients and lymphoma murine models (65), but serum IL-10 could also be produced by lymphoma cells and contribute to an increased number of M-MDSC (66). In DLBCL patients, the release of IL-10, together with S100A12 and an increased PD-L1 expression, could explain the M-MDSC mediated T-cell immunosuppression (31). Accordingly, monocytes from NHL patients (including DLBCL, FL, MCL) induce less T-cell proliferation *in vitro* than HD monocytes, and the removal of these monocytes restore T cell functions (44). The role of arginine metabolism in the suppressive activity of MDSC from the blood of NHL patients is debated (31, 36, 44), as well as PMN-MDSC pathogeny. Even though no immunosuppressive effect of PMN-MDSC has been reported in a study including only DLBCL patients (31), another study including both HL and B-cell NHL patients showed that *in vitro* depletion of lymphoma PMN-MDSC restored the proliferation of autologous T cells (36). This could be due to the different phenotypic definition of the cells studied, or to the pathologies included. In CLL, the efficacy of the depletion of monocytes to reduce tumorigenesis has been shown in mice models (67). CLL cells induce IDO^{high} MDSC *in vitro*, and CLL patients display an increase in M-MDSC, with suppressing T cell activity, *via* IDO and Treg expansion (33). Accordingly, monocytes from CLL patients inhibit T cell proliferation, an effect abrogated by anti-TGFβ, anti-IL10 antibodies and IDO inhibitor, and support Treg expansion (68).

Overall, the sometimes conflicting results of the different works focusing on explaining the functional roles of myeloid cells in B-cell lymphomas highlight the need for a better and clearer definition of these different cells subsets.

DISCUSSION

Over the last few years, an increasing interest has been shown in the myeloid regulatory compartment in B cell lymphomas, and notably the circulating monocytic population. MDSC in particular were shown to be involved in disease prognosis and could have a pathogenic role. However, there is still no consensus on the phenotypic definition of the different circulating myeloid populations. Moreover, the monocytic compartment is more of a continuum than constituted of distinct subtypes, as highlighted by our results, resulting in sometimes contradictory conclusions on the prognostic impact of the different MDSC populations. We confirm here previous results, such as HLA-DR down regulation in B-cell lymphomas, and provide a first exhaustive phenotypic evaluation of the monocytic compartment in different B-cell lymphomas, identifying for example S100A9^{high} monocytic cells as a potential biomarker in DLBCL. This proof-of-concept preliminary study underlines the need to further assess the circulating myeloid compartment at the phenotypic level, on a larger panel of patients and possibly with more markers, to validate clinically relevant prognostic signatures.

The therapeutic management of lymphomas today largely relies on immunotherapy, including rituximab and immune checkpoint blockade. The efficacy of immunotherapy could be modulated by MDSC, as it has been shown in TAM, and their monitoring in the blood could help evaluate response to treatment. However, the characterization of MDSC is currently based on functional assays (39), preventing their monitoring in personalized medicine. Linking easily accessible and monitorable surface phenotypic markers to the functionality of these cells would help overcome this issue. A refined characterization through deep phenotyping of the myeloid compartment could also help identify potential new therapeutic targets (69) and study the immune response to treatment (70).

Altogether, the observations discussed in this article support the idea that circulating myeloid regulatory cells in B-cell lymphomas are promising diagnostic and prognostic biomarkers, and that a comprehensive phenotypic evaluation could serve as a surrogate biomarker of their pathological activity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB Rennes University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JF analyzed data and wrote the manuscript; FL, SLG performed experiments; JMI analyzed data; MR designed and supervised research and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Single-Cell Transcriptome Analysis Identifies Ligand–Receptor Pairs Associated With BCP-ALL Prognosis

Liang Wu[1†], Minghao Jiang[1†], Ping Yu[1], Jianfeng Li[1,2], Wen Ouyang[1], Chong Feng[1,2], Wei Li Zhao[1], Yuting Dai[1,2*] and Jinyan Huang[1*]*

[1] Shanghai Institute of Hematology, State Key Laboratory of Medical Genomics, National Research Center for Translational Medicine, Shanghai Rui Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China

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Martina Seiffert,
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Laura Llaó Cid,
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Deepshi Thakral,
All India Institute of Medical Sciences, India

***Correspondence:**
Wei-Li Zhao
zhao.weili@yahoo.com
Yuting Dai
forlynna@sjtu.edu.cn
Jinyan Huang
huangjy@sjtu.edu.cn

[†]These authors have contributed equally to this work

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B cell precursor acute lymphoblastic leukemia (BCP-ALL) is a blood cancer that originates from the abnormal proliferation of B-lymphoid progenitors. Cell population components and cell–cell interaction in the bone marrow microenvironment are significant factors for progression, relapse, and therapy resistance of BCP-ALL. In this study, we identified specifically expressed genes in B cells and myeloid cells by analyzing single-cell RNA sequencing data for seven BCP-ALL samples and four healthy samples obtained from a public database. Integrating 1356 bulk RNA sequencing samples from a public database and our previous study, we found a total of 57 significant ligand–receptor pairs (24 upregulated and 33 downregulated) in the autocrine crosstalk network of B cells. Via assessment of the communication between B cells and myeloid cells, another 29 ligand–receptor pairs were discovered, some of which notably affected survival outcomes. A score-based model was constructed with least absolute shrinkage and selection operator (LASSO) using these ligand–receptor pairs. Patients with higher scores had poorer prognoses. This model can be applied to create predictions for both pediatric and adult BCP-ALL patients.

Keywords: BCP-ALL, scRNA-seq, ligand–receptor pairs, machine learning, prognosis

INTRODUCTION

B cell precursor acute lymphoblastic leukemia (BCP-ALL) is a hematological malignant neoplasm caused by the abnormal proliferation and accumulation of B-lymphoblastic progenitor cells in the bone marrow (1). Although the 5-year survival rate of pediatric BCP-ALL has surpassed 90% in some developed countries, it remains a main factor in cancer-related death in children and has high morbidity (2, 3). Chemotherapy and targeted therapy are effective treatments for the majority of incipient BCP-ALL patients. However, about 15–20% of such patients will relapse within 5 years, become drug resistant, and eventually die (4). This is in part due to the high heterogeneity of BCP-ALL and to extensive remodeling of the immune microenvironment (5).

Bulk RNA sequencing (RNA-seq) is widely used to analyze the transcriptomic landscape of BCP-ALL. It can reflect the average expression level of various cell types in bone marrow or peripheral blood as a whole. However, our knowledge of the microenvironment of leukemia cells is limited to only bulk RNA-

seq data. As single-cell RNA sequencing (scRNA-seq) technology in cancer research becomes increasingly promoted and applied, it has come to provide insights into the analysis of the complexity of cellular composition as well as the heterogeneity of the tumor microenvironment (TME) (6, 7). The use of scRNA-seq can help us gain a deep understanding of the pathogenesis of BCP-ALL (5).

TME plays a crucial role in tumorigenesis and tumor progression, drug tolerance, and immune infiltration (8). The process of tumor development is inhibited by immune cells, and conversely, tumor cells secrete immunoregulatory factors and constantly reshape the microenvironment, leading to a change in the microenvironment in favor of tumor growth and invasions (9–12).

The communication among various cells in TME is mainly mediated through ligand–receptor interactions either in soluble or membrane bound form (13). Checkpoint inhibitors that operate based on the ligand–receptor interaction have become powerful tools for clinical therapy (14). In recent years, several studies have been conducted on the cell–cell crosstalk of TME based on scRNA-seq. For example, Kumar et al. characterized cell–cell communication across all cell types in the microenvironment of mouse tumor models, including melanoma, breast cancer, and lung cancer, and found that the expression of individual ligand–receptor pairs was closely linked to tumor growth rate (15). By analyzing single-cell data in glioma, Shi et al. found that cellular interactions between glioma stem cells and tumor-associated macrophages could affect the prognosis of glioma patients (16). These works provide the references and analytical workflow for cell–cell communications.

However, current research on cell–cell communication focuses on solid tumors. Our understanding of intercellular interactions in leukemia, such as BCP-ALL, remains limited. Previous research has found the extensive remodeling of the TME in BCP-ALL, and a non-classic mononuclear subpopulation is enriched within the myeloid compartment. This subpopulation has prognostic implications for BCP-ALL (5). How myeloid cells affect tumorigenesis and the communication between myeloid and neoplastic B cells in the BCP-ALL TME has not been fully explored. To investigate cell–cell communication in BCP-ALL in depth, we analyzed scRNA-seq data of seven BCP-ALL samples and four healthy samples. Among the seven BCP-ALL samples, five of them are *ETV6-RUNX1* fusion. They belong to low-risk subtype and occurs mostly in children. Two of them are *BCR-ABL1* fusion (also called Ph+), which belong to high-risk subtype (17, 18). Totally 57 ligand–receptor pairs were found in the autocrine crosstalk network of tumor-related B cells, and 29 were detected in the paracrine crosstalk network between B cells and myeloid cells. A robust least absolute shrinkage and selection operator (LASSO) regression model was constructed using ligand–receptor pairs to predict prognoses for both pediatric and adult BCP-ALL patients.

MATERIALS AND METHODS

Datasets

The scRNA-seq data related to BCP-ALL in recent five years was searched from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and only the dataset GSE134759 was found. Bulk RNA-seq and clinical data of BCP-ALL used for survival analysis and prognostic model construction was downloaded from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET, https://ocg.cancer.gov/programs/target). The TARGET ALL P2 cohort with 532 samples was obtained by R package TGCAbiolinks (v2.16.3). And 133 primary diagnosis BCP-ALL samples whose definition was primary blood derived cancer (bone marrow) were used in the downstream analysis. Another bulk RNA-seq and the clinical dataset was collected from five significant patient cohorts (19–26), including 1,223 BCP-ALL cases available from our previous study (17). This dataset was used for Spearman's correlation calculation and prognostic model validation. The 36 tumor cohorts of The Cancer Genome Atlas (TCGA) used for validating the model were downloaded *via* R package TGCAbiolinks (v2.16.3). Ligand–receptor pairs were collected from several public databases (13, 27).

scRNA-seq Data Analysis

All steps for scRNA-seq data processing and cell–cell communication analysis as well as for the machine learning model development described below were performed with R (v4.0.1). For the seven BCP-ALL and four healthy samples, cells for which less than 500 genes or over 10% genes derived from the mitochondrial genome were first filtered out. To remove doublets, cells with more than 5,000 genes were also filtered. All of the 11 samples were preprocessed and normalized using SCTransform, with default parameters implemented in Seurat (v3.5.1) package individually (28, 29). Seurat anchor-based integration method was used to correct the batch and merge multiple samples (30). Cell-type annotation was performed by R package cellassign (v0.99.21) in conjunction with manual comparison of the expression of marker genes among different clusters (31). The pheatmap (v1.0.12) was used to plot heatmap for cell-type annotation using 5,000 randomly selected cells. This was only done to plot the heatmap. The inferCNV (v1.4.0) was used to calculate the copy number variation (CNV) levels of tumor samples.

Cell–Cell Communication Analysis

The differential expression of genes between the BCP-ALL samples and healthy samples separately for B cells and myeloid cells was compared using MAST (v1.14.0) (32). Significant genes with adjusted P-value < 0.05 were mapped to ligand–receptor pair databases. To further investigate the correlations in the ligand–receptor pairs, Spearman's correlation coefficient was calculated to check the co-expression level of individual pairs. Any pair with an adjusted P-value < 0.05 and coefficient > 0.3 was considered to be significant. Gene set enrichment analysis (GSEA) was performed using fgsea (v1.14.0). Pathway enrichment analysis was performed using clusterProfiler (v3.16.1) (33).

Survival Analysis

Kaplan-Meier and log-rank tests were performed using the survival (v3.2-3) and survminer (v0.4.8) packages to construct

and compare survival curves for the LASSO prediction model or specific genes. For specific genes, the patients were divided into high- or low-expression groups according to the mean expression of this gene, and P-value < 0.05 was considered to denote significance.

Machine Learning Model Development

The LASSO regression model implemented in the glmnet (v4.0-2) package was fitted to predict the patient prognosis based on ligand–receptor pairs between B cells and myeloid cells. LASSO regression penalizes the data-fitting standard by eliminating predictive variables with less information to generate simpler and more interpretable models. To evaluate the variability and reproducibility of the estimates produced by the LASSO Cox regression model, we repeated the regression fitting process for each of the 1,000 leave-10%-out cross-validation evaluations. Genes with non-zero coefficient estimates were retained across all 1,000 evaluations. For each of these genes, the final model coefficient was taken as the average of the coefficient estimates obtained for the set of cross-validation evaluations. The recursive partitioning survival model available in the rpart (v4.1-15) package was used to dichotomize patients into low- and high-score groups. Multivariable Cox-proportional hazard model was used to check the independent prognostic effect. The risk group was defined by our previous study (17). In pediatric BCP-ALL, patients with *TCF3-PBX1*, *ETV6-RUNX1*/-like, *DUX4* fusions, *ZNF384*/*ZNF362* fusions, and high hyperdiploidy (51–65/67 chromosomes) were defined as low-risk. Patients with hyperdiploidy (≤50 chromosomes), *PAX5* and *CRLF2* fusions were defined as intermediate-risk. While patients with *MEF2D* fusions, *BCR-ABL1*/Ph-like, and KMT2A fusions were defines as high-risk. And in adult BCP-ALL, patients with *DUX4* fusions, *ZNF384*/*ZNF362* fusions, and hyperdiploidy were defined as intermediate-risk, and patients with *MEF2D* fusions, *TCF3-PBX1*, *BCR-ABL1*/Ph-like, and *KMT2A* fusions were defined as high-risk (17).

RESULTS

Cellular Heterogeneity Within the Immune Microenvironment of BCP-ALL

To delineate the cellular diversity of the BCP-ALL microenvironment, we analyzed the scRNA-seq data for seven newly diagnosed BCP-ALL samples (five with *ETV6-RUNX1* and two with *BCR-ABL1*, Ph+) and four healthy samples. After initial quality control was conducted (see methods), and all samples were merged using anchor-based integration, 58,518 cells (**Figure 1A**) were enrolled for downstream analyses (38,860 from BCP-ALL, 19,658 from healthy samples). Little difference was seen in the cell distribution of tumor and normal samples (**Figure 1B**). This may be due to the special sample preparation method for BCP-ALL, where 20% CD19+ B cells was mixed with 80% CD19-CD45+ non-B cells (5). The profiles separated by subtype of BCP-ALL were also very similar (**Figure S1A**). Cell-type annotation was performed using cellassign (31), and then the top genes upregulated in each cluster were examined and visualized (**Figure S1B**).All 58,518 cells were assigned to six distinct cell types: B cells (25.2%), erythrocytic cells (0.7%), hematopoietic stem and progenitor cells (HSPC 3.1%), myeloid cells (11.1%), natural killer (NK) cells (6.3%), and T cells (53.5%, **Figures 1C, D**). All 11 samples contained each of the six cell types (**Figure 1E**). After assessing the differences of non-tumor cell subsets between BCP-ALL and healthy samples, only the proportion of myeloid cells was significantly different (**Figure S1C**), which could imply a special role for myeloid in the bone marrow of BCP-ALL. According to the expression level of *MME* (an important cell surface marker in the diagnosis of human ALL), the vast majority of B cells present in neoplastic samples were leukemic cells of a pre-B phenotype (**Figures 1F**, **S1B**). The malignity of these B cells was also confirmed by inferred CNV level. Among the different cell types in the seven BCP-ALL samples, B cells had the highest CNV level (**Figure S1D**). A comparison of the CNV level of B cells between BCP-ALL and healthy samples found a significant difference (**Figure 1G**).

Specific Ligand–Receptor Pairs Reveal an Autocrine Crosstalk Network in BCP-ALL

The cell–cell communication level can be reflected in the expression of ligands and their special receptors. For this reason, first, we detected the intracellular communication network of B cells. Only those ligand–receptor pairs in B cells of BCP-ALL samples that had significantly high or low expression passed the filtration. We supposed that these pairs were more closely associated with leukemogenesis. As shown in **Figure S2A**, we performed differential expression testing between tumor B cells and non-tumor B cells. Then, these genes were mapped to public ligand–receptor databases (see *Materials and Methods*) (13, 27). And 152 upregulated and 206 downregulated genes were identified. Finally, the expression correlation between the individual ligands and their corresponding receptors was examined using bulk RNA-seq data obtained from our previous study (17). Only the 296 samples with *ETV6-RUNX1* and *BCR-ABL1* subtypes were used. After these strict criteria were applied, 24 upregulated and 33 downregulated ligand–receptor pairs were detected in total (see *Materials and Methods*, **Figures 2A, B**, **S3A, B, Tables S1, S2**).

In the upregulated pairs, the B-cell leukemogenesis gene *FZD6* and its ligand *CTHRC1* were upregulated in several solid tumors, associated with increased cell migration and tumor invasion (34, 35). The analytical results showed that *FZD6* and *CTHRC1* were both highly expressed in the B cells of tumor samples (**Figure 2C**). It should be noted that *APP* is highly expressed in acute myeloid leukemia (AML), which may promote cancer cell proliferation and metastasis (36). In our results, we found that *APP* and its binding partner *TNFRSF21* were also highly expressed in tumor-related B-cells (**Figure S2B**). *MDK* (a cytokine and growth factor with complex biological functions involved in cancer development and progression) (37), together with its two receptors (*SDC1* and *GPC2*) were highly expressed in the B-cells of BCP-ALL samples (**Figures S2C, D**).

Among the downregulated pairs, the receptor genes *TLR4*, *ITGB2*, and *LRP1*, located in the center of the ligand–receptor

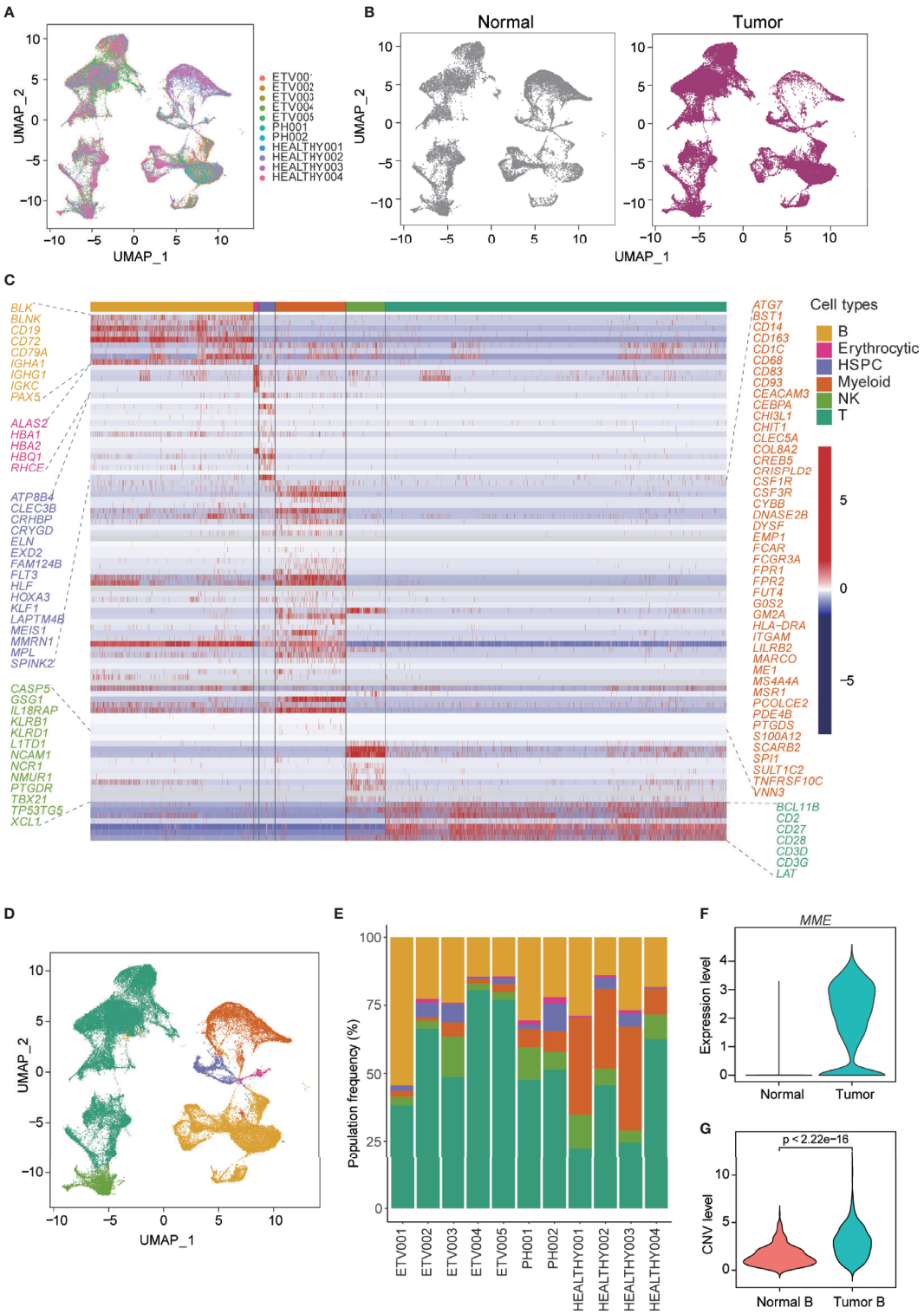

FIGURE 1 | Single-cell profiling and cell-type identification in both healthy and BCP-ALL samples. **(A)** Distribution of 58,518 cells from 11 samples shown by uniform manifold approximation and projection (UMAP). **(B)** UMAP plot showing similar cell distributions in normal and tumor samples. **(C)** Gene expression heatmap of marker genes for the identification of six cell types. **(D)** UMAP visualization of six marker-based cell types. Cell types are colored as in **(C)**. **(E)** Stacked barplots showing the frequencies of six cell types in all of the 11 samples. Cell types are colored as in **(C)**. **(F)** Expression level of *MME* of B cells from normal and tumor samples. **(G)** Inferred CNV level of B cells from normal and tumor samples.

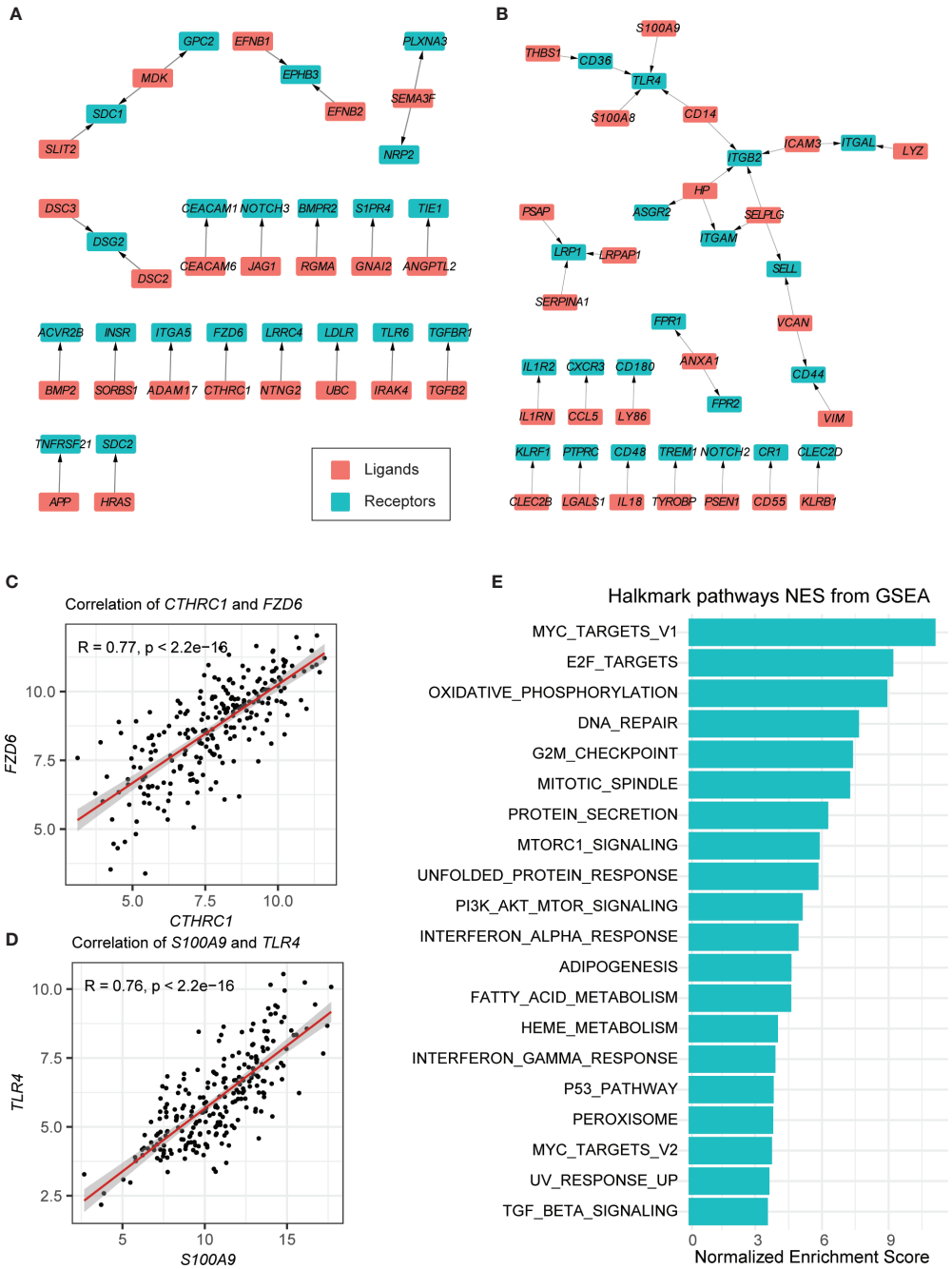

FIGURE 2 | Autocrine ligand–receptor pairs network in tumor-related B cells. **(A, B)** Ligand–receptor pairs that were upregulated **(A)** and downregulated **(B)** in B cells. Red and green squares represent ligands and receptors, respectively, and arrows point from ligands to receptors. **(C, D)** Spearman correlation coefficients of two ligand–receptor pairs (*CTHRC-FZD6* and *S100A9-TLR4*). **(E)** GSEA of the hallmark pathways in tumor-related B cells.

network, with three to four ligands connected respectively (**Figure 2B**). This may imply that they play an important role in anti-tumorigenesis. Previous studies on these genes suggested that *TLR4* is required for protective immune response and to kill cancer cells (**Figure 2D**) (38). *ITGB2* has been found to participate in cell adhesion and cell-surface mediated signaling (39). Lower expression of *LRP1* is associated with the aggressive phenotypes and inferior clinical outcomes in some cancers (40, 41). It should be noted that *ITGAM* also has low expression in the B cells of tumor samples (**Figure S2E**). This has been reported as negative regulator of immune suppression and a target for cancer immune therapy (42).

We also conducted GSEA on B cells in BCP-ALL and healthy samples (**Figure 2E**). The enriched pathways in the HALLMARK database of neoplastic B cells were correlated with cell cycle progressions, such as the E2F targets and the G2M checkpoint, suggesting that most B cells in neoplastic samples are immature B cell progenitors. Other canonical tumor-related pathways, such as the MYC targets and the p53 pathway, were also enriched in neoplastic B cells (**Figure 2E**).

Cell–Cell Communication From B Cells to Myeloid Cells

Previous studies have reported that myeloid cells might play a central role in the immune microenvironment of BCP-ALL (5, 43). Investigation of the crosstalk of B cells with myeloid cells is important for understanding the BCP-ALL TME. Thus, we performed differential expression testing between the myeloid cells of tumor samples and healthy samples. Ligands that were highly expressed in B cells and the receptors that were highly expressed in myeloid cells were selected. After calculating the Spearman's correlation coefficient, 11 ligand–receptor pairs were identified (**Figures 3A**, **S5A, B, Table S3**). Interestingly, we found that some of these 11 ligand–receptor pairs were the same as those found in the autocrine crosstalk of B cells, such as *UBC-LDLR* and *MDK-GPC2* (**Figures S4A, B**). This partly indicates the consistency in the process of leukemogenesis within the bone marrow environment. Of note, patients with higher expression level for *UBC* tend to have worse clinical outcomes (**Figure 3B**). *MDK* has similar survival trends (**Figure 3C**). The other ligand–receptor pairs that were specifically present in the crosstalk of B cells to myeloid cells, also have a crucial influence on tumorigenesis. For example, *ABCA1* is an auspicious therapy target in prostate cancer (**Figure S4C**) (44). A previous study has shown that high expression of *ADRB2* is significantly linked to early treatment failure in ALL (45) (**Figure S4D**). In the survival analyses of these specially expressed ligand–receptor pairs, patients with higher expression of *LIN7C* or *NRTN* are prone to poor prognosis (**Figures 3D, E**). Gene Ontology (GO) analysis indicated that these 11 ligand–receptor pairs are mainly associated with the biological processes of cell migration and cell development (**Figure 3F**).

Cell–Cell Communication From Myeloid Cells to B Cells

We also further identified cell–cell communication from myeloid cells to B cells, built on the expression of differentially expressed ligand–receptor pairs. Ligands and receptors that were separately highly expressed in myeloid and B cells were tested. In all, 18 ligand–receptor pairs passed the strict criteria (**Figures 4A**, **S6A, B, Table S4**), and about half of them match autocrine pairs of tumor-related B cells. This suggested that many interactions could be simultaneously activated by malignant or normal cells in the process of leukemogenesis. Intriguingly, the ligand *B2M* had three receptors, indicating its important role in crosstalk from myeloid cells to B cells (**Figures S4E–G**). And patients with higher expression level for *B2M* tended to have worse OS (**Figure 4B**). We also found that *LAMB1* and its receptor *ITGB4* were overexpressed in myeloid cells and B cells, respectively (**Figure S4H**). Patients with higher expression of *LAMB1* have a superior prognosis (**Figure 4C**). *ITGB4* is also a significant prognostic indicator tested by the TARGET cohort (**Figure 4D**). Besides, patients with higher expression of *HRAS* and VEGFB have worse prognoses (**Figures 4E, F**, **S4I, J**). Both of them are closely related to tumorigenesis and progression. GO analysis indicated that these ligand–receptor pairs in the crosstalk from myeloid cells to B cells were mainly related to leukocyte migration, cell proliferation, and cell activation (**Figure 4G**).

LASSO Model Based on Ligand–Receptor Pairs Precisely Predicted BCP-ALL Patient Prognosis

The results of cell–cell communication in BCP-ALL revealed that significantly expressed ligand–receptor pairs might play a key role in leukemogenesis and progression. A machine learning model was built to predict the prognosis for BCP-ALL patients based on these pairs identified above. The principal component analysis was performed, with the expression level of ligand–receptor pairs in 14 different BCP-ALL subtypes which were classified in our previous study (17). The result showed little difference in the expression level of these ligand–receptor pairs across all the 14 BCP-ALL subgroups (**Figure S7A**).

To develop the prognostic model, a curated TARGET cohort with 133 BCP-ALL samples was used as training cohort and samples from our previous BCP-ALL cohort were used as validation cohort (see methods). The overall process is shown in **Figure 5A** (46, 47). First, we fitted a LASSO regression model using the expression levels of ligand–receptor pairs. After performing 1,000 leave-10%-out cross-validation replications, the coefficients of 18 genes were found to be non-zero in at least one of these 1,000 evaluations (**Table S5**). And the coefficients of 11 genes were presented in at least 950 of 1,000 analyses (**Figure S7B**). Then we calculated an LR (ligand–receptor) score for each patient using the expression of these 15 genes, weighted by the regression coefficients, as defined in the LASSO model. The equation is LR score = (*ITGB4* × −0.263) + (*SDC1* × 0.177) + (*GPC2* × −0.13) + (*TLR6* × −0.0838) + (*CEACAM1* × −0.0607) + (*JAG1* × 0.058) + (*NOTCH3* × 0.0501) + (*LDLR* × −0.0469) + (*ACVR2B* × −0.0511) + (*SLIT2* × −0.0191) + (*TIE1* × −0.00592). We further used a recursive partitioning Cox regression model to dichotomize patients. After pruning the regression tree, patients in the curated TARGET cohorts with different LR scores were divided into a low-LR score group (n = 65, 50%), and a high-LR score group (n= 65, 50%). The overall survival (OS) of these two groups is remarkably different. Higher LR scores were predictive of inferior OS in TARGET cohort (HR = 8.27, 95% CI = 4.27–16.04, $p < 0.0001$) (**Figure 5B**).

To further confirm the robustness of the LASSO model, an independent cohort with 295 pediatric and 85 adult BCP-ALL patients was used as a validation cohort (19, 24, 25, 48). The LR score was computed with the equation defined above. A similar result was observed in pediatric patients. Based on the recursive

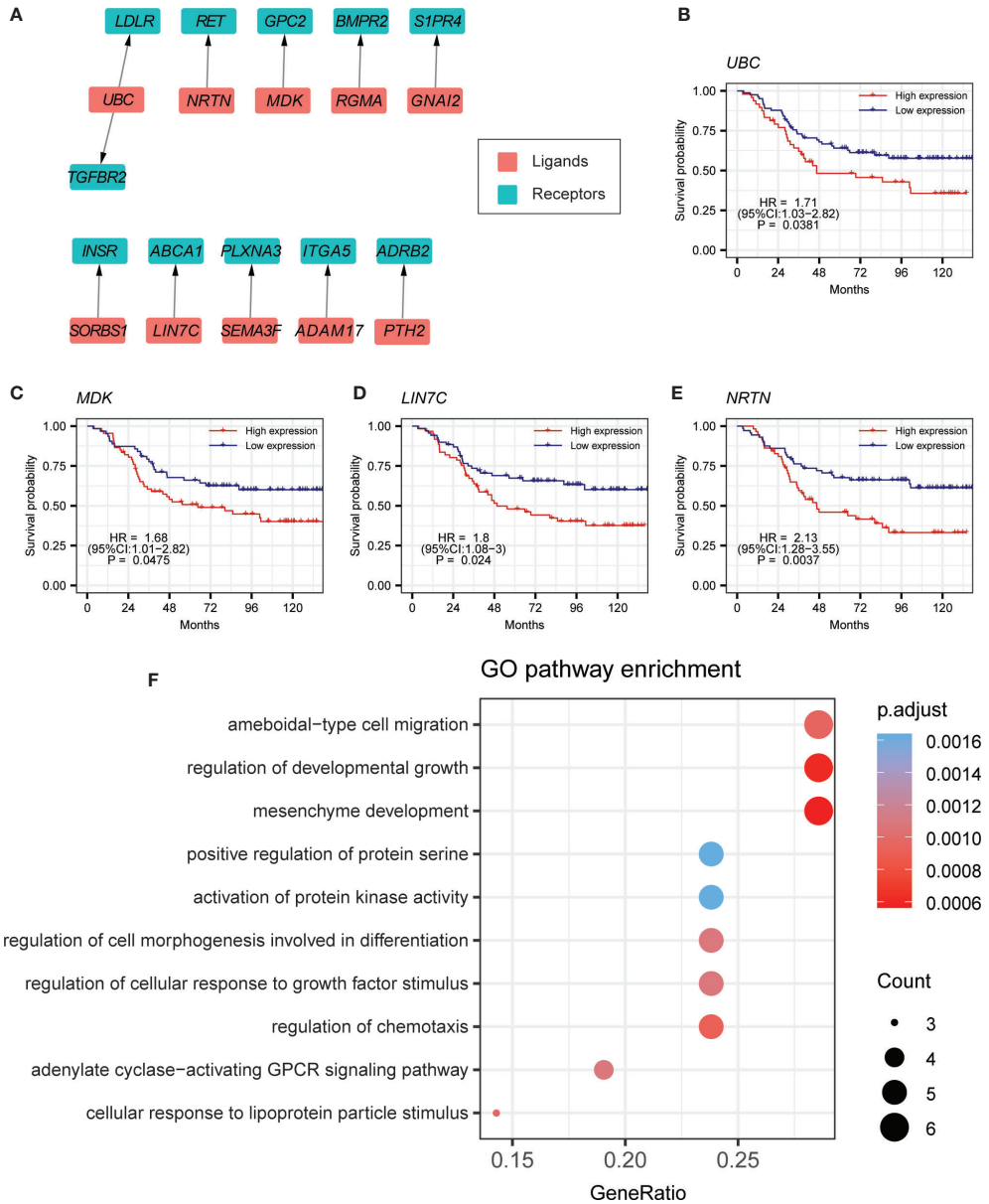

FIGURE 3 | Cell–cell-communication from B cells to myeloid cells. **(A)** Ligand–receptor pairs of the signaling network from B cells to myeloid cells. Red and green squares represent ligands and receptors, respectively, and arrows point from ligands to receptors. **(B–E)** Kaplan-Meier survival for *UBC*, *MDK*, *LIN7C*, and *NRTN* in curated TARGET BCP-ALL P2 cohort. **(F)** GO pathway enrichment analysis for ligand–receptor pairs in the crosstalk from B cells to myeloid cells.

portioning cutoff, the high-LR score group (n=61, 21%) demonstrated worse OS than the low-LR score group (n = 234, 79%), and the range of HR was 4.56 (95% CI = 2.08–10, p < 0.0001, **Figure 5C**). Although these ligand–receptor pairs were identified using scRNA-seq data of pediatric BCP-ALL patients, the prognostic power of the LR score in the 97 adult BCP-ALL patients (17) was also significant (HR = 2.99, 95% CI = 1.26–7.14, p = 0.009, **Figure S7C**). Multivariate analysis was performed with the Cox-proportional hazard model to check the individual risk factor. In the pediatric validation cohort, after adjusting for gender and risk group, the LR score remained an independent predictor of worse OS (HR = 2.45, 95% CI = 1.06–5.7, p = 0.036 **Figure 5D**). The same was true for the adult validation cohort (HR = 2.8, 95% CI = 1.18–6.8, p = 0.019, **Figure S7D**). All of these results demonstrate that the robust machine learning model built with ligand–receptor pairs has promise for identifying high-risk BCP-ALL patients and may have a role as a primary consideration for developing different treatment strategies.

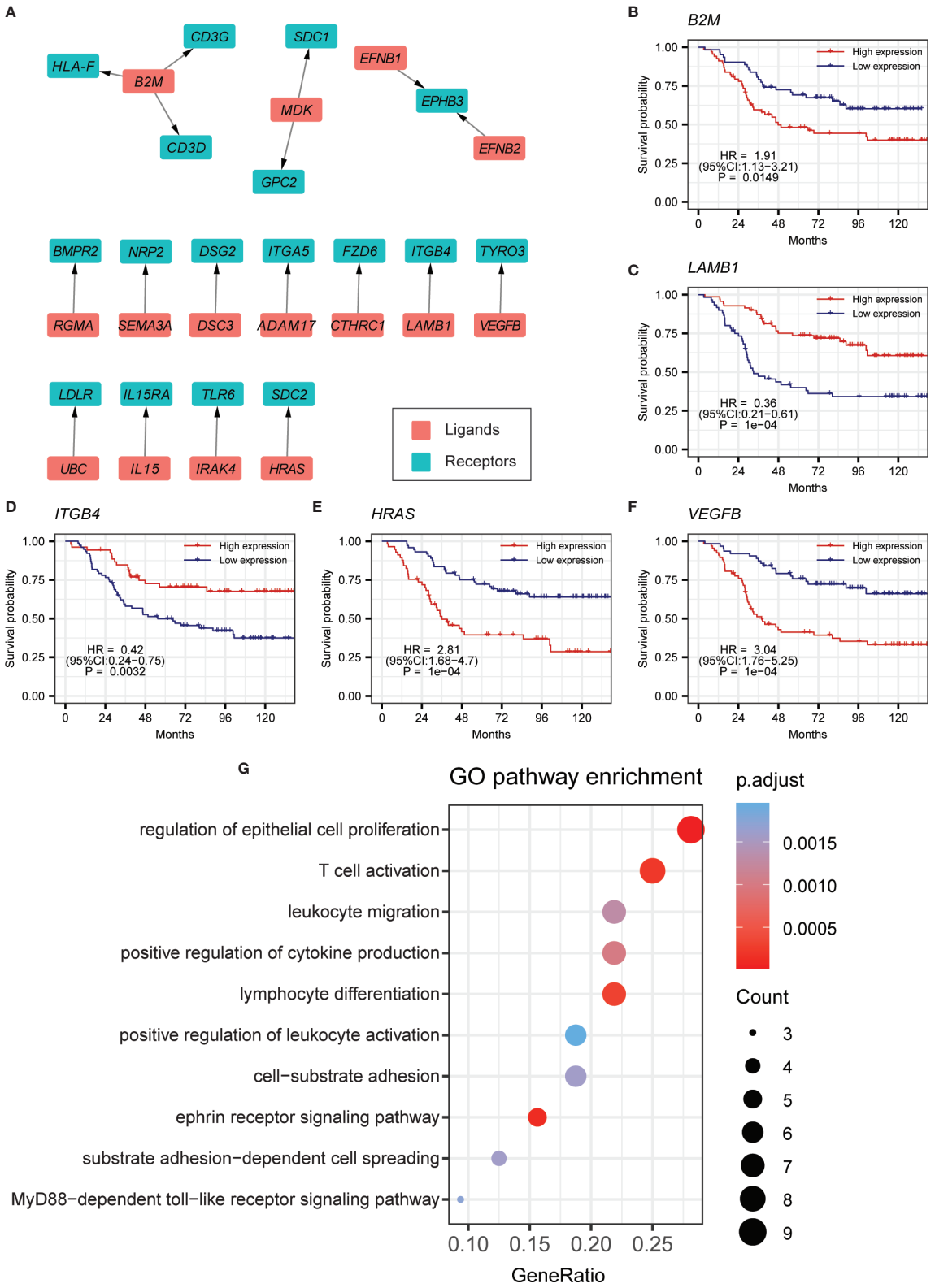

FIGURE 4 | Cell–cell-communication from myeloid cells to B cells. **(A)** Ligand–receptor pairs of the signaling network from myeloid cells to B cells. Red and green squares represent ligands and receptors, respectively, and arrows point from ligands to receptors. **(B–F)** Kaplan-Meier survival for *B2M*, *LAMB1*, *ITGB4*, *HRAS*, and *VEGFB* in the curated TARGET BCP-ALL P2 cohort. **(G)** GO pathway enrichment analysis for ligand–receptor pairs in the crosstalk from myeloid cells to B cells.

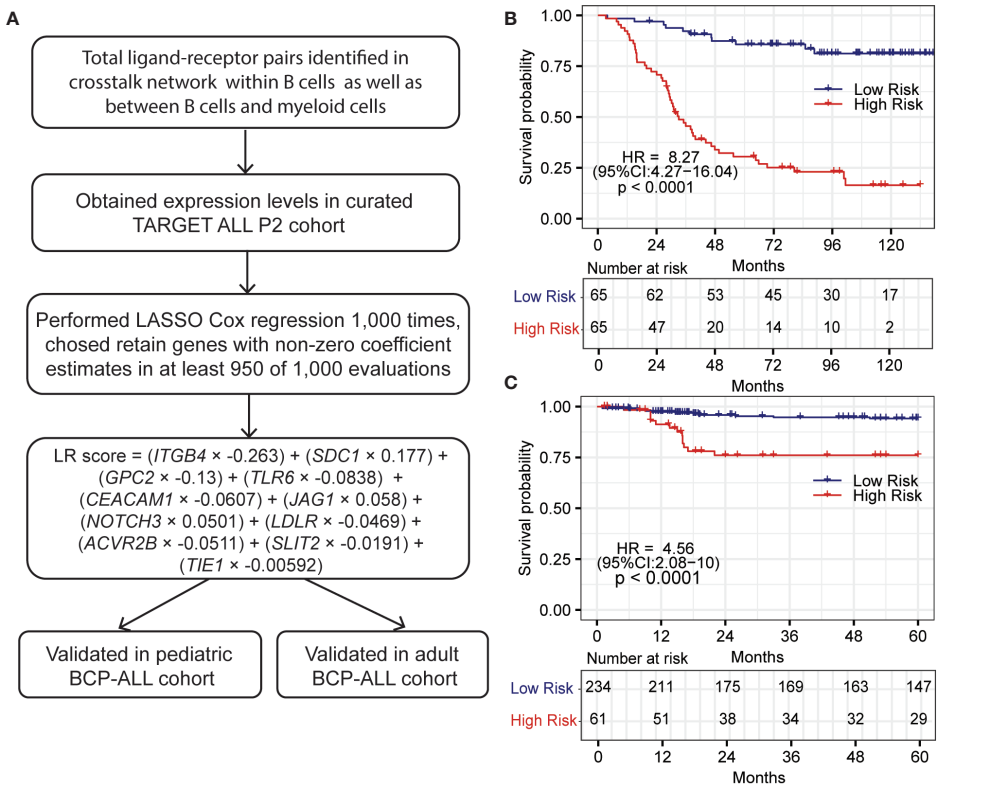

FIGURE 5 | LR score based on LASSO regression model predicts inferior OS in pediatric BCP-ALL patients. **(A)** Overall scheme for constructing LASSO prognostic model. **(B, C)** High LR scores predict poor OS in the TARGET and pediatric validation cohort, respectively. **(D)** Forest plot of multivariable Cox-proportional hazard model showing LR score as an independent prognostic factor for OS in the pediatric validation cohort. Within forest plot, * indicates P-value < 0.05, *** indicates P-value < 0.001.

DISCUSSION

Cellular composition and cell–cell communication are two important aspects of TME. In hematological malignant neoplasms, such as BCP-ALL, a deep understanding of cell–cell interactions in the bone marrow can help us to investigate the leukemogenesis and progression and support the development of new drugs and therapies. In the current omics studies of BCP-ALL, which mainly focus on bulk RNA-seq, the promotion of scRNA-seq reveals the landscape of TME at cellular level resolution and make it possible to investigate the cell–cell communications. In this work, we analyzed a large scale of scRNA-seq profile in seven BCP-ALL pediatric samples and four healthy samples. By classifying and identifying each cell cluster (**Figures 1D, E**), we found B cells from both BCP-ALL and healthy samples were mixed. This may indicate that the biological characteristics of proliferating tumor B cells were presented in a way that was similar to normal B cells. However, compared to both B cells from healthy samples and other cell types from BCP-ALL samples, tumor B cells had higher CNV level (**Figure 1G**), revealing that the accumulation of genetic abnormalities was mainly focused on B cells during leukemic progression.

T cells appeared in the largest proportion in the TME of BCP-ALL. Although previous studies of solid tumors have explored the interaction between T cells and tumor cells (49, 50), other work revealed that myeloid cells also play an important role in the TME of BCP-ALL (5). However, our understanding of the interactions involved in myeloid cells in TME remains limited. In this study, we focused on the two ways to explore the cell–cell communication: the autocrine way for B cells of tumor samples, and the paracrine way between myeloid cells and malignant B cells. Interestingly, this revealed that a considerable number of ligand–receptor pairs were closely associated with tumorigenesis and progression. For example, the *CTHRC1-FZD6* pair and the *APP-TNFRSF21* pair may significantly promote tumorigenesis and the proliferation of cancer cell (34–36). And the *LAMB1-ITGB4* pair has been hypothesized to be involved in tumor invasion and EMT (51). *LAMB1* has also been shown to be a potential biomarker for some cancers, such as colorectal cancer and multiple myeloma (52, 53). Pairs of *UBC-LDLR* and *MDK-GPC2* are widely overexpressed in various cell types of the BCP-ALL bone marrow microenvironment, participating in many processes of tumor development (37, 54). The ligand gene *B2M* takes center stage in the crosstalk from myeloid cells to B cells. It has been demonstrated in several studies that the elevated expression level of *B2M* is historically associated with poor outcome in several lymphoproliferative disorders, such as AML, myelodysplastic syndrome, and ALL (55). Similar results were found in our study (**Figure 4B**). Several genes in ligand–receptor pairs showed significant correlations with the clinical outcomes of pediatric BCP-ALL patients. To better predict prognosis, a machine learning model based on LASSO regression was built based on the determined ligand–receptor pairs. In the pediatric validation cohort, the prognosis for the high-LR score group was significantly worse than for the low-LR score group. Although these ligand–receptor pairs were assessed with pediatric BCP-ALL samples, our prognostic model achieved good performance in the adult validation cohort. This suggests that the prognostic model could help support the clinical decisions for both adult and pediatric BCP-ALL patients. And to further test the predictive efficiency of LR score, we applied our model in 36 tumor cohorts of TCGA. The results showed that LR score had good predictive power in a considerable number of tumors, such as acute myeloid leukemia (AML), skin cutaneous melanoma (SKCM) and uveal melanoma (UVM), of which AML was the most significant. It may indicate that LR score has strong predictive potential for prognosis in hematological malignant neoplasms (**Figure S8**).

In conclusion, *via* integrated analyses of scRNA-seq and bulk RNA-seq data for BCP-ALL, we presented a comprehensive landscape of the autocrine crosstalk network of neoplastic B cells and the paracrine communication network between B cells and myeloid cells. Based on the significant ligand–receptor pairs, a LASSO regression model was built to predict the prognoses for both pediatric and adult patients. These identifications shed light on BCP-ALL pathogenesis and have the potential to improve the clinical diagnosis for BCP-ALL patients.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134759.

AUTHOR CONTRIBUTIONS

JH conceived, designed, and supervised the study with WZ, LW, and YD. LW collected and analyzed data, wrote the draft of the manuscript. MJ, PY, JL, WO, WZ, and CF analyzed the data and reviewed the manuscript. JH and YD oversaw the bioinformatics data analyses and modified and improved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.639013/full#supplementary-material

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Post-Transformation IGHV-IGHD-IGHJ Mutations in Chronic Lymphocytic Leukemia B Cells: Implications for Mutational Mechanisms and Impact on Clinical Course

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Edited by:
Jérôme Paggetti, Luxembourg Institute of Health, Luxembourg

Reviewed by:
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Christopher C. Oakes, The Ohio State University, United States

***Correspondence:**
Nicholas Chiorazzi
NChizzi@Northwell.edu

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*Davide Bagnara[1,2], Catherine Tang[3], Jennifer R. Brown[4], Siddha Kasar[4], Stacey Fernandes[4], Monica Colombo[5], Stefano Vergani[1], Andrea N. Mazzarello[1], Fabio Ghiotto[2,5], Silvia Bruno[2], Fortunato Morabito[6,7], Kanti R. Rai[1,8], Jonathan E. Kolitz[1,8], Jacqueline C. Barrientos[1,8], Steven L. Allen[1,8], Franco Fais[2,5], Matthew D. Scharff[9], Thomas MacCarthy[3] and Nicholas Chiorazzi[1,8]**

[1] The Feinstein Institutes for Medical Research, Institute for Molecular Medicine, Northwell Health, Manhasset, NY, United States, [2] Department of Experimental Medicine, University of Genoa, Genoa, Italy, [3] Department of Applied Mathematics and Statistics, State University of New York at Stony Brook, Stony Brook, NY, United States, [4] Chronic Lymphocytic Leukemia Center, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, United States, [5] Molecular Pathology, IRCCS Ospedale Policlinico San Martino, Genoa, Italy, [6] Biotechnology Research Unit, Azienda Ospedaliera of Cosenza, Cosenza, Italy, [7] Hematology and Bone Marrow Transplant Unit, Hemato-Oncology Department, Augusta Victoria Hospital, East Jerusalem, Israel, [8] Department of Medicine, Zucker School of Medicine at Hofstra/Northwell, Hempstead, NY, United States, [9] Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY, United States

Analyses of IGHV gene mutations in chronic lymphocytic leukemia (CLL) have had a major impact on the prognostication and treatment of this disease. A hallmark of IGHV-mutation status is that it very rarely changes clonally over time. Nevertheless, targeted and deep DNA sequencing of IGHV-IGHD-IGHJ regions has revealed intraclonal heterogeneity. We used a DNA sequencing approach that achieves considerable depth and minimizes artefacts and amplification bias to identify IGHV-IGHD-IGHJ subclones in patients with prolonged temporal follow-up. Our findings extend previous studies, revealing intraclonal IGHV-IGHD-IGHJ diversification in almost all CLL clones. Also, they indicate that some subclones with additional IGHV-IGHD-IGHJ mutations can become a large fraction of the leukemic burden, reaching numerical criteria for monoclonal B-cell lymphocytosis. Notably, the occurrence and complexity of post-transformation IGHV-IGHD-IGHJ heterogeneity and the expansion of diversified subclones are similar among U-CLL and M-CLL patients. The molecular characteristics of the mutations present in the parental, clinically dominant CLL clone (CDC) differed from those developing post-transformation (post-CDC). Post-CDC mutations exhibit significantly lower fractions of mutations bearing signatures of activation induced deaminase (AID) and of error-prone repair by Polη, and most of the mutations were not ascribable to those enzymes. Additionally, post-CDC

mutations displayed a lower percentage of nucleotide transitions compared with transversions that was also not like the action of AID. Finally, the post-CDC mutations led to significantly lower ratios of replacement to silent mutations in VH CDRs and higher ratios in VH FRs, distributions different from mutations found in normal B-cell subsets undergoing an AID-mediated process. Based on these findings, we propose that post-transformation mutations in CLL cells either reflect a dysfunctional standard somatic mutational process or point to the action of another mutational process not previously associated with IG V gene loci. If the former option is the case, post-CDC mutations could lead to a lesser dependence on antigen dependent BCR signaling and potentially a greater influence of off-target, non-IG genomic mutations. Alternatively, the latter activity could add a new stimulatory survival/growth advantage mediated by the BCR through structurally altered FRs, such as that occurring by superantigen binding and stimulation.

Keywords: chronic lymphocytic leukemia, immunoglobulin genes, somatic mutations, mutation mechanisms, activation-induced deaminase

INTRODUCTION

Chronic lymphocytic leukemia is a relatively common, yet broadly divergent disease (1, 2). Some patients survive for decades without therapy, while others succumb soon after diagnosis. At the molecular level, this heterogeneity is also prominent and linked to the clinical condition. Most relevant, the level of mutations in the IGHV genes expressed by leukemic clones segregate CLL patients into two groups (3), and patients fitting into these categories have divergent clinical courses and outcomes (4, 5). In general, those patients with leukemic clones expressing an IGHV with somatic mutations exceeding a defined threshold (IGHV-mutated, M-CLL) have better prognoses, whereas those patients whose leukemic clones express an IGHV without or with minimal numbers of mutations (IGHV-unmutated, U-CLL) experience more severe disease. For this reason, it is currently recommended that IGHV-mutation status be determined at the time of diagnosis to help physicians identify and more closely follow patients at greater risk (6).

The IGHV-mutation status used in the clinic is usually determined by standard Sanger DNA sequencing (7), and rarely changes at the clonal level over time. Because the sensitivity of the Sanger sequencing approach is low, the likelihood of detecting minor intraclonal variants differing in IGHV mutations is small and can be inconclusive. Despite this, by sequencing relatively large numbers of IGHV-IGHD-IGHJ molecular clones across a panel of patients (8, 9) or within a distinct stereotyped subset (10) or in single cells (11), intraclonal IGHV-IGHD-IGHJ heterogeneity has been found. This was subsequently confirmed when next generation, deep DNA sequencing (NGS) became available (12).

In addition to corroborating that intraclonal V-region diversity exists, the latter study highlighted several important considerations that could be incorporated into future studies of the process (12). These emphasized that the DNA sequencing approach employed needs to have considerable sensitivity ("sequencing depth") and contain sufficient safeguards to insure accuracy (e.g., avoidance of PCR and DNA sequencing artifacts). This was seen as essential since the number of cells with unique mutations that develop in the IGHV-IGHD-IGHJ sequence might be small. The authors also indicated the necessity to study a patient cohort with a large number of patients differing in IGHV-mutation status to determine if the generation of intraclonal IGHV-IGHD-IGHJ variants differed between U-CLL and M-CLL clones. Additionally, there should be enough unique reads to determine if the mutations identified bear the characteristics of somatic hypermutation (SHM) caused by activation-induced deaminase (AID) (13) and error prone mismatch repair (14), and if there is in silico evidence for antigen-selection of the sequence variants. Finally, the CLL cases studied should have an adequate period of follow-up to establish if the intraclonal process links with clinical course and outcome.

In light of this background, we have addressed the frequency and degree of intraclonal IGHV-IGHD-IGHJ heterogeneity in CLL, the extent that the subclones exhibiting this contribute to the clonal burden, the characteristics of the post-transformation mutations, and the association of intraclonal IGHV-IGHD-IGHJ diversity with clinical outcome. To tackle these issues, we used an NGS approach of considerable depth that incorporates Unique Molecular Identifiers (UMIs) to minimize technical artefacts and PCR amplification bias (15) to study the IGHV-IGHD-IGHJ regions in CLL clones from a cohort of well characterized, untreated patients, for whom clinical and laboratory features and temporal follow-up were available.

Our study extends previous findings and documents that: the intraclonal IGHV-IGHD-IGHJ diversification process is virtually ubiquitous and takes place with equal frequency and extent in both U-CLL and M-CLL patients; certain subclones expressing new IGHV-IGHD-IGHJ mutations can be significantly expanded *in vivo*; the fraction of mutations bearing SHM marks is considerably less than in the parental clone and these are inconsistent with selection for enhanced antigen binding; and finally some of the features of the post-

transformation mutations might be useful adjuncts to the standard U-CLL vs. M-CLL IGHV mutation status prognostic approach.

METHODS

Samples

This study was approved by the Institutional Review Boards of Northwell Health and of Dana Farber Cancer Center. Written informed consent was obtained in accordance with the Declaration of Helsinki. PBMCs were isolated from untreated CLL patients by density gradient centrifugation (Ficoll, GE Healthcare), suspended in in RPMI1640 medium with 50% FBS, and stored in liquid nitrogen until used.

Cell Separation

PBMCs from 45 CLL samples collected at Northwell Health were incubated with V500 anti-CD19 (BD Biosciences) and PE-cy7 anti-CD5 (Invitrogen). After excluding dead cells using Sytox Blue (ThermoFisher), live $CD19^{+}CD5^{+}$ B cells were sorted directly into tubes containing Dynabeads Oligo(dT) (ThermoFisher) lysis buffer and stored at −80°C.

For the 15 CLL PBMC samples collected at Dana Farber Cancer Institute, B cells were isolated using RosetteSep negative selection prior to density gradient centrifugation if the white blood cell count was less than 25,000/ml or the absolute lymphocyte count was less than 20,000/ml.

Library Preparation and Sequencing

Library preparation and sequencing were performed as described (15) and illustrated in **Supplemental Figure S1**. Briefly, mRNA was isolated with Dynabeads Oligo (dT) in 200 µl PCR tubes as instructed for the Dynabeads macknowledRNA DIRECT Micro Kit (ThermoFisher). cDNA was synthesized in solid phase using SuperScript III Enzyme (ThermoFisher). Second strand synthesis was performed with a mix of IGHV leader sequence specific primers, and Unique Molecular Identifiers (UMIs) and Illumina adaptors were introduced at this point. Purified ds-cDNA was used as template to amplify, by a semi-nested approach, the entire IGHV-IGHD-IGHJ rearrangement and a portion of the constant region sufficient to identify IgM, IgG and IgA.

The product was quantified with Qubit (ThermoFisher) and 1–10 ng was used to add the Illumina Index with the Nextera XT kit (Illumina). Pooled libraries were sequenced with MiSeq Illumina (v3.2 × 300 kit, Illumina MS-102-3003). Raw data are available at SRA (BioProject ID PRJNA673787-https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA673787&o=acc_s%3Aa).

Bioinformatic and Statistical Analyses

Processing of raw reads was performed with pRESTO (16). Processed sequences were submitted to IMGT/HighV-QUEST. Data handling was carried out using ChangeO (17) and R. Prism 8 and R were used for statistical analyses. All box plots in the figures represent medians and quartiles. Non-productive IGHV-IGHD-IGHJ rearrangements were excluded from all analyses, except those dealing with mutation targeting and selection.

A Cox proportional hazards regression analysis was used to test mutation, complexity and their interaction (mutation × complexity) in the model. The "interaction" between mutation and complexity measures whether the effect of complexity in the *IGHV*-mutated group is the same or different from its effect in the *IGHV*-unmutated group. To visualize these results, the Kaplan–Meier curves for TTFT and survival were generated for the four groups ($M\text{-}CLL^{High}$, $M\text{-}CLL^{Low}$, $U\text{-}CLL^{High}$, $U\text{-}CLL^{Low}$). Pairwise multiple comparisons were carried out for differences among the four groups without adjustment for multiple testing. All results were considered statistically significant if $P < 0.05$.

Subclonal Expansion Sensitivity Threshold

For 27 samples, we sequenced four or eight replicates, each containing 25,000 cells from the same blood draw (**Supplemental Table S1**); replicates were used to calculate the sensitivity threshold to identify expanded subclonal variants. The approach is based on the principle that, if a subclonal variant is repeatedly observed in several sequencing replicates, it derives from expanded B cells with the identical IGHV-IGHD-IGHJ sequence. **Supplemental Figure S2** shows the frequency distribution of the subclones observed in at least ¾ of the replicates. We chose the 25th percentile (0.008%) of the distribution as a conservative threshold to identify expanded variants. This threshold corresponds to two cells in a 25,000 cell replicate; this number is possible because, as described (15), our library preparation and sequencing approach exhaustively determines the repertoire of all starting B cells. The calculated sensitivity threshold for subclonal amplification was then applied to all samples analyzed.

Clonal Inference and Phylogeny

For each sample, we identified the CLL clonal family with ChangeO (17), using a distance threshold of 0.07. Since the repertoire diversity of $CD5^{+}$ B cells in a CLL patient is very low, we were able to perform a "manual" assessment of the clonal attribution to correct possible false positives, false negatives, and PCR artifacts such as chimeras that might have escaped error correction. The phylogenetic tree for each CLL clonal family was inferred with the R package Alakazam (18), and the trees were plotted with igraph (Csardi G, Nepusz T. 2006. The igraph software package for complex network research. InterJournal, Complex Systems, 1695. https://igraph.org).

Mutation Targeting Analysis

We used the dominant, consensus IGHV-IGHD-IGHJ sequence found for each patient as the clinically-dominant clone (CDC), and used this as the reference for all variant mutation calls ("post-CDC mutations"). In order to evaluate whether mutations in the post-CDC sequences were targeted to hotspots or not, we assumed that the mutations occurred in the CDC sequence background. For each sample and category, we added up the mutations from each unique sequence, then considered the context in which they occurred (hotspot or non-hotspot). To evaluate mutational

enrichment to AID (WRC/GYW) hotspots, we compared the ratio (h:n) of mutations targeting hotspots (h) to non-hotspots (n) to the expected proportion (number of hotspots/number of G:C sites) using a binomial test (19). Benjamini–Hochberg correction was then applied at the sample level. Using the same method, we also considered hotspots for APOBEC3A/B (TC/GA) and Polη (WA/TW), where the expected proportion used A:T sites in denominator); these are similar to Signatures 2 and 13 and to Signature 9, respectively, as defined in the Catalogue of Somatic Mutations in Cancer (COSMIC) database [Tate et al. (20)] (cancer.sanger.ac.uk/cosmic). Finally, we compared the observed mutations to those ascribed by the COSMIC database to Signatures 1 ("Aging signature") and 5 ("Cancer signature"). Signature 1 is the result of an endogenous mutational process initiated by spontaneous deamination of 5-methylcytosine (N(C>T)G), and Signature 5 exhibits transcriptional strand bias for T > C substitutions at ApTpN context. One-sided tests were used to evaluate higher targeting to hotspots. Finally, those sequences representing a non-productive IGHV-IGHD-IGHJ rearrangement, which were present at a frequency of 0.008% (25th percentile of the intraclonal subclone distribution used to define subclonal amplification) and hence more likely derived from CDC subclones (n = 17), were used in mutation analysis as mutations that could not undergo antigen selection.

Selection Analysis

The selection pressure on mutations in the VH Complementarity Determining Regions (CDRs) and Framework Regions (FRs) was calculated using the Bayesian estimation of Antigen-driven SELectIoN (BASELINe) (21, 22). This algorithm compares observed and expected mutations estimated with an Ig-specific targeting model of SHM and calculates the posterior probability density function represented in the plots.

RESULTS

Approach

We carried out NGS of full length IGHV-IGHD-IGHJ rearrangements in 62 CLL patients to define intraclonal variants differing in cDNA sequences from those found in the CDC. We define the CDC as the overriding IGHV-IGHD-IGHJ sequence determined by Sanger sequencing of circulating CLL cells and confirmed by NGS. In some cases, additional subclones, unrelated to the CDC based on VH CDR3 sequence differences, were identified; these were excluded from the following analyses.

We used our sequencing methodology that employs Unique Molecular Identifiers (UMIs) to label each mRNA molecule and high sequencing depth, thereby improving error correction capacity, attainment of high quality sequences, and enhanced sequencing sensitivity (15) (**Supplemental Figure S1**). In addition, to minimize the artificial diversity inevitably arising from errors in DNA sequencing and PCR, here we have also included only those unique sequences that were observed with ≥3 distinct UMIs (corresponding to three different mRNA molecules sequenced) and that came from the consensus of ≥5 reads. Thus, this is a very high bar for error correction and filtering and further avoids misinterpretations based on inaccuracies or randomness. Using these cutoffs, on average for each sample 2.3 million reads met our filtering criteria (effective "sequencing depth" = 2,300,000×) carrying 0.4 million distinct UMIs representing 540 unique nucleotide sequences (intraclonal subclones) for an individual CLL IGHV-IGHD-IGHJ rearrangement. Details of each sample are provided in **Supplemental Table S1**. All frequencies reported below refer to the abundance of mRNA molecules sequenced as quantified by UMIs.

Detection of Intraclonal IGHV-IGHD-IGHJ Rearrangement Variants in Patients With CLL

We defined an "intraclonal IGHV-IGHD-IGHJ subclone" as an IGHV-IGHD-IGHJ sequence that is clonally-related to the CDC based on HCDR3 similarity and use of the same IGHV and IGHJ genes but differing from the CDC by at least one nucleotide somewhere in the entire IGHV-IGHD-IGHJ region. These clonally related progeny downstream of the CDC are hereafter referred to as post-CDC intraclonal subclones. Despite the stringent error correction and filtering of our approach, all but one patient sample (61/62, 98%) bore IGHV-IGHD-IGHJ rearrangements differing in sequence from the CDC. The one case in which significant variants were not detected had much lower sequencing depth compared with the average (194,000× vs. 2,300,000×). **Figure 1A** provides representative examples of the types of differences uncovered; these changes reflect not only the number of IGHV-IGHD-IGHJ mutations that developed post-transformation but also the complexity of that new mutational load, illustrated by the existence and extent of branching found in certain cases.

Considering all patient samples, the median frequency of IGHV-IGHD-IGHJ intraclonal subclones differing from the CDC was 8% (distribution 0–34%) (**Figure 2A**). The most abundant intraclonal subclone ("predominant subclone", PSC) was defined by the percentage of the total defined IGHV-IGHD-IGHJ repertoire that the PSC represented. This was estimated to be on average 0.6%, ranging in frequency from 0 to 29% (**Figure 2B**). Additionally, we calculated the PSC frequency among only the members of each subclone whose sequences differed from that CDC. This ranged from 1.8–88% with a median of 9% of the variant fraction (**Figure 2C**).

Finally, after dividing the samples based on IGHV-mutation status, both M-CLL and U-CLL clones exhibited similar frequencies of intraclonal variants (**Figure 2A**). Moreover, the percentage of the variants represented by the PSC was not significantly different between the two CLL subtypes (**Figure 2B**). When the CDC were excluded, the average frequency of unique sequences did not differ between M-CLL and U-CLL cases (**Figure 2C**).

In summary, IGHV-IGHD-IGHJ sequences differing from that of the CDC exist within virtually all CLL clones (~98% here). Additionally, when focusing on the PSCs, the ratio of these intraclonal variant sequences varies among patients. Although in most instances the ratio is relatively low, at times it can be sizeable. Notably, each finding is similar in

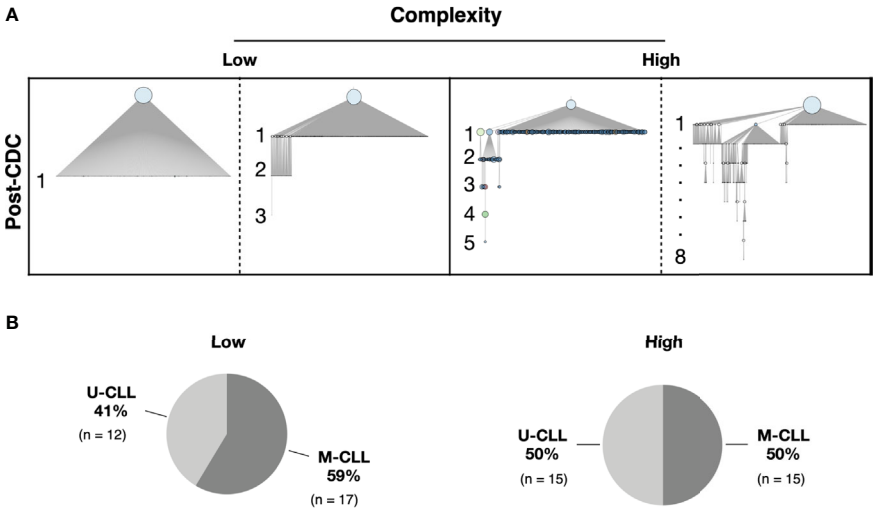


FIGURE 1 | Complexity of intraclonal IGHV-D-J variants. **(A)** Examples of the degree of intraclonal diversity in CLL samples based on NGS sequencing data. The phylogenetic trees were inferred with the R package Alakazam and plotted with igraph (see *Methods*). The size of the CDC (light blue circles at top of each example) is proportional to its abundance within the entire leukemic clonal family. IGHV-D-J complexity is defined and represented by the number of distinct sequences downstream of the CDC. Each downstream sequence has all the mutations of the upstream sequence plus at least one additional. Low complexity contains one to three mutation-defined sequences downstream of the initial branch from the CDC. High complexity represents four or more unique sequences downstream of each initial branch, some involving intricate branching. **(B)** Pie graphs indicating the representation of U-CLL and M-CLL cases in the Low and High complexity categories.

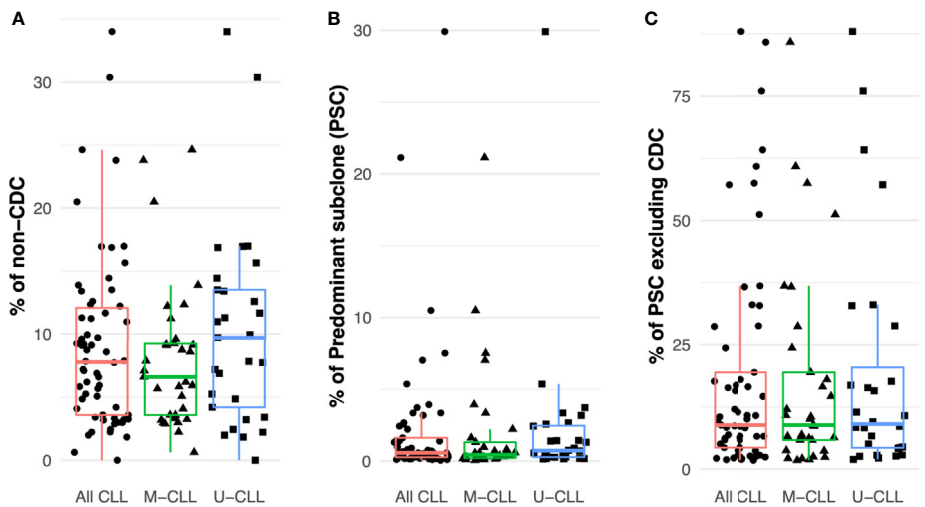


FIGURE 2 | Intraclonal IGHV-D-J diversity is relatively common in CLL. **(A)** Fraction of the intraclonal IGHV-D-J variant sequences among the total number of mRNA molecules coding the CLL clonal family in the 61 patients examined. **(B)** Frequency of PSCs among the total number of mRNA molecules encoding the CLL clonal family. **(C)** Fraction of the subclonal IGHV-D-J variants that the PSCs represent after excluding the CDC sequences.

M-CLL and U-CLL, indicating that these intraclonal phenomena occur in all CLL clones, regardless of IGHV-mutation status.

Expansion of Subclones Downstream of the CDC

Next we determined the relative sizes of the CLL subclones by assigning a sensitivity threshold to minimize the likelihood of random sampling of sequences carried by expanded B cells. This was possible because when CLL cells were sorted, for 28 samples at least four aliquots of 25,000 cells were collected (**Supplemental Table S1**), and each aliquot was sequenced separately. Using the data from these replicates, we calculated the frequency distribution of the unique IGHV-IGHD-IGHJ sequences found in at least 75% of the aliquots (*Methods* and **Supplemental Figure S2**), and used the 25th percentile of the

distribution (0.008%) as a cut-off for B-cell expansion. **Figure 3A** shows that all post-CDC PSCs are well above this cut-off.

Next, using the absolute number of lymphocytes in each patient's blood sample, we inferred the numerical size of individual subclones in the blood. This calculation revealed that almost all PSCs exceeded a count of 10 cells/μl, in comparison to the median count of the entire set of subclones which was 1.5 cells/μl (distribution 0.01–3,616 cells/μl; **Figure 3B**). **Figure 3C** illustrates representative examples of the calculated size of the PSC compared to the CDC and to the other IGHV-IGHD-IGHJ intraclonal subclones.

Thus, many of the intraclonal subclones, especially the most frequent fraction (PSC), are numerically expanded, although the relative sizes vary. Therefore, the PSCs appear to be "advantaged" in some way.

Extent of Intraclonal Complexity Occurring Downstream of the CDC

The previous analyses were based solely on the presence or absence of IGHV-IGHD-IGHJ mismatches from the CDC sequence. Next, we divided the cases by the extent of intraclonal complexity/architecture using branching from the CDC. Fifty-nine of the samples were included in this analysis; for the three cases excluded, no subclones were found in one, and the software used could not construct phylogenetic intraclonal IGHV-IGHD-IGHJ trees for the other two.

To perform this analysis, we partitioned the samples into two complexity categories. The "Low" group contains one to three mutations downstream of the initial branch from the CDC (see **Figure 1A** for examples). The "High" group exhibited four or more unique sequences downstream of each initial branch (**Figure 1A**). Of the 59 patients, 29 fell into the Low category

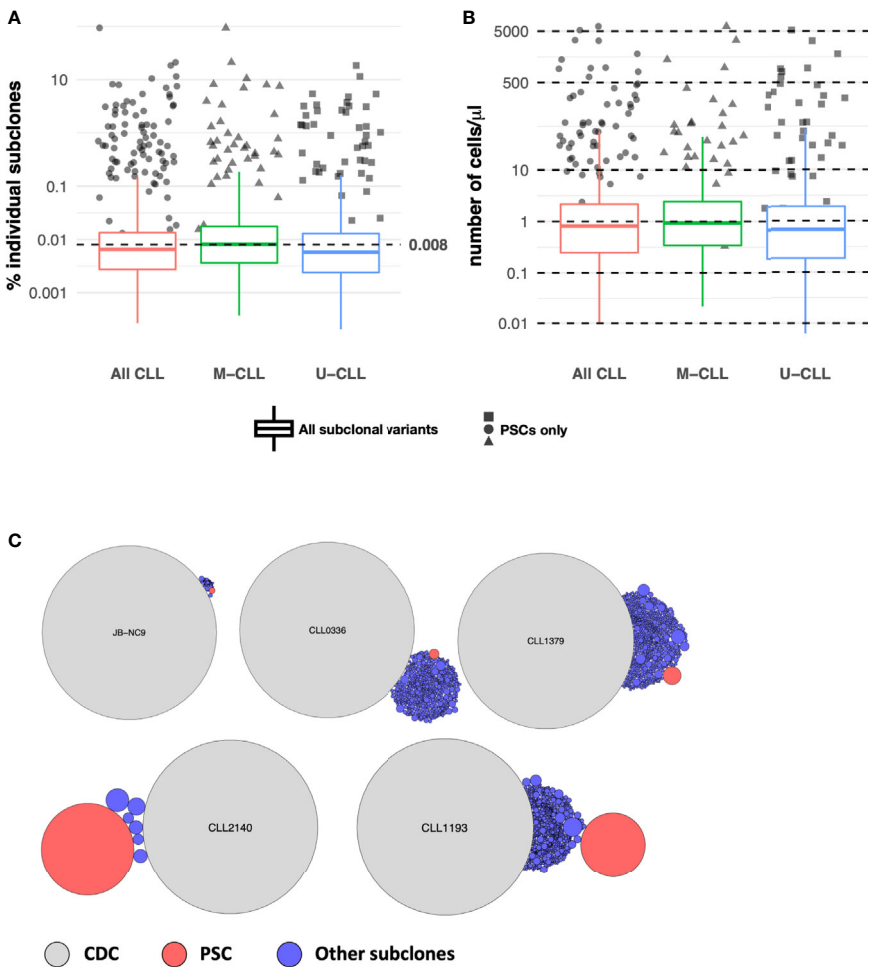


FIGURE 3 | Subclonal variant cell counts. **(A)** PSCs are numerically expanded. Box plots indicate the percentage distribution of all post-CDC subclones. The solid colored dots represent the percentages of individual PSCs. The dotted horizontal line indicates the 0.008% sensitivity threshold used to define subclonal expansion (see *Methods*). **(B)** Absolute numbers of distinct IGHV-D-J subclones per ul of individual patient blood samples estimated from sequencing data, flow cytometry, and absolute blood lymphocyte counts. Box plots and solid colored dots as in **(A)**. **(C)** Representative examples of the relative numbers of the post-CDC subclones (blue circles) and the PSCs (red circles) in relation to the CDC of five samples analyzed in this study.

and 30 into the High category (**Figure 1B**). Notably, the U-CLL and M-CLL patients were relatively uniformly distributed based on complexity levels (Low complexity—12 M-CLL and 17 U-CLL; High complexity—15 M-CLL and 15 U-CLL; **Figure 1B**).

Thus, U-CLL and M-CLL clones display intraclonal differences at similar frequencies, and the presence and extent of post-CDC complexity are very similar among them as well.

Characterization of Mechanisms Inducing and Repairing the Post-CDC IGHV-IGHD-IGHJ Mutations

Next, we analyzed the specific post-CDC IGHV-IGHD-IGHJ rearrangements for evidence that the mutations were the consequences of mutational mechanisms associated with SHM (targeting AID hotspots and avoiding AID coldspots [SYC: s (G/C; C/T; C) (23)] or bearing features of error prone repair by Polη. We also looked for mutational signatures found outside IG loci as listed in COSMIC v86 (20), focusing on Signatures 1 (Aging), 2 and 13 (APOBEC) and 5 (Cancer). The mutation signatures for these are listed in *Methods*.

Notably, although there were some sequences bearing APOBEC and the other COSMIC signatures, none of the samples as a whole bore significant features consistent with these mechanisms (not shown). As expected, AID and Polη signatures were identified. Twenty-nine percent of the samples exhibited significant AID targeting, with no significant differences between U-CLL and M-CLL (**Figure 4A**). When incorporating the degree of intraclonal IGHV-IGHD-IGHJ complexity into the analysis, samples with significantly more High complexity displayed significantly more AID targeting compared with Low complexity samples (**Figure 4B**), with M-CLL cases being the majority (**Figures 4C, D**). When examining Polη-related mutations, many fewer samples exhibited significant targeting (3.4%) (**Figure 4E**), and all of these were from U-CLL cases with High complexity (**Figure 4H**). No M-CLL and no U-CLL with Low complexity exhibited significant Polη signatures; hence the findings were not obviously influenced by the degree of IGHV-IGHD-IGHJ complexity (**Figures 4F, G**). So, the dominant identifiable mutational signature found in the post-CDC samples is AID-related. Nevertheless, this represented only ~25% of the samples.

Next, we carried out similar analyses to determine if the mutational targeting found in the parent CDCs was like that found in the post-CDC samples and how these compared with those in non-productive IGHV-IGHD-IGHJ rearrangements found within the same patients. Notably, for the non-productive sequences, AID mutations accounted for 33% of the CDC mutations (targeting hotspots: 21%; avoiding coldspots: 12%) and Polη for 20% (**Figure 4I**). For the parental CDC, AID mutations accounted for a significantly greater level (35%) (hotspot: 30%; coldspot: 5%) and Polη for 26% (**Figure 4I**). In our CDC cohort, 13 samples did not bear *IGHV* mutations. Therefore, to assure that these findings were not influenced by the number of samples analyzed (n = 49), we also used an extended database containing 2,084 CLL IGHV-IGHD-IGHJ CDC sequences. When employing the larger data, still no samples bore significant evidence for APOBEC and COSMIC Signatures 1 and 5.

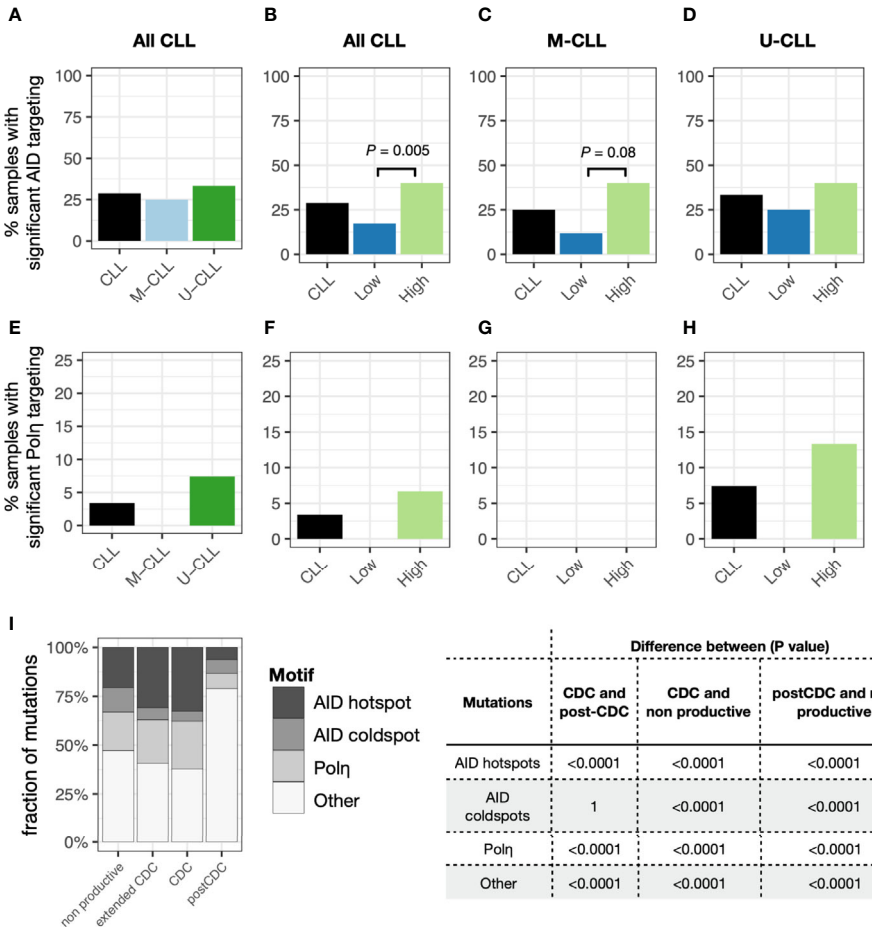
Next, we compared the distribution of mutations in the non-productive rearrangements and in the CDC sequences with those in the post-CDC (**Figure 4I**). This illustrated a striking change in the attribution of mutations to SHM between the CDC and post-CDC periods. Specifically, there were highly significant decreases in targeting of AID hotspots and in mutations resembling Polη action in relation to both the non-productive and productive (CDC) rearrangements. Additionally, there were highly significant increases of mutations targeting AID coldspots. Consequently, there was a very significant increase in the fraction of mutations that could not be assigned to SHM (**Figure 4I**).

Since AID induced mutations usually result in more base transitions (Ts) than transversions (Tv), usually ~1.5 Ts to 1 Tv (24–27), we analyzed this parameter as well. Overall, the median Ts : Tv for the post-CDC mutations grouped by sample was 0.80 (**Figure 5A**). Notably, this ratio was significantly less than that of the 49 CDC samples with IGHV mutations (median = 1.2; P = 0.0012) and of the IGHV-mutated cases in the extended CDC set (median = 1.3; P <0.0001). When analyzing the degree of IGHV-IGHD-IGHJ complexity to the post-CDC mutations, the High complexity samples had a significantly lower Ts : Tv than the Low complexity samples (0.79 *vs.* 1.09; P <0.01; **Figure 5B**). When analyzing those mutations occurring only at AID hot spots (**Figure 5C**), the Ts : Tv was 0.92, and no difference was seen based on IGHV-IGHD-IGHJ complexity (**Figure 5D**). Finally in the non-productive rearrangements, the Ts : Tv was 0.63 (**Figure 5E).** So, whereas the CDC samples bearing IGHV mutations have Ts : Tv similar to that expected, the post-CDC samples as well as the non-productive sequences have ratios very significantly lower than these.

In summary, these analyses suggest that the majority of mutations occurring in the post-CDC period are much less attributable to AID based SHM based on their type and targeting and the repair mechanisms employed than those mutations that developed in the CDC.

Analyses of Selection Among the Post-CDC IGHV-IGHD-IGHJ Mutations

Finally, we asked if there was evidence for antigen selection in the post-CDC mutations using the BASELINe algorithm that detects and quantifies mutation selection using large datasets such as those generated here by NGS (**Figure 6**). Of note, the program extends the traditional approach based on replacement to silent ratio (R:S), refining it by considering the intrinsic mutational biases observed for SHM. The Bayesian approach used also quantifies the uncertainty for each estimate. Specifically, the program provides a distribution that measures "Selection Strength" for a set of sequences, together with an associated P value. The selection strength measure is analogous to the standard R:S ratio, with positive values indicating greater than expected R mutations, and negative values indicating fewer than expected R mutations. In an adaptive immune response, therefore, an increase in R:S would be expected in the VH CDRs, leading to a structural change in these regions and implying positive selection by antigen and a decreased R:S



Mutations	Difference between (P value)		
	CDC and post-CDC	CDC and non productive	postCDC and non productive
AID hotspots	<0.0001	<0.0001	<0.0001
AID coldspots	1	<0.0001	<0.0001
Polη	<0.0001	<0.0001	<0.0001
Other	<0.0001	<0.0001	<0.0001

FIGURE 4 | Relative targeting of mutational mechanisms. **(A–D)** Percentage of samples exhibiting significant targeting to AID hot spots in subclones, based on IGHV-mutation status **(A)**, IGHV-mutation status + level of IGHV-D-J complexity for all CLL samples **(B)** or for only M-CLL **(C)** or only U-CLL cases **(D)**. **(E–H)** Percentage of samples exhibiting significant features of Polη repair in subclones, based on IGHV-mutation status **(E)**, IGHV-mutation status + level of IGHV-D-J complexity for all CLL samples **(F)** or for only M-CLL **(G)** or only U-CLL cases **(H)**. **(I)** Fraction of post-CDC mutations attributable to the actions of AID at hot spots (red), AID at cold spots (green), of Polη repair (blue), or other/unclear (purple). For each motif type (AID hotspot, AID coldspot, Polη hotspot, all others), we calculated the fraction of mutations targeting the motif. Within each sample, this was done separately for the CDC, post-CDC, and non-productive sequences, giving a pair of fractions per sample. After calculating pairs for each sample, then CDC, post-CDC and non-productive sequences were compared across all samples using a paired t-test. Table to the right indicates the mean fractional differences of mutations between the CDC and the post-CDC settings in regard to mutations targeting the three motifs and not attributable to the any of the three.

would be expected in the VH FRs, implying negative selection and hence avoidance of structural changes in the Ig as a whole. Thus, true underlying selection for antigen is reflected by a structurally intact Ig with positive selection strength values in CDRs and negative values in FRs.

Since the normal cellular equivalent of a CLL cell is still a matter of debate (28–31), we used a database containing IGHV-IGHD-IGHJ sequences from several normal B-cell types generated with the same methodology, i.e., memory B cells (isotype-switched, sMem, and IgM only, mMem), marginal zone B cells (MZ), and $IgD^{-}CD27^{-}$ ("double negative", DN) B cells, as controls (32) (**Figure 6**). DN B lymphocytes are a memory population that exhibits IGHV mutations (33), albeit at a level less than classical memory cells, that are expanded in autoimmune settings (34) and upon aging (35).

As expected, both memory B cell subsets displayed significantly positive selection strength in the CDRs and significantly negative values in the FRs. The DN B cell subset exhibited similar findings. The extended group of CDC sequences showed a significantly reduced selection strength in the CDRs than memory and DN cells, with some evidence of negative selection (broadly, silent mutations outnumbering replacement mutations). This pattern resembled that of MZ B cells. However, the selection strength in the FRs was like the mMem, sMem, and DN subsets. In conspicuous contrast, there was a striking difference for the post-CDC mutations, both High and Low complexity, in that there was a markedly reduced and negative selection in the CDRs and positive selection strength for changes in the FRs. Finally, the IGHV-IGHD-IGHJ non-productive rearrangements bear the least evidence for VH

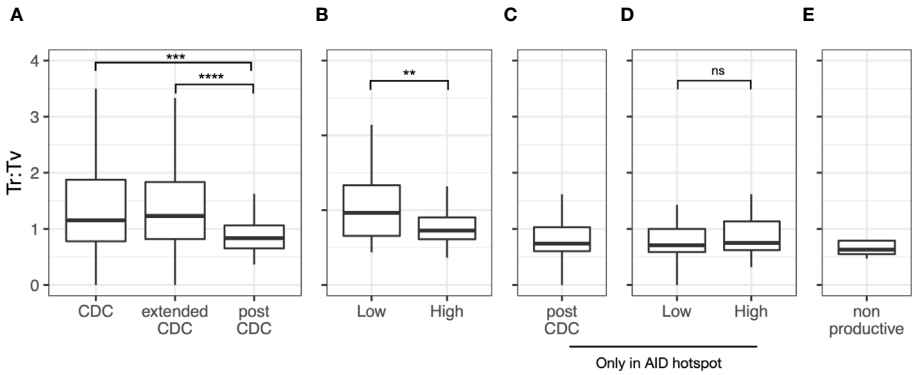


FIGURE 5 | Transition to transversion ratios in the post-CDC mutations. Transition to transversion ratios (Ts : TV) analyzed for: **(A)** all post-CDC mutations, **(B)** all post-CDC mutations based on levels of complexity, **(C)** only those post-CDC mutations involving AID hotspots, **(D)** post-CDC mutations involving AID hotspots based on levels of complexity and **(E)** non-productive IGHV-IGHD-IGHJ rearrangements (**$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$). NS, Not statistically significant.

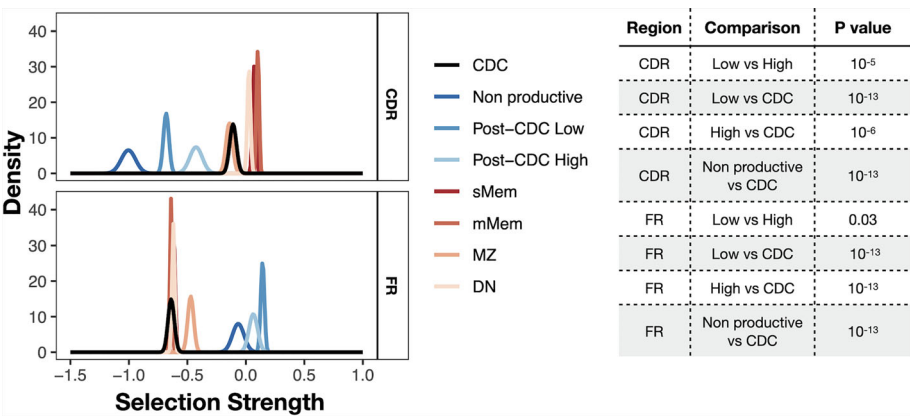
CDR selection for mutations but a selection for VH FR mutations somewhat less than that for the post-CDC mutations.

Together, the R:S in the VH CDRs and FRs of the post-CDC mutations are categorically inconsistent with selection for enhanced antigen binding as a reason for the distribution of non-synonymous mutations.

IGHV-IGHD-IGHJ Heterogeneity and Mutation Extent and Targeting Have Implications for Patient Clinical Courses

Since the presence or absence of significant numbers of IGHV gene mutations has proven to be an excellent prognostic indicator, we asked if intraclonal differences in IGHV-IGHD-IGHJ sequence measured throughout the entire rearrangement might provide additional information. For the 61 cases that displayed intraclonal heterogeneity, neither the percentage of intraclonal IGHV-IGHD-IGHJ variants nor the percentage of the PSCs, including or excluding the CDC, correlated significantly with time to first treatment (TTFT) (**Supplemental Figure S3**).

When the clinical course (TTFT) of the cohort was analyzed using Cox regression, no significant interaction between mutation and complexity was observed ($P = 0.22$). Accordingly, when the interaction term was deleted from the model and the cases were divided into the U-CLL and M-CLL subgroups, there was, as expected a significant difference in TTFT between U-CLL and M-CLL ($P = 0.0125$; **Figure 7A**).



Region	Comparison	P value
CDR	Low vs High	10^{-5}
CDR	Low vs CDC	10^{-13}
CDR	High vs CDC	10^{-6}
CDR	Non productive vs CDC	10^{-13}
FR	Low vs High	0.03
FR	Low vs CDC	10^{-13}
FR	High vs CDC	10^{-13}
FR	Non productive vs CDC	10^{-13}

FIGURE 6 | Selection strength on mutations in VH CDRs and the VH FWRs. The BASELINe program was used to calculate Selection Strength (Σ; see *Methods*) starting from observed and expected replacement to silent mutation ratios (R:S). Selection strength of mutations found in CDC and post-CDC productive and non-productive rearrangements based on complexity (post-CDC^{High} and post-CDC^{Low}) as well as those of a series of normal human mature B cell subsets (IgM memory cells, mMem; isotype-class switched memory cells, sMem; marginal zone B cells (MZ); and IgD^{-}CD27^{-} double negative B cells (DN). Σ indicates selection strength favoring selection for (right of 0) or against (left of 0) replacement mutations. Table to the right indicates the statistical analyses for selected differences between comparisons in the VH CDRs and VH FRs involving mutations detected in CDC and post-CDC productive and non-productive sequences divided based on post-IGHV-IGHD-IGHJ diversity into High and Low as described in **Figure 1**. All comparisons were significantly different except for the following: for CDR: CDC *vs*. MZ; mMem *vs*. sMem; and sMem *vs*. DN; for FR: post-CDC^{High} *vs*. post-CDC^{Low}; post-CDC^{High} *vs*. non-Productive; CDC *vs*. mMem; CDC *vs*. sMem; CDC *vs*. DN; mMem *vs*. sMem; mMem *vs*. DN; sMem *vs*. DN. A complete list of statistical comparisons can be found in **Supplemental Table S2**.

However, when the cohort was divided based on IGHV-IGHD-IGHJ complexity (High and Low), TTFT was not different between the groups ($P = 0.15$; **Figure 7B**). Although we did not find evidence for a significant interaction between the IGHV-mutation groups and the complexity groups, those U-CLL patients in the High complexity group (U-CLL^{High}) had a significantly shorter TTFT compared to both the M-CLL^{Low} ($P < 0.0102$) and M-CLL^{Hi} ($P < 0.008$) complexity groups (**Figure 7C**). Moreover, the estimates of the hazard ratios for U-CLL^{High} *vs.* U-CLL^{Low} and M-CLL^{High} *vs.* M-CLL^{Low} we were 2.59 and 1.02, respectively. This numerically suggests an interaction effect, i.e., that complexity is associated with TTFT in the U-CLL group but not in the M-CLL group). See **Supplemental Information** for details of the analyses.

Additionally, there was no significant mutation × complexity interaction ($P = 0.7$) for OS. Although the estimates of the hazard ratios for U-CLL^{High} *vs.* U-CLL^{Low} and M-CLL^{High} *vs.* M-CLL^{Low} appeared to differ numerically (0.40 and 0.62, respectively), the insignificant *P*-values, wide confidence intervals, and lack of pairwise differences between groups do not support a mutation × complexity interaction for OS.

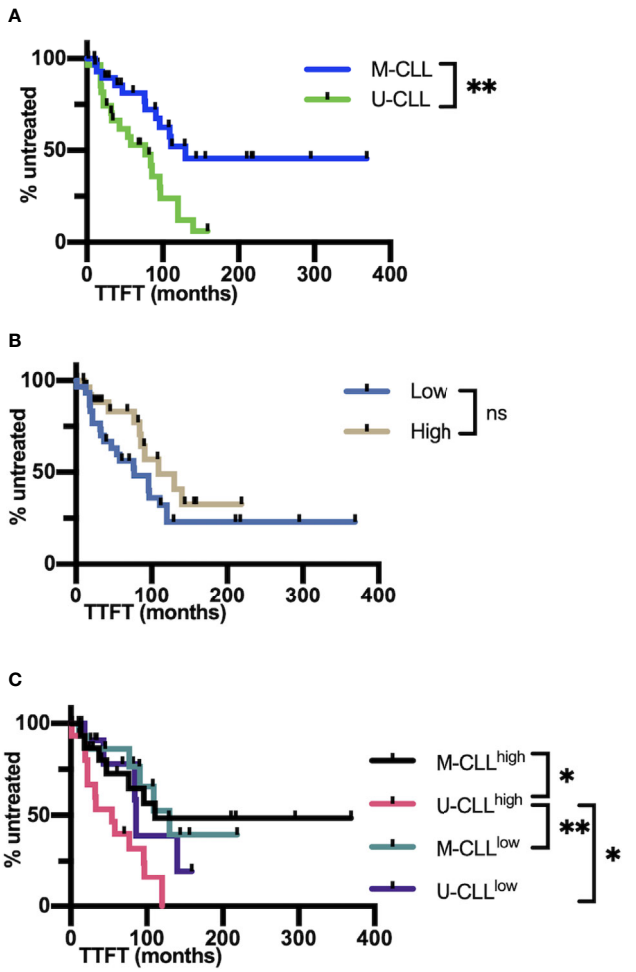


FIGURE 7 | IGHV-D-J complexity correlates with clinical course based on time to first treatment. **(A)** Time to first treatment (TTFT) in months of the patients falling into the *IGHV*-mutated (M-CLL; blue) and *IGHV*-unmutated CLL subsets (U-CLL; green). **(B)** TTFT in months of the patients falling into the post-CDC complexity categories, Low and High. **(C)** TTFT (months) of the patients based on a combination of IGHV-mutation status plus the post-CDC complexity categories, U-CLL^{High}, U-CLL^{Low}, M-CLL^{High}, and M-CLL^{Low}. Statistics are displayed only when *P-value* ≤0.05 (**P-value* ≤0.05, ***P-value* ≤0.01). NS, Not statistically significant.

DISCUSSION

We have examined the frequency and characteristics of intraclonal IGHV-IGHD-IGHJ diversification occurring in the progeny of the CLL clones defined in the clinic by Sanger sequencing. This has been a question of interest for several decades, and the answer that has slowly but progressively emerged as DNA sequencing technologies have become more sensitive and precise is that such diversification occurs. Using an NGS approach that achieved an average sequencing depth of 2,300,000× and allowed improved error correction due to the use of UMIs, we addressed the problem in 62 well characterized, untreated CLL patents. Our approach indicated that IGHV-IGHD-IGHJ sequence diversity occurs in virtually every CLL clone (~98%), with an average of 540 clonal variants observed per sample.

In addition to the surprising frequency at which intraclonal heterogeneity occurred, the size that the PSC can achieve in a given patient was also unexpected. When comparing the frequency of individual PSCs to the other intraclonal sequences (excluding the CDC), this reached 9% and was much larger (up to 88%) in some patients. Additionally, when using the absolute lymphocyte count in a patient's blood at the time of sample collection to calculate numerical size, the PSC was almost always ≥10 cells/μl and could reach >3,600 cells/μl. In a healthy person, the latter level of clonal B cells would be consistent with monoclonal B-cell lymphocytosis (36), a documented pre-CLL disease (37). Moreover, the level of complexity of IGHV-IGHD-IGHJ intraclonal diversification was unanticipated, ranging from a single mutation to a series of mutations, often with several and occasionally multiple branch points.

Finally, the presence, frequency, and extent of post-transformation IGHV-IGHD-IGHJ mutations occurred equally in U-CLL and M-CLL. This indicates that the initial mutation load does not affect the ability of CLL clones to develop IGHV-IGHD-IGHJ diversity after leukemia develops. The finding also suggests strongly that the lack of mutations found in a U-CLL clone is not due to an inherent inability to carry out SHM. Rather, it strongly implies that the absence/scarcity of IGHV mutations in the normal B lymphocyte that converted to a CLL cell was dictated by signals delivered to the cell *via* the microenvironment prior to transformation.

These findings also suggested that when individual IGHV-IGHD-IGHJ subclones are found in larger numbers,

exemplified by the PSCs, these cells bear a biologic advantage. We, therefore, analyzed the characteristics of the mutations occurring in the post-CDC setting, trying to define the mutation machinery responsible for the downstream mutations. We focused not only on mechanisms involved in targeting the IG loci, and thereby responsible for the generation of antigen-binding diversity in normal B lymphocytes, but also on mechanisms that could account for mutations found outside IG loci in CLL clones. The former included AID and error prone Polη, and the latter APOBEC and Signatures 1 and 5 in the COSMIC database. Although certain clones significantly expressed AID and/or Polη characteristics, none significantly exhibited signatures in the IGHV-IGHD-IGHJ region consistent with the actions of APOBEC or COSMIC Signatures 1 and 5. The approach also revealed that the percent of the post-CDC mutations within a given sample that could be assigned to the actions of AID and Polη was decreased very significantly when transitioning from the CDC to the post-CDC settings, suggesting that most of the mutations occurring post-leukemic transformation were not the consequences of an AID/Polη mechanism(s).

Because of these findings, we carried out similar analyses on the CLL sequences in the CDC set. Since not all the CDC samples bore IGHV somatic mutations (49 of 62), we also scrutinized a larger database of CLL clones comprised of 2,084 sequences. Both the CDC set directly related to this study and the expanded CLL set revealed the same features as the post-CDC mutations, with samples exhibiting significance for AID and Polη (AID > Polη), and none for the mutation mechanisms that affect non-IG loci in CLL. However, the percentage of mutations attributable to AID and Polη was very significantly higher in both CDC sets than for the post-CDC mutations, confirming the impression that the influence of AID/Polη mechanisms decreased substantially after leukemic transformation. When including comparisons of mutations detected in clonally expanded, non-productive IGHV-IGHD-IGHJ rearrangements, these mutations bore less evidence for AID and Poln actions than the CDC but more than the post-CDC mutations.

Since AID and Polη are essential and important components of SHM, respectively, we also investigated another feature of SHM, the ratio of mutations that represent transitions to transversions. In the normal SHM setting, the Ts : Tv is ~ 1.5:1. Notably, the Ts : Tv for all post-CDC mutations, productive or not, was considerably lower than this, and when the degree of mutational complexity was added to this analysis the High complexity samples had a more significantly lower Ts : Tv than the low complexity samples. Notably, when restricting the analysis to mutations bearing an AID targeting signature, the Ts : Tv was still below that expected for SHM.

Finally, we investigated if the post-CDC mutations were consistent with selection for improved antigen binding, based on comparing the expected with the observed R to S mutation ratio (R:S), using as controls normal mature B cell subsets as well as the non-productive IGHV-IGHD-IGHJ rearrangements identified. This indicated that, unlike the mutations found in memory and DN B cells and to a somewhat lesser extent in the starting CDC and MZ B cells samples, mutations in the productive post-CDC and non-productive IGHV-IGHD-IGHJ rearrangements showed a marked decrease of the R:S in CDRs and a significant increase in the R:S in FRs. These features are the opposite of what is expected for the selection of AID-mediated mutations that improve the affinity of antigen binding and for the preservation of an intact membrane IG and B-cell receptor signaling.

Collectively, the analyses of the type and targeting of the post-CDC mutations and the predicted consequences of these on selection for antigen reactivity suggest that many of these mutations do not reflect outcomes seen with typical, AID-based SHM that, in the normal setting, would be subsequently selected for improved antigen binding and associated adaptive protection. The findings also suggest that the mutations that develop after leukemic transformation differ, in multiple ways (inducing mechanism, motif targeting, Ts : Tv, and antigen binding), from those that occurred before/up to the time of transformation.

Thus, are those mutations occurring downstream of the transformation event the result of deranged SHM mediated by AID or of another process? If the former, the derangement might be the consequence of a direct effect on the leukemic B cell occurring upon transformation. Additionally, the discrepancy might reflect a change in microenvironmental influences in play up to the time of transformation compared to subsequently. Certainly, the lymphoid microenvironment in which the normal B lymphocyte that converted into a CLL cell developed and was shaped is different from the one to which the subclones were exposed after leukemic transformation. Simplistically, the former was normal, structurally and functionally, whereas the latter was likely dysregulated in both ways. This change is at least due to the expansion of the leukemic clone, resulting in major degradation of the architecture of lymph nodes (38), thereby allowing access of CLL subclones to different microenvironmental inputs. This would be especially relevant if the normal B lymphocyte precursors of CLL develop outside of normal germinal center structures (39) and if the influences delivered at extrafollicular sites and the responses to these are distinct from those in classical GCs. Studies in mice indicate that such differences are likely between extrafollicular and follicular sites (40). Additionally, the quality and level of these signals could be affected by the leukemic cells themselves. This has been documented in CLL for T cells (41) and follicular dendritic cells (42), key constituents of mutation initiation and subclonal variant selection. The consequences of these structural and function changes in lymphoid tissue might increase mutation frequency and decrease selection of mutation variants in a normal physiologic manner. In this scenario, mutations occurring in the IGHV-IGHD-IGHJ region would decrease the BCR's influence on survival and growth of the intraclonal subclones, shifting the advantage to subclones developing mutations outside IG loci and leading to more dysregulated B-cell function and resulting in more aggressive disease.

Alternatively, the detected post-transformation mutations could, in part, be physiologic. In this case, the strict avoidance of replacement mutations in the CDRs and the deduced selection for replacement mutations in the FRs could provide the post-CDC subclones an alternative way to receive beneficial interactions, such as allowing superantigen binding to structurally altered FRs. In this regard, Staphylococcal superantigen can bind to FR3 (43) or to a discontinuous epitope created by FRs 1 and 3 and CDR2 (44). Similarly, HIV gp120 can bind IG through an unconventional binding site comprised of an intact hypervariable loop and alterations in the FRs (45). Additionally, FR mutations could potentially promote self-association (46) which can result in autonomous signaling (47), thereby promoting subclonal survival and expansion. In this situation, mutations occurring in the IGHV-IGHD-IGHJ region post-transformation would enhance the BCR's ability to advance survival and growth of the intraclonal subclones. The apparent selection for replacement mutations in IGHV subregions that differ from those targeted by AID suggest that CLL cells when diversifying in an altered tissue microenvironment might employ a different mutational mechanism with distinct target sites.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI Bio Project; PRJNA673787.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards of Northwell Health and of Dana Farber Cancer Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB and NC conceived the project and designed the experimental approach. DB, CT, SV, and TM performed experiments and data analyses. MC supplied IGHV-IGHD-IGHJ sequences from normal human B cell subpopulations. JB, SK, SF, KRR, JK, JB, and SA provided clinical samples and clinical correlations. AM, FG, SB, MC, FM, and MS offered interpretations and conceptual insights. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.640731/full#supplementary-material

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Prognostic Significance of Systemic Immune-Inflammation Index in Patients With Diffuse Large B-Cell Lymphoma

Zanzan Wang[1], *Jiawei Zhang*[1], *Shuna Luo*[2] *and Xiaoying Zhao*[1]*

[1] Department of Hematology, The Second Affiliated Hospital of Zhejiang University School of Medicine, Zhejiang University, Hangzhou, China, [2] Department of Hematology, The Fourth Affiliated Hospital of Zhejiang University School of Medicine, Zhejiang University, Yiwu, China

Objective: The systemic immune-inflammation index (SII) based on neutrophil, platelet and lymphocyte counts, is a prognostic biomarker in some solid cancers. However, the prognostic value of SII has not yet been validated. This study was to evaluate the role of SII in predicting survival for patients with diffuse large B cell lymphoma (DLBCL).

Methods: We retrospectively investigated 224 patients with DLBCL between August 2005 and October 2018. Kaplan–Meier analysis and Cox proportional hazard models were used to assess the prognostic value of SII.

Results: In the ROC curve analysis, SII had the highest AUC and was more accurate as a prognostic factor. Patients with higher SII tended to have higher level of LDH, more advanced stage, poor PS, and high IPI score compared with low SII group. In univariate analyses, SII, PLR and NLR were all prognostic for progression-free survival and overall survival. Moreover, only SII, older age, HBSAg-positive and IPI were the independent prognostic factors for patients in multivariate analysis. The nomogram based on SII, older age, HBSAg status and IPI showed accurate prognostic ability for predicting 3-years and 5-years survival rates (c-index, 0.791) compared to the IPI alone (c-index, 0.716).

Conclusion: SII was a powerful tool for predicting outcome in patients with DLBCL. It might assist the separation of high-risk patients among patients with the same IPI.

Keywords: diffuse large B-cell lymphoma, systemic immune-inflammation index, prognosis, neutrophil, lymphocyte, platelet, nomogram

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***Correspondence:**
Xiaoying Zhao
zrxz@zju.edu.cn

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common histologic subtype of non-Hodgkin's lymphoma (NHL), accounting for 30-40% of new cases. Over the recent years, due to the introduction of rituximab into treatment regimens, more than half of untreated DLBCL patients can be better cured (1). However, approximately 30%-40% patients still have recurrence or develop refractory disease that remain poor outcome (2). Therefore, it is significant to distinguish the

patients with poor prognosis in the early stage and select the effective therapeutic regimen accordingly.

During the pre-rituximab era, International Prognostic Index (IPI) was the most powerful prognostic clinical tool for DLBCL (3). After the extensive use of rituximab, the outcome of DLBCL patients has been improved so much that it is difficult to identify high-risk groups using IPI alone (4). In an effort to risk-stratify patients treated with R-CHOP, the revised IPI (R-IPI) and NCCN-IPI were generated. The R-IPI redistribute the IPI clinical scores to form three groups, which has better prediction of clinical outcomes compared with IPI (5). The NCCN-IPI is also calculated based on various clinical characteristics with better definition of extranodal site involvement and re-evaluation of age and LDH to divide patients into four groups (6). Recently, molecular genetic markers (7) and gene expression profiling (8) have been identified as new prognostic parameters. However, these indicators are inconvenient and expensive, so it is essential to seek readily available and inexpensive parameters to stratify the prognosis of patients with DLBCL and provide appropriate treatment measures.

Inflammation plays a vital role in the tumor progression and therapeutic response. Peripheral blood counts which in some degree reflect inflammation status are closely related to the progress of cancers (9, 10). Numerous studies published in recent years have demonstrated that inflammation indicators such as pretreatment neutrophil-lymphocyte ratio (NLR) (11) and platelet-lymphocyte ratio (PLR) (12) play crucial roles in influencing the outcomes of the DLBCL patients. Moreover, compared with molecular genetic markers, blood biomarkers are cheaper and easier to obtain.

High systemic immune-inflammation index (SII), which is associated with neutrophil, platelet and lymphocyte counts, has been reported to be a poor prognostic indicator in several solid malignancies, such as pancreatic cancer (13), breast cancer (14), lung cancer (15) and gastrointestinal cancer (16). But the cutoff of SII in different cancers is different. To the best of our knowledge, the relationship between SII and the outcome of DLBCL has not been explored. Hence, we first conducted this study to evaluate the clinical and prognostic value of SII in patients with DLBCL.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the data of 436 patients with DLBCL at the Second Affiliated Hospital of Zhejiang University School of Medicine between August 2001 and October 2018. 55 patients with incomplete data and 157 patients received CHOP or CHOP-like chemotherapy were excluded. Included in the study were 224 patients with DLBCL between August 2005 and October 2018. A total of 124 DLBCL patients from 2005 to 2018 from the Second Affiliated Hospital of Zhejiang University School of Medicine were reviewed as the validation cohort. We included 90 patients with complete data, while 22 patients received CHOP or CHOP-like chemotherapy and 12 patients with incomplete data were excluded. Selection criteria were as follows (1): patients were confirmed CD20+ DLBCL according to the 2016 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue (17) (2); patients who received at least 4 cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone),or R-CEOP (rituximab, etoposide, cyclophosphamide, vincristine, and prednisone) and R-EPOCH (rituximab, etoposide, cyclophosphamide, doxorubicin, vincristine, and prednisone) (3). the availability of complete information in laboratory tests and medical records. All patients who were suspected of being infected must receive examinations including CRP, procalcitonin, CT of the infected site, etc. to

TABLE 1 | Baseline characteristics of the study population categorized by systemic immune-inflammation index(SII).

Characteristics	Overall N (%)	SII ≥1046.1N (%)	SII<1046.1N (%)	p
N	224	55	169	
Age (years)				
<60	114 (50.9)	29 (52.7)	85 (50.3)	0.754
≥60	110 (49.1)	26 (47.3)	84 (49.7)	
Gender				
Male	121 (54.0)	32 (58.2)	89 (52.7)	0.476
Female	103 (46.0)	23 (41.8)	80 (47.3)	
LDH				
Increased	121 (54.0)	47 (85.5)	74 (43.8)	**<0.001**
Normal	103 (46.0)	8 (14.5)	95 (56.2)	
B-symptoms				
No	159 (71.0)	34 (61.8)	125 (74.0)	0.085
Yes	65 (29.0)	21 (38.2)	44 (26.0)	
Ann Arbor stage				
I/II	76 (33.9)	11 (20.0)	65 (38.5)	**0.012**
III/IV	148 (66.1)	44 (80.0)	104 (61.5)	
Performance status				
0-1	170 (75.9)	30 (54.5)	140 (82.8)	**<0.001**
≥2	54 (24.1)	25 (45.5)	29 (17.2)	
Bulky disease				
0	215 (96.0)	52 (94.5)	163 (96.4)	0.532
1	9 (4)	3 (5.5)	6 (3.6)	
Extranodal involvement				
<1	45 (20.1)	9 (16.4)	36 (21.3)	0.427
≥1	179 (79.9)	46 (83.6)	133 (78.7)	
HBSAg				
Negative	181 (80.8)	44 (80.0)	137 (81.1)	0.862
positive	43 (19.2)	11 (20.0)	32 (18.9)	
IPI				
Low	70 (31.3)	6 (10.9)	64 (37.8)	**<0.001**
Low-intermediate	63 (28.1)	14 (25.5)	49 (29.0)	
High-intermediate	46 (20.5)	17 (30.9)	29 (17.2)	
High	45 (20.1)	18 (32.7)	27 (16.0)	
NLR				
<3.554	142 (63.4)	5 (9.1)	137 (81.1)	**<0.001**
≥3.554	82 (36.7)	50 (90.9)	32 (18.9)	
PLR				
<216.00	162(72.3)	5(9.1)	157(92.9)	**<0.001**
≥216.00	62(27.7)	50(90.9)	12(7.1)	
Response to treatment				
CR+PR	192 (85.7)	42 (76.4)	150 (88.8)	**0.023**
SD+PD	32 (14.3)	13 (23.6)	19 (11.2)	

GC, germinal center; LDH, lactate dehydrogenase; IPI, International Prognostic Index; CR, complete remission; PR, partial response; SD, stable disease; PD, progressive disease. Significant p value are represented in bold.

determine whether they were infected. Patients with infection at initial diagnosis were excluded. Patients were excluded if they were HIV positive. Transformed indolent lymphoma and primary central nervous systems (CNS) B cell lymphoma were excluded. Primary mediastinal large B-cell lymphoma, high grade cell lymphoma and intravascular large B-cell lymphoma were also excluded. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine in line with the principles of the Declaration of Helsinki.

Data Collection

We retrieved patient characteristics including gender, age, LDH level, Ann Arbor stage, B symptoms, serum β_2-microglobulin, IPI, Eastern Cooperative Oncology Group Performance Status (ECOG PS), bulky disease(≥7.5cm), hepatitis B surface antigen status, number of extranodal involvement and chemotherapy regimen. The laboratory data and full blood cell counts were obtained before 1 week of the treatment of DLBCL. SII was defined as platelet counts × neutrophil counts/lymphocyte counts. PLR and NLR were calculated by dividing the absolute platelet counts and neutrophil counts by the absolute lymphocyte counts, respectively. Follow-up of all patients ended on December 20, 2020. During the follow-up time, 38 patients in the group were lost to follow-up in the entire cohort. The missing rate was 17.0%. These patients who lost to follow-up were censored on their own last follow-up visit. Overall survival (OS) was calculated as the interval between diagnosis and death or date of last follow-up and progression free survival (PFS) was calculated from diagnosis to recurrence, progression, death from any cause or last follow-up.

Statistical Analyses

ROC curves were used to determine the optimal cut-off values and measure the area under curve (AUC) of NLR, SII and PLR. The categorical variables were analyzed by the Pearson Chi-squared test. The association between the different groups with OS and PFS was analyzed using the Kaplan–Meier curves and compared using the log-rank test. Univariate and multivariate analysis were used to evaluate prognostic values of each variable. The Cox proportional hazards regression model was used to examine the hazard ratio (HR) and 95% confidence interval (95% CI). Predictors with $p < 0.05$ were considered statistically significant, which were incorporated into the nomogram. The concordance index (c index) was calculated to evaluate the ability of the nomogram. Next, model calibration was used to verify the accuracy of nomogram by comparing the predicted OS with the

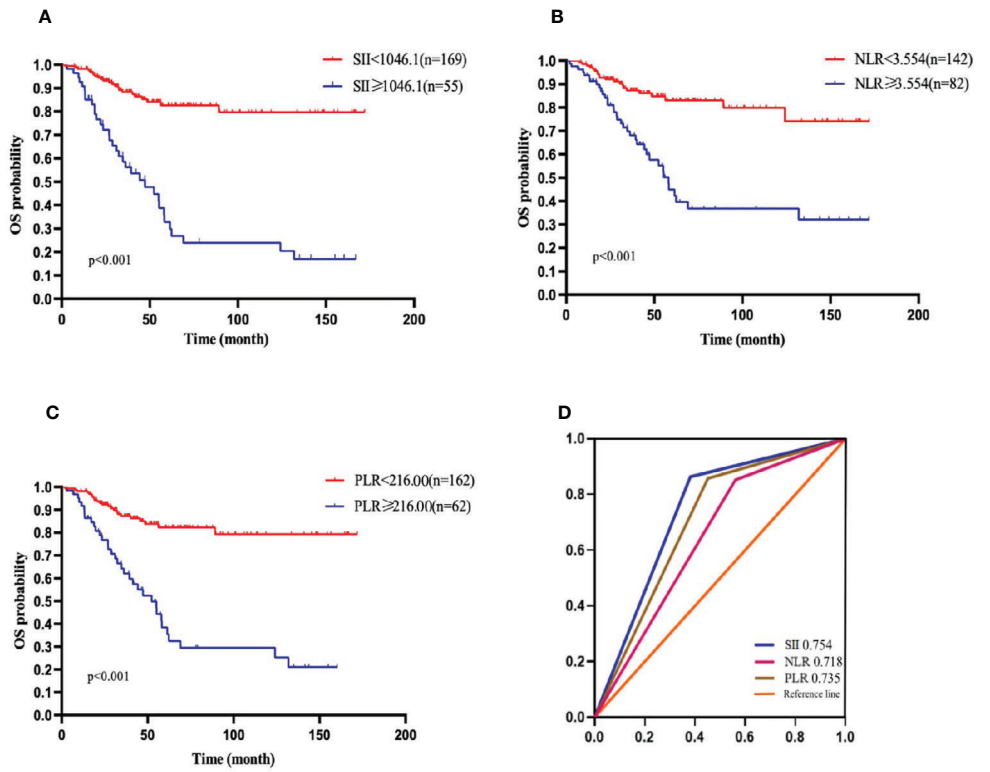

FIGURE 1 | OS of patients with diffuse large B cell lymphoma (DLBCL). **(A)** OS in patients affected by DLBCL with SII at diagnosis < 1046.1 and ≥ 1046.1. **(B)** OS in patients affected by DLBCL with NLR at diagnosis < 3.554 and ≥ 3.554. **(C)** OS in patients affected by DLBCL with PLR <216.00 and ≥ 216.00 at diagnosis. **(D)** Predictive ability of the SII in DLBCL was compared with PLR and NLR by ROC curves in 3-years in the primary cohort.

actual OS. SPSS statistical software (SPSS statistics 25.0), R software (version 3.6.3) and GraphPad Prism software were used for statistical analyses.

RESULTS

Patient Cohorts and Characteristics

The clinical characteristics of 224 patients with confirmed DLBCL included in this study were showed in **Table 1**. The median age was 59 years (range 22 – 80 years), with 49.1% of patients older than 60 years of age. Female/male ratio was 0.85:1. B symptoms was observed in 65 (29.0%) patients. 148 (66.1%) patients had more advanced stage, and 54 (24.1%) patients had performance status ECOG ≥2. The patients for low, low-intermediate, high-intermediate, high IPI scores were 70 (31.3%), 63 (28.1%), 46 (20.5%) and 45 (20.1%), respectively. R-CHOP was the most commonly used regimen (n=199, 88.9%), followed by R-CEOP (n=24, 10.7%), R-EPOCH (n=1, 0.4%). The median values of absolute neutrophil counts, absolute lymphocyte counts, absolute platelet counts were 3.795 (1.08~21.07)×10^9/L, 1.295(0.2~6.8)×10^9/L and 210 (53~456)×10^9/L. Most patients (192, 85.7%) obtained complete remission (CR) or partial response (PR), while relapse after response occurred in 48 (25.0%) patients. During the median follow-up time of 51.3 months, 57 (25.4%) died before the end of the follow-up period.

According to the ROC curve, the optional cutoff points of SII, NLR and PLR were 1046.1, 3.554 and 216.00 (**Figure 1D**). The area under the ROC curves for SII, NLR and PLR were 0.754, 0.718 and 0.735, respectively, demonstrating that the prognostic value of SII was better than that of PLR and NLR.

Patients were divided into two groups according to the cutoff value: 169 patients had SII <1046.1 and 55 patients had SII ≥ 1046.1. Patients with high SII tended to have high level of LDH, more advanced stage, poor PS, and high IPI score. Moreover, 42 (76.4%) patients with high SII achieved CR or PR and 150 (88.8%) patients with low SII achieved CR or PR, which showed the patients with high SII had statistically significant poor response (p<0.001). However, there were no significant differences between two group in sex, age, B-symptoms, bulky disease, extranodal involvement and HBSAg status.

Univariate and Multivariate Analysis of OS

In the present study, the 1-, 2-,3-, and 5-year OS rates were 96.4%, 88.2%, 81.1%, and 68.8%, respectively. Patients with SII <1046.1 (n=169) had a 3-year OS probability of 88.3%, significantly higher than the 3-year OS rate of 58.6% in patients with SII ≥ 1046.1 (n=55) (p=0.001) (**Figure 1A**). The survival curves of PLR and NLR also showed similar trends

TABLE 2 | Univariate and multivariate analysis of prognostic factors for OS in DLBCL patients.

Variable	Parameter	Univariate analysis		Multivariate analysis	
		HR (95% CI)	p value	HR (95% CI)	p value
Age	<60	1	**0.018**	1	**0.023**
	≥60	1.864 (1.105-3.145)		2.109 (1.109-4.010)	
Gender	Female	1	**0.035**	1	0.351
	Male	1.828 (1.086 -3.078)		1.327 (0.732-2.407)	
B symptoms	Absent	1	0.084		
	Present	1.607(0.883-2.922)			
Performance status	0-1	1	**<0.001**	1	0.967
	≥2	3.002 (1.536-5.867)		1.014(0.533-1.928)	
LDH	Normal	1	**0.003**	1	0.073
	Increased	2.299 (1.367-3.864)		1.963(0.940-4.102)	
IPI	Low	1		1	
	Low-intermediate	4.525 (1.606-12.747)	**0.004**	3.583 (1.209-10.614)	**0.021**
	High-intermediate	11.293(4.131-30.873)	**<0.001**	7.091(2.307-21.978)	**0.001**
	High	11.352(4.114-31.328)	**<0.001**	6.620(1.863-23.515)	**0.003**
Bone marrow involvement	No	1	0.100		
	Yes	2.002 (0.644-6.223)			
Extranodal involvement	<1	1	0.689		
	≥1	1.143 (0.678-2.152)			
Ann Arbor stage	I/II	1	0.085		
	III/IV	1.711 (0.988-2.965)			
HBsAg	Negative	1	**0.005**	1	**0.002**
	Positive	2.167 (1.095-4.289)		2.656 (1.414.976)	
SII	<1046.1	1	**<0.001**	1	**0.029**
	≥1046.1	5.659(2.982-10.740)		3.850 (3.850-12.941)	
NLR	<3.554	1	**<0.001**	1	0.534
	≥3.554	3.608(2.070-6.289)		1.299(0.570-2.960)	
PLR	<216.00	1	**<0.001**	1	0.919
	≥216.00	4.686(2.551-8.607)		1.058(0.356-3.141)	

IPI International Prognostic Index; LDH lactic dehydrogenase; SII systemic immune-inflammation index; HR hazard ratio; CI confidence interval.
The bold values indicate that p < 0.05 and the corresponding factors are significantly associated with survival.

(**Figures 1B, C**). Furthermore, the univariate analysis revealed that older age, male, high level of LDH, high IPI score, ECOG≥2, HBsAg- positive, high NLR, high PLR and high SII had significantly inferior OS.

Multivariate Cox proportional hazard models were used to analyze whether the above parameters were effective predictors. In multivariate analyses, older age, HBsAg -positive, high IPI score and high SII were independent prognostic factors for poor OS (**Table 2**).

Univariate and Multivariate Analysis of PFS

Similarly, the 1-, 2-, 3-, and 5-year PFS rates were 86.5%, 76.4%, 65.1%, and 59.1%, respectively. Patients with SII <1046.1 (n=169) had a 3-year PFS probability of 74.4%, higher than the 3-year PFS rate of 33.7% in patients with SII ≥ 1046.1 (n=55) (p<0.001). The survival curve also showed that the PFS of patients with high NLR and high PLR values were shorter than that of patients with low values (**Figures 2 A–C**). The univariate analysis demonstrated that older age, male, B symptoms, high level of LDH, ECOG≥2, high IPI score, advanced stage (stage III/IV), high NLR, high PLR and high SII were independent prognostic predictors of PFS. Multivariate Cox regression analysis indicated that high IPI score, high SII and high level of LDH were related to poor PFS (**Table 3**).

Nomogram Predicting Survival

A nomogram, including significant independent risk factors such as SII, IPI, age and HBSAg status, was established to predict the 3- and 5-years OS (**Figure 3**). The C-index of the nomogram was 0.791, which showed excellent role of predicting the outcome compared to the IPI (C-index, 0.716). The calibration curve of the nomogram showed great concordance between the predicted and actual survival rates for 3- and 5-years(**Figures 4A, B**). Moreover, according to the ROC curve, the AUC of the nomogram was 0.840, which was higher than that of IPI (AUC=0.718, p<0.001), illustrated that this nomogram was suited for predicting the outcome of DLBCL patients (**Figure 4C**).

Validate the Prognostic Value of SII

We verified the role of SII in survival of the patients in the validation cohort. A total of 90 DLBCL patients were enrolled in this study as the validation cohort. The median age of the patients was 58 (22–80) years. All patients received R-CHOP

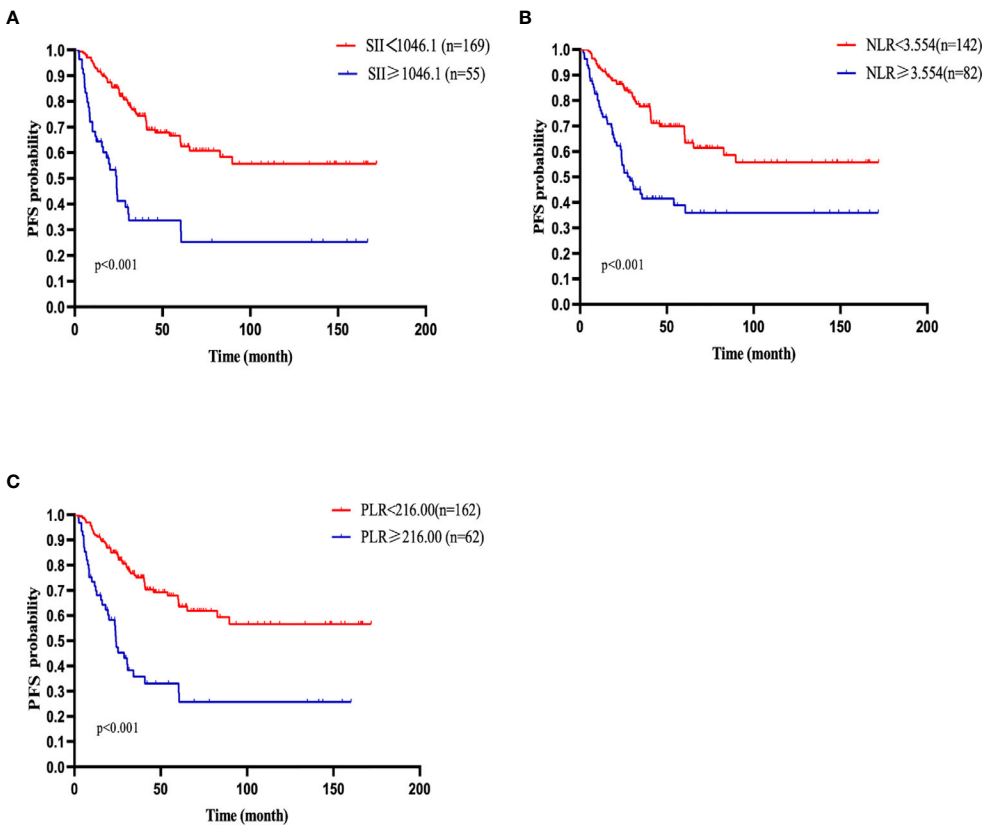

FIGURE 2 | PFS of patients with diffuse large B cell lymphoma (DLBCL). **(A)** PFS in patients affected by DLBCL with SII at diagnosis < 1046.1 and ≥ 1046.1. **(B)** PFS in patients affected by DLBCL with NLR at diagnosis < 3.554 and ≥ 3.554. **(C)** PFS in patients affected by DLBCL with PLR < 216.00 and ≥ 216.00 at diagnosis.

TABLE 3 | Univariate and multivariate analysis of prognostic factors for PFS in DLBCL patients.

Variable	Parameter	Univariate analysis		Multivariate analysis	
		HR (95% CI)	p value	HR (95% CI)	p value
Age	<60	1	**0.003**	1	0.153
	≥60	1.961 (1.267-3.036)		1.434 (0.874-2.354)	
Gender	Female	1	**0.048**	1	0.205
	Male	1.553 (1.004 -2.402)		1.338 (0.853-2.101)	
B symptoms	Absent	1	**0.018**	1	0.507
	Present	1.701(1.096-2.640)		1.172 (0.733-1.875)	
Performance status	0-1	1	**<0.001**	1	0.627
	≥2	2.341 (1.492-3.674)		1.149(0.656-2.012)	
LDH	Normal	1	**0.019**	1	**0.023**
	Increased	1.294 (1.043-1.606)		1.926(1.094-3.392)	
IPI	Low	1		1	
	Low-intermediate	3.072 (1.536-6.143)	**0.002**	2.952 (1.330-6.551)	**0.008**
	High-intermediate	6.044(2.984-12.244)	**<0.001**	5.377(1.728-16.736)	**0.003**
	High	6.401(3.183-12.870)	**<0.001**	6.292(2.556-15.490)	**<0.001**
Bone marrow involvement	No	1	0.072		
	Yes	1.953 (0.942-4.052)			
Extranodal involvement	<1	1	0.924		
	≥1	1.026 (0.610-1.723)			
Ann Arbor stage	I/II	1	**0.019**	1	0.417
	III/IV	1.801 (1.102-2.943)		1.311 (0.682-2.520)	
HBsAg	Negative	1	0.101		
	Positive	1.509 (0.923-2.466)			
SII	<1046.1	1	**<0.001**	1	**0.043**
	≥1046.1	1.835(1.476-2.280)		2.253 (1.026-4.950)	
NLR	<3.554	1	**<0.001**	1	0.275
	≥3.554	1.609(1.302-1.987)		1.381(0.773-2.465)	
PLR	<216.00	1	**<0.001**	1	0.471
	≥216.00	1.785(1.441-2.212)		1.289(0.646-2.573)	

IPI International Prognostic Index; LDH lactic dehydrogenase; SII systemic immune-inflammation index; HR hazard ratio; CI confidence interval.
The bold values indicate that p < 0.05 and the corresponding factors are significantly associated with survival.

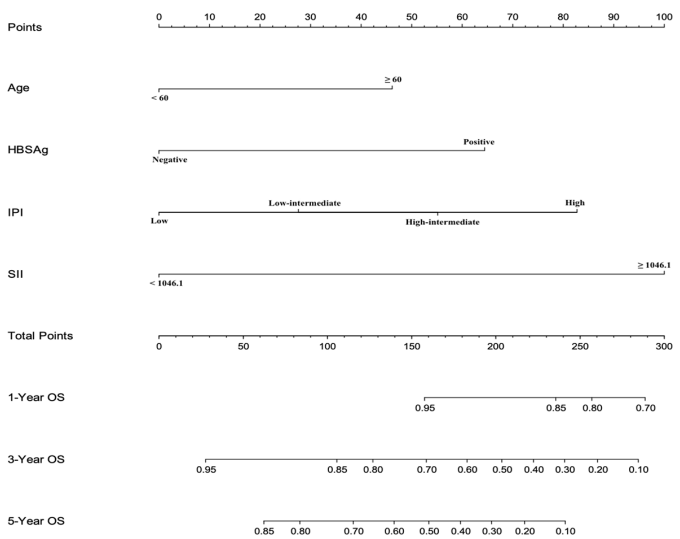

FIGURE 3 | Evaluation of nomogram integrated age, SII, IPI and HBsAg status in patients with DLBCL.

regimen. Survival curve showed that patients with lower SII levels had better OS than those with higher levels of SII (p<0.001), which was consistent with the primary results. In univariate analysis, we found that older age, ECOG≥2, high level of LDH, HBsAg positive, high IPI scores, high NLR, high PLR and high SII were associated with poor OS. And in multivariate analyses, older age and high SII were shown to be independently associated with poor OS (**Table 4**). These results demonstrated

that the SII could be used as an accurate prognostic factor for predicting the survival of patients.

DISCUSSION

Inflammation is closely linked to tumor development (18). Immune cells always secrete cytokine and chemokine to shape tumor growth in tumor microenvironment.

Neutrophils which exist in tumor microenvironment are capable of producing angiogenic chemokines and cytokines including CCL2,CCL3, CCL5, CCL10, IL4 and IL10 to promote tumor growth, invasion and angiogenesis (19). Therefore, high levels of neutrophils could affect the survival of DLBCL. In solid tumors, it has been found that platelets can protect circulating cancer cells from NK cell-mediated killing, promote cancer cells metastasis and secrete cytokines to stimulate proliferation of tumor cells (20, 21). Tumor-infiltrating lymphocytes could inhibit tumor cell proliferation and metastases, and low lymphocytes could weaken the immunological response to tumor (22). Lymphocytes are the basis of cytotoxic effect of rituximab on tumor cells (23). Previous studies have shown that a low lymphocyte count played an adverse impact on outcome in DLBCL patients (24). In the study by Kusano and colleagues, low CD4+ T-cell count at diagnosis was significantly related to OS and PFS, and the CD4+ T-cell count might be an independent predictive marker in patients with DLBCL (25). Dehghani et al. (26) also demonstrated that in patients with DLBCL receiving R-CHOP, the elevated level of Treg cell was associated with improved prognosis. Therefore, the amounts of peripheral hematologic cells can reflect the inflammatory and immune changes.

Numerous studies showed that inflammatory-based prognostic indexes, such as PLR and NLR, were considered to have prognostic value for DLBCL. A retrospective study indicated that high PLR value was significantly related to poor prognosis to R-CHOP for DLBCL (27). Meanwhile, a meta-analysis revealed that high PLR was related to poor tumor behavior and poor OS with 1931 individuals, and the PLR might be an effective factor of poor prognosis for patients with DLBCL (28). A study of 505 patients proved that NLR may be a useful prognostic marker in OS and could predict the

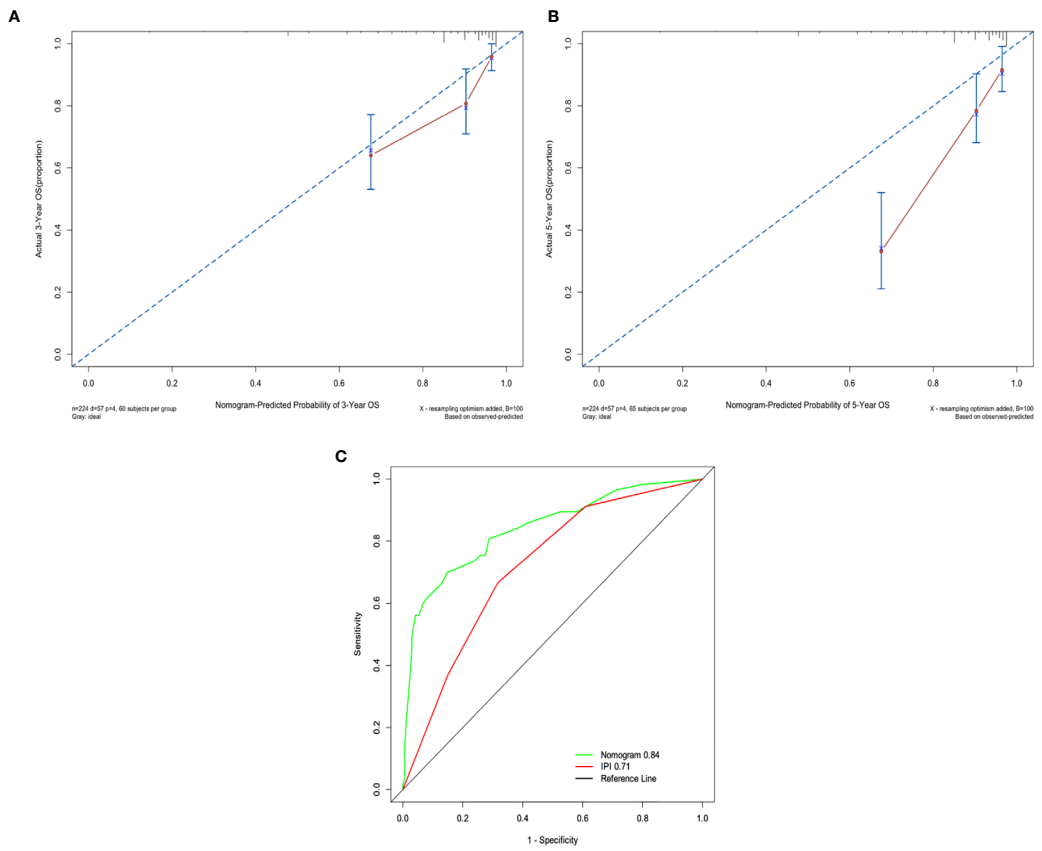

FIGURE 4 | **(A)** The 3-years survival rate of DLBCL predicted by nomogram is consistent with the actual observed values. **(B)** The 5-years survival rate of DLBCL predicted by nomogram is consistent with the actual observed values. **(C)** The ability of ROC analysis nomogram to predict the 3-years survival rate of the DLBCL, the nomogram has a larger AUC than IPI.

TABLE 4 | Univariate and multivariate analysis of prognostic factors for OS in DLBCL patients in validation cohort.

Variable	Parameter	Univariate analysis		Multivariate analysis	
		HR (95% CI)	p value	HR (95% CI)	p value
Age	<60	1	**<0.001**	1	**0.038**
	≥60	6.063 (2.287-16.071)		3.869 (1.076-13.917)	
Gender	Female	1	0.725		
	Male	1.146 (0.538-2.441)			
B symptoms	Absent	1	0.063		
	Present	2.075(0.962-4.478)			
Performance status	0-1	1	**0.034**	1	0.372
	≥2	2.286 (1.065-4.907)		1.535(0.599-3.934)	
LDH	Normal	1	**<0.001**	1	0.462
	Increased	4.494 (1.933-10.450)		1.501(0.509-4.427)	
IPI	Low	1		1	
	Low-intermediate	14.851 (1.713-128.715)	**0.014**	3.433(0.318-37.101)	0.310
	High-intermediate	34.050(4.238-273.577)	**0.001**	10.028(0.873-115.164)	0.064
	High	70.155(8.519-577.710)	**<0.001**	12.763(0.968-168.286)	0.053
Bone marrow involvement	No	1	0.141		
	Yes	2.224 (0.767-6.449)			
Extranodal involvement	<1	1	0.924		
	≥1	1.026 (0.610-1.723)			
Ann Arbor stage	I/II	1	0.084		
	III/IV	2.555 (0.883-7.395)			
HBsAg	Negative	1	**0.006**		
	Positive	3.344 (1.405-7.961)			
SII	<1046.1	1	**<0.001**	1	**0.002**
	≥1046.1	4.340(2.760-6.824)		79.091 (5.279-118.959)	
NLR	<3.554	1	**<0.001**	1	0.443
	≥3.554	2.259(1.493-3.418)		1.686(0.444-6.402)	
PLR	<216.00	1	**<0.001**	1	0.078
	≥216.00	2.148(1.433-3.218)		6.600(0.809-53.868)	

IPI International Prognostic Index; LDH lactic dehydrogenase; SII systemic immune-inflammation index; HR hazard ratio; CI confidence interval.
The bold values indicate that p < 0.05 and the corresponding factors are significantly associated with survival.

progression in patients with DLBCL (11). A recent meta-analysis revealed that the NLR was associated with worse OS and regarded as an inexpensive prognostic factor with 2297 patients with DLBCL (29). SII, based on neutrophil, platelet and lymphocyte, may reflect the inflammatory status and tumor activity more accurately than PLR and NLR, and SII has been proved to be an independent predictor in several malignancies, such as breast cancer and lung cancer (14, 15). However, data in non-Hodgkin's lymphoma are limited and only one study has been performed to assess the impact of pretreatment SII in DLBCL. Yang et al. (30) enrolled 28 patients with testicular DLBCL and found that pretreatment SII was a negative prognostic factor for PFS. But it should be noted that the most common chemotherapy regimen was CHOP without rituximab and the population was small. Whether SII could influence the outcome of DLBCL patients is still unclear, so we conducted this retrospective study and hope that SII could be useful for predicting the prognosis in DLBCL.

In the present study, we reviewed 224 patients with DLBCL for their clinical and laboratory data and evaluated the prognostic impact of SII in DLBCL. We determined the cutoff value of 1046.1 for SII. Consistent with other solid cancers (16, 31), our results showed high SII was associated with more aggressive clinical features including high level of LDH, more advanced stage, poor PS, and high IPI score. Advanced disease, higher LDH and higher IPI score reflect a high tumor burden, leading to a negative prognosis (32). Neutrophils which exist in tumor microenvironment are capable of producing IL10 and CCL3 to promote tumor growth (19). Ji et al. (33) found that the serum CCL3 and IL-10 levels were significantly increased in DLBCL patients with high LDH levels. The above indicated that elevated neutrophils which mainly stimulated by cytokines produced by tumor cells in turn induced the release of chemokines and cytokines, thus promoted cancer growth. Meanwhile, The high SII at diagnosis negatively correlated with the overall response rate and the complete response rate significantly ($p<0.001$). These evidences prove that SII could reflect a higher tumor burden and predict chemosensitivity.

High SII had a significant negative impact on the 3-year PFS and the OS as compared with low SII. Other inflammation factors including PLR and NLR also showed the similar result. In univariate analysis, the NLR, PLR and SII could be used to judge the prognosis of patients. However, according to the multivariate analysis, only the SII was a prognostic factor for

patients with DLBCL. The ROC curve analysis showed that the SII was more accurate and effective in predicting the outcomes of patients when compared with NLR or PLR.

Due to the high prevalence of hepatitis B infection in the Chinese population, we studied the prognosis of patients with HBsAg-positive. We found that high SII, high IPI score, HBsAg-positive and older age were significant prognostic factors for OS in patients with DLBCL. Based on this finding, a new nomogram included high SII, high IPI score, HBsAg-positive and older age was established to predict the prognosis of patients. The ability of the nomogram to predict the outcome was more accurate than that of IPI(c index 0.791 *vs*. 0.716). This result suggested that SII, when combined with IPI, age and HBsAg status, could provide more accurate prognostic information in patients with DLBCL, identify high-risk patients early and provide support for selecting individualized treatment strategy.

Nonsteroidal anti-inflammatory drugs (NSAIDs) could inhibit the enzyme cyclooxygenase (COX) to reduce inflammation (34). Additionally, aspirin, a kind of NSAIDs, have been found to induce apoptosis and inhibit tumor invasion by decreasing the expression of B-cell lymphoma 2 (BCL-2) and inhibiting NFκB signaling pathway (35). Previous studies have reported that NSAIDs is associated with reduced risk of cancer-related death by inhibit tumor growth in several malignancies (36, 37). Further research is necessary to investigate the therapeutic effect of NSAIDs in improving prognosis in patients with DLBCL.

There were some limitations in this study. First, it was a single-center and retrospective study and the number of patients was not large enough. Therefore, there were some selection and analytical biases. Second, there were no consistent cutoff values for SII in different studies, which led to the difficulty of comparing our results with other studies. Third, this study did not evaluate the association between prognosis and the changes of SII before and after treatment, which could reflect the change of inflammatory status to predict the survival and recurrence. Fourth, we did not investigate the link between SII and recurrent mutations reported in DLBCL.

In conclusion, this is the first analysis to demonstrate that the high SII as a reproducible and easily assessable prognostic biomarker is able to predict poor outcome in DLBCL patients. Maybe SII can be combined with other biomarkers to identify high risk patients and instruct treatment. But larger prospective clinical trial is warranted to validate this result and explore the mechanistic explanation for association between the SII and clinical outcome in DLBCL patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZW designed the study, performed the data analysis, and drafted the manuscript. JZ performed the data analysis and drafted the manuscript. SL participated in the data acquisition and drafted the manuscript. XZ designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Oncology

BRIEF RESEARCH REPORT
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In Vitro Sensitivity to Venetoclax and Microenvironment Protection in Hairy Cell Leukemia

Alexia Vereertbrugghen[1†], Ana Colado[1†], Ernesto Gargiulo[2], Raimundo Fernando Bezares[3], Horacio Fernández Grecco[4], Gregorio Cordini[1,5], Maria del Rosario Custidiano[6], Jean-Hugues François[7], Guy Berchem[2,8], Mercedes Borge[1,9], Jerome Paggetti[2], Etienne Moussay[2], Romina Gamberale[1,9], Mirta Giordano[1,9] and Pablo Elías Morande[1,2]*

[1] Instituto de Medicina Experimental (IMEX)–CONICET–Academia Nacional de Medicina (ANM), Buenos Aires, Argentina, [2] Tumor Stroma Interactions, Department of Oncology, Luxembourg Institute of Health, Luxembourg, Luxembourg, [3] Sección Hematología, Hospital General de Agudos Dr. Teodoro Álvarez, Buenos Aires, Argentina, [4] Sección Hematología, Sanatorio Municipal Dr. Julio Méndez, Buenos Aires, Argentina, [5] Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Buenos Aires, Argentina, [6] Servicio de Hematología y Transplante de Médula Ósea, Instituto Alexander Fleming, Buenos Aires, Argentina, [7] Laboratory of Hematology, Centre Hospitalier de Luxembourg, Luxembourg, Luxembourg, [8] Department of Hemato-Oncology, Centre Hospitalier de Luxembourg, Luxembourg, Luxembourg, [9] Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Current standard treatment of patients with hairy cell leukemia (HCL), a chronic B-cell neoplasia of low incidence that affects the elderly, is based on the administration of purine analogs such as cladribine. This chemotherapy approach shows satisfactory responses, but the disease relapses, often repeatedly. Venetoclax (ABT-199) is a Bcl-2 inhibitor currently approved for the treatment of chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) in adult patients ineligible for intensive chemotherapy. Given that HCL cells express Bcl-2, our aim was to evaluate venetoclax as a potential therapy for HCL. We found that clinically relevant concentrations of venetoclax (0.1 and 1 μM) induced primary HCL cell apoptosis *in vitro* as measured by flow cytometry using Annexin V staining. As microenvironment induces resistance to venetoclax in CLL, we also evaluated its effect in HCL by testing the following stimuli: activated T lymphocytes, stromal cells, TLR-9 agonist CpG, and TLR-2 agonist PAM3. We found decreased levels of venetoclax-induced cytotoxicity in HCL cells exposed for 48 h to any of these stimuli, suggesting that leukemic B cells from HCL patients are sensitive to venetoclax, but this sensitivity can be overcome by signals from the microenvironment. We propose that the combination of venetoclax with drugs that target the microenvironment might improve its efficacy in HCL.

Keywords: hairy cell leukemia, ABT-199, cell death, leukemia microenvironment, venetoclax

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****Correspondence:***
Pablo Elías Morande
pabloelias.morande@lih.lu;
pabloemorande@gmail.com

†These authors have contributed equally to this work

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INTRODUCTION

Hairy cell leukemia (HCL) is an incurable lymphoproliferative B cell malignancy of low incidence characterized by the presence of pancytopenia, splenomegaly, and infiltration of leukemic cells in the bone marrow, spleen, and liver (1, 2). Current standard treatment with the purine analog cladribine shows a partial good response, but patients relapse repeatedly and develop refractoriness

that raises up to 40% of cases (3). Novel therapy strategies being presently tested include immune toxin-based targeting of tumor cells and kinase inhibitors, among others (4–6).

HCL cells express B cell lymphoma 2 (Bcl-2) (7, 8), an anti-apoptotic protein that plays a central role in evading programed cell death, promoting tumor growth and disease progression in cancer (9). A rising number of clinical trials based on Bcl-2 inhibition are currently ongoing for different hematological malignancies and also for solid cancers (10). The BH3 mimetic venetoclax (ABT-199) is a potent Bcl-2 selective inhibitor (11) approved for the use in chemotherapy-unfit patients with chronic lymphocytic leukemia (CLL) and older acute myeloid leukemia (AML), able to induce rapid apoptosis in blood-derived leukemic cells *in vitro* and showing promising results in the clinic (12–14). Despite this, the efficacy of drugs that selectively inhibit Bcl-2 has not been studied in HCL yet.

The tumor microenvironment exerts a protective role that withholds the levels of Bcl-2-induced cell death. In CLL, the complex cross-talk between leukemic cells and their milieu drives proliferation, disease progression, and therapy refractoriness (15). The pro-survival supportive niche localizes in the lymph nodes and bone marrow of patients, the anatomical site where CLL cells proliferate, and is composed of accessory myeloid cells, stromal cells, and T lymphocytes, among others (16). Activation of CLL cells through interactions provided by autologous T cells and TLR8 signaling accompanied by IL-2 stimulation lead to resistance to venetoclax-induced apoptosis of the leukemic clone (17, 18). In HCL, the niche interactions between tumor cells and the normal counterpart cells play a key role as well (19). HCL patients present a fibrotic and infiltrated bone marrow, and leukemic cells show high affinity to mesenchymal bone marrow stromal cells (BMSCs) in *in vitro* studies (20). The cross-talk between HCL cells and BMSCs leads to activation of signaling pathways such as mitogen activated protein (MAP) kinases and the nuclear factor κB (NF-κB) pathway and to inhibition of apoptosis induced by treatment with BRAF inhibitors (21). Peripheral blood T lymphocytes and also those present in the characteristic leukemia infiltrated spleen of HCL patients are expanded and can recognize autologous HCL cells (22). It has been proposed that rather than the growth control of neoplastic cells, such T-cell recognition favors HCL cell survival and thus disease progression (23).

In this work, we studied the effect of venetoclax in HCL to test its therapeutic potential. We evaluated the capacity of the drug to induce cell death in patient-derived leukemic cells from the blood and also studied its effect in non-malignant NK cells and T lymphocytes. Because the cross-talk between tumor cells and their milieu is relevant for understanding the mechanisms that lead to drug resistance, we also evaluated the effect of venetoclax-induced cell death on microenvironment-activated HCL cells.

MATERIALS AND METHODS

Patients

The eight HCL peripheral blood samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and with Institutional Review Board approval from the National Academy of Medicine, Buenos Aires, Argentina, and the Comité National d'Ethique de Recherche (Luxembourg). HCL was diagnosed according to standard criteria. At the time of analysis, all patients were free from clinically relevant infectious complications and had not been treated for at least two years. To obtain the peripheral blood mononuclear cells (PBMCs) from HCL patients, a density gradient centrifugation (Ficoll-Paque, GE Healthcare) was developed. PBMC samples were used fresh in cases #8, #14, #15, #18, and #23 or frozen in cases #1, #2, and #9 (in 10% DMSO, 45% FBS, and 45% RPMI) and stored in liquid nitrogen until thawed to be used.

Primary Cultures and HS-5 Cell Line

PBMCs (2.5×10^5 cells in 150 µl) from HCL patients were cultured using 96-well plates in RPMI 1640 + 10% FCS alone (control), or in the presence of PAM3 used at 300 ng/ml (Invivogen, San Diego, California, USA #tlrl-pms), CpG used at 5 µM (Invivogen, San Diego, California, USA #tlrl 2216), or immobilized anti-CD3 (αCD3) used at 0.5 µg/ml (Biolegend, San Diego, California, USA # 300302). After 24 h of culture, a part of HCL cells was collected to assess their activation, and another fraction was incubated with DMSO as control or with venetoclax (ABT-199, MedKoo, Morrisville, North Carolina, USA) at 0.1 or 1 µM, in all the conditions detailed above. After another 24 h of culture, HCL cells were collected to further assess activation and to evaluate cell death. HS-5 cell line was purchased from ATCC, Manassas, Virginia, USA (CRL-11882).

Flow Cytometry Studies

For surface antigen staining, cells were incubated for 30 min at 4°C with the corresponding antibodies diluted in PBS supplemented with 0.5% BSA and washed twice in the same buffer before acquisition. Activation of HCL cells was evaluated by measuring the expression of CD69 (BD, Bergen, New Jersey, USA Biosciences #555531) at 24 h of culture, or CD86 (BD, Bergen, New Jersey, USA Biosciences #555658, Biolegend #305412 or # 374203) at 48 h of culture, and anti-CD19 (clone J3-119, Beckman Coulter, Brea, California, USA # A07771 or Biolegend #302254). For immunoglobulin light chain usage determinations, anti-Kappa PE and anti-Lambda PE antibodies were purchased from BD, Bergen, New Jersey, USA Biosciences (#555792 and 555797, respectively). Antibodies used to detect CD25, CD4, and CD56 were purchased from BD Biosciences, Bergen, New Jersey, USA (#555431, 555347 and 555517, respectively). Antibodies used for CD103 and CD8 were purchased from Biolegend (#121406 and 300908, respectively). Annexin V staining and Apotracker Green (Biolegend #427402) were used to evaluate the percentage of cell death induced by venetoclax. After the incubation with the drug, PBMCs were stained for CD19 and next incubated for 20 min at room temperature with Annexin V-FITC (Biolegend #640905) in the corresponding binding buffer to discriminate viable and dead cells. Flow cytometry analysis was performed immediately after incubation, by gating HCL cells or normal lymphocytes in the forward *versus* side scatter and by studying the CD19+ and CD19

negative populations. Cell death was corroborated by flow cytometric alterations of light-scattering properties. Cells were acquired on a FACScalibur cytometer (Becton Dickinson) or FACSARIA III (BD Biosciences). Data were analyzed with FlowJo 10.6.1 software (Tree Star, Inc).

Statistical Analysis

Paired one-way analysis of variance (ANOVA) using correction followed by multiple comparison tests or two-tailed paired Student's *t*-tests was performed to evaluate statistical significance. Data sets that did not pass normality tests were analyzed using Friedman test followed by Dunn's multiple comparisons. Variables with $P < .05$ were considered to be significant. All analyses were performed using GraphPad Prism 8 software version 8.0.01 (GraphPad, San Diego, CA).

RESULTS

Venetoclax Induces Cell Death in Primary HCL Leukemic Cells

To test the effect of venetoclax in HCL, we developed primary cultures using PBMC samples obtained from eight diagnosed patients. Six of them belong to the classic HCL group, whereas two were variant HCL cases. Additional characteristics of the cohort enrolled in this study are detailed in **Table 1**. History of previous treatment/s and infection/s is included in **Supplementary Table S1**. Using the flow cytometry FSC and SSC parameters, HCL cells can be distinguished from normal lymphocytes based on increased size and granularity (24) (**Figure 1A** and **Supplementary Figure S1**). More than 95% of cells within this gate were CD19+ kappa+ or CD19+ lambda+, corroborating their leukemic origin. The cohort approached the expected frequency of immunoglobulin light chain gene usage in HCL of 50% (25) (**Table 1**).

PBMCs were exposed to clinically relevant concentrations of venetoclax (0.1 and 1 μM) for 24 h, and cell death was evaluated by Annexin V binding and flow cytometry analysis (**Figure 1A**). Venetoclax significantly augmented cell death in HCL cells at both concentrations tested in a dose-dependent manner (**Figure 1B**). Three samples were additionally evaluated at 48 and 96 h of incubation, showing increased cell death in leukemic cells that reached up to 90% of positivity (**Supplementary Figure S2**). Non-malignant lymphocytes (T + NK cells) were sensitive to venetoclax at 1 μM, but not when the drug was added at 0.1 μM (**Figure 1C**). Since it was previously reported that T cells from CLL patients and healthy donors are less sensitive to venetoclax (17, 26), we compared the levels of cell death induced in HCL cells and in T + NK cells without finding a significant differential sensitivity to this drug in our experimental settings (**Figure 1D**).

Activation of HCL Cells Enhances Resistance to Venetoclax-Induced Cell Death

We next determined whether activation of HCL cells through signals from a supportive microenvironment could impair venetoclax-induced cell death. Since CD40–CD40L triggering plus IL-4 represents a known activator of HCL cells (27), we first tested if αCD3 stimulation could mimic such phenomenon. After 24 h of stimulation, HCL cells upregulated CD69 due to the presence of autologous activated T cells by CD3 engagement (**Supplementary Figure S3A**), and after 48 h of culture, a significant increase in CD86 positive HCL cells was observed (**Figure 2A**). Upon stimulation, HCL cells were highly resistant to venetoclax-induced death at both doses evaluated (**Figure 2B**).

TABLE 1 | Characteristics of HCL patients included in this study.

Patient ID/ code	Sex	Age	HCLc/ HCLv	Flow cytometry phenotype	WBC count (μl)	Hematocrit (%)	Platelets (μl)	% HCL Cells in PBMC	Light chain usage	IGHV gene usage	BRAF V600E mutation
HCL#1 ◊	M	88	Variant [molecular]	$CD19^{+}$, $CD20^{++/+}$, $CD10^{-}$ $CD38^{-}$, $CD5^{-}$, $CD103^{+}$ $LAIR^{++}$, $CD25^{+}$	3,840	22	8,000	15.1	λ	IGHV 4-34	No
HCL#2 □	M	58	Variant [phenotypic]	$CD45^{++}$, $CD19^{++}$, $CD20^{++}$ $CD10^{+}$, $CD38^{-/dim}$, $CD5^{-}$ $CD81^{+}$, $CD43^{-}$, $CD103^{+v}$ $LAIR^{++v}$, $CD11c^{++v}$, $CD25^{-}$	1,570	22	21,000	34.2	λ	IGHV 2–70	ND
HCL#8 ○	M	61	Classic	$CD19^{+}$, $CD20^{++/+}$, $CD10^{+}$ $CD38^{-}$, $CD5^{-}$, $CD305^{++}$ $CD103^{++}$, $CD11c^{++}$, $CD25^{+}$	2,500	35	53,000	28.4	λ	IGHV 5–51	ND
HCL#9 Δ	M	32	Classic	$CD19^{+}$, $CD20^{+}$, $CD10^{-}$, $CD38^{-}$, $CD5^{-}$, $CD43^{-}$, $LAIR^{++}$ $CD103^{+}$, $CD11c^{+}$, $CD25^{+}$	3,800	42	85,000	24.3	κ	IGHV 3–30	Yes
HCL#14 ▽	M	62	Classic	$CD19^{+}$, $CD20^{+}$, CD10 $CD38^{-}$, CD5–, $CD81^{-}$, $CD305^{++}$, $CD103^{+}$ $CD11c^{+}$, $CD25^{+}$	2,500	42	137,000	44	κ	ND	Yes
HCL#15 ⬡	M	70	Classic	$CD19^{++}$, $CD20^{++}$, $CD103^{+}$ $CD25^{+}$,LAIR 1^{++}, $CD11c^{++}$ $CD45^{++}$, $CD38^{-}$, $CD10^{+/-}$ $CD43^{-}$, $CD23^{-}$, $CD5^{-}$	3,000	37.3	83,000	10.3	λ	ND	Yes
HCL#18 ⊠	M	46	Classic	$CD19^{+}$, $CD20^{+}$, $CD11c^{+}$,$CD25^{+}$, $CD103^{+}$, $CD123^{+}$	3,680	38.5	75,000	10.8	κ	ND	Yes
HCL#23 ⊗	M	58	Classic	$CD19^{+}$, $CD20^{+}$, $CD11c^{+}$,$CD25^{+}$, $CD103^{+}$, $CD123^{+}$	8,800	33.6	46,000	50.4	λ	ND	Yes

M, male; WBC, white blood cell count; κ, kappa immunoglobulin light chain; λ, lambda immunoglobulin light chain; IGHV, immunoglobulin heavy chain variable; ND, not determined. Symbols assigned to each patient in this table are depicted in the subsequent figures to allow tracking of particular cases.

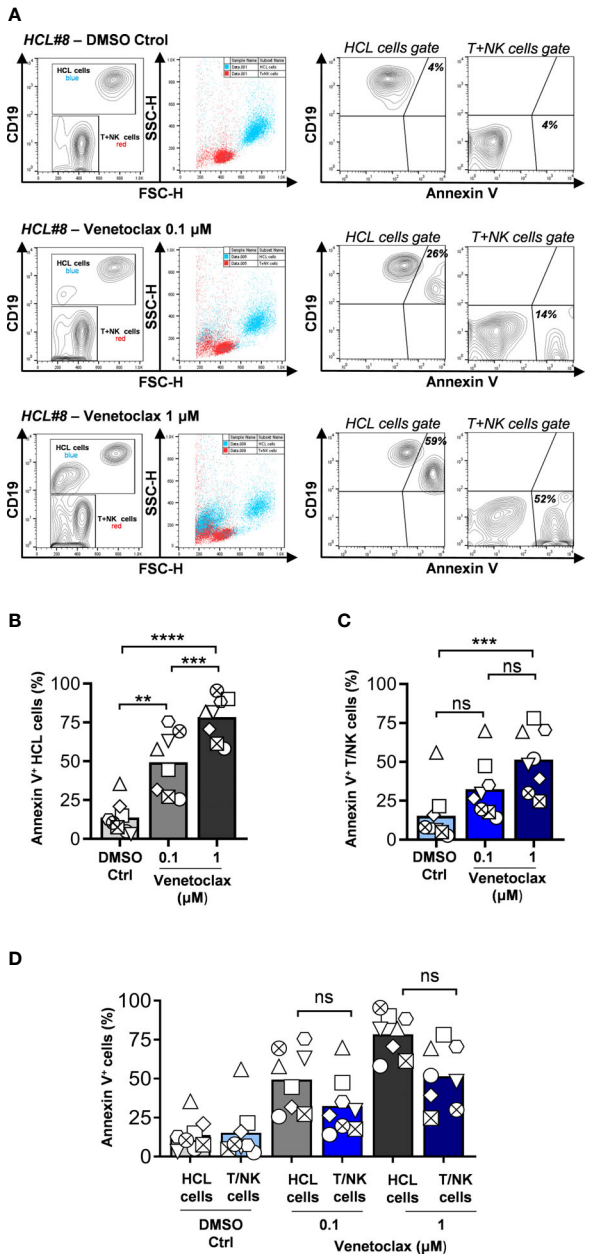

FIGURE 1 | HCL cells are sensitive to venetoclax-induced cell death *in vitro*. **(A)** PBMCs were obtained by Ficoll-Paque centrifugation from peripheral blood of eight HCL patients, incubated with venetoclax for 24 h, and cell death was analyzed by flow cytometry. Leukemic cells or T + NK cells were discriminated according to CD19 expression and forward side scatter, and next gated to determine binding of Annexin V. Results from a representative sample (HCL#8) are shown. **(B)** HCL cell death induced by venetoclax for the whole cohort evaluated. **(C)** T + NK cell death induced by venetoclax for the whole cohort evaluated. **(D)** Comparison of cell death induced in HCL cells and T + NK cells. For all the group graphics, each symbol represents a single patient (n = 8, **p < 0.01, ***p < 0.001, ****p < 0.0001, paired one-way ANOVA using correction for **(B)** and Friedman test followed by Dunn's multiple comparisons test for **(C, D)**, ns, not significant).

This was also the case when HCL-derived PBMC samples were co-cultured with the stromal cell line HS-5. As shown in **Figures 2C, D** and in **Supplementary Figure S3B**, HCL co-cultured with the HS-5 cells increased their size and granularity, upregulated CD86, and showed diminished values of Annexin V positivity when treated with venetoclax at 0.1 and 1 µM.

Finally, we evaluated the role of Toll-Like Receptor (TLR) engagement in the activation of primary HCL samples and studied their potential to affect venetoclax-induced cell death of tumor cells. For such aim, we incubated patient-derived PBMCs with CpG (5 µM) or PAM3 (300 ng/µl), ligands for TLR9 and TLR2, respectively. A significant increase in the expression of CD86 in HCL cells was observed after 48 h of stimulation with PAM3 (**Figure 2E**), whereas CpG did not induce statistically significant effects of activation markers in our cohort (**Supplementary Figure S3A**). **Figure 2F** shows the levels of death induced by venetoclax on HCL exposed to PAM3 or CpG for 24 h. A consistent decrease of Annexin V+ HCL cells for both stimuli was detectable when venetoclax was used at 0.1 or 1 µM, suggesting a protective role for TLR9 and TLR2 signaling in venetoclax-induced cell death of HCL cells.

DISCUSSION

In the present work, we focused on studying the effect of venetoclax in HCL *in vitro* and chose to work with patient-derived samples. We forwarded into this approach because previous reports using HCL cell lines detected absence of key hallmarks of this disease such as BRAF-V600E mutation (28, 29) and differences in sensitivity to drugs such as Ibrutinib as compared to their effect on primary HCL samples (30). Additionally, inherent resistance to Bcl-2 inhibition by venetoclax has been previously reported in tumor-derived human B cell lines (31). In our series of eight HCLs, two patients belong to the variable HCL group and showed similar values of venetoclax-induced cell death, of activation by the three stimuli tested, and of their protection capacity towards cell death induction, compared to the six classic HCL patients enrolled. The same occurred with the only patient that belongs to the VH4-34 molecular variant group of HCL, sample where the parameters evaluated were comparable to the remaining three in which we could identify the immunoglobulin (IG) variable heavy chain gene of use. Similarly, venetoclax-induced cell death was achieved independently of the IG light chain usage, the percentage of circulating HCL cells, white blood cell or platelet counts, sex, or age of the patient.

The main limitation of our study is the low number of samples analyzed. Testing primary HCL samples remains a difficult task mainly due to the low frequency of the disease. Besides being a rare neoplasia, as most patients develop pancytopenias, a limited amount of leukemic cells is usually obtained from peripheral blood, and extraction during bone marrow biopsies faces the additional complication of fibrosis. Furthermore, cladribine-treated patients remain with undetectable circulating leukemic

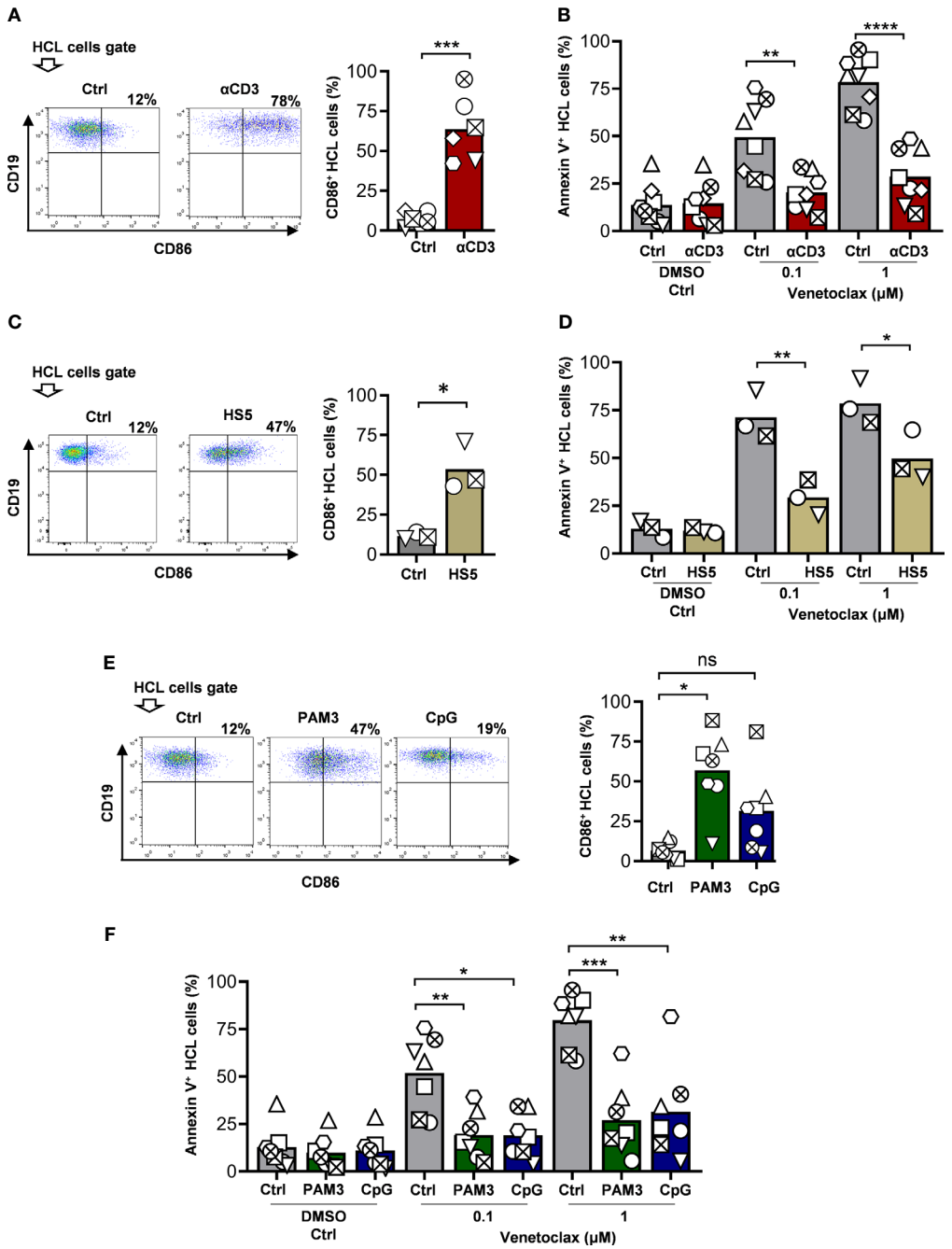

FIGURE 2 | Activation of HCL cells confers resistance to venetoclax-induced cell death. **(A)** PBMCs from HCL patients were incubated with medium alone (Ctrl) or on anti-CD3-coated wells (25 ng). At 48 h, cells were labeled with anti-CD19 and anti-CD86. Shown are representative dot plots (left) and mean values (right) for n = 6 samples (two-tailed paired *t*-test). **(B)** PBMCs from HCL patients were incubated with medium alone (Ctrl) or on anti-CD3-coated wells for 24 h. Then venetoclax (0.1 or 1uM) or 0.01% DMSO as vehicle was added for additional 24 h. HCL cell death was analyzed by flow cytometry as described in the legend of **Figure 1**. Shown are the results for the entire cohort (n = 8). **(C)** PBMC from HCL patients were co-cultured with HS-5 stromal cells for 48 h and labeled with anti-CD19 and anti-CD86. Shown are representative dot plots (left) and mean values (right) for n = 3 (two-tailed paired *t*-test). **(D)** PBMCs from HCL patients were co-cultured with HS-5 for 48 h. Then venetoclax (0.1 or 1uM) or 0.01% DMSO as vehicle was added for additional 24 h, and HCL cell death was analyzed as described above. **(E)** PBMCs from HCL patients were incubated with medium alone (Ctrl) or in the presence of PAM3 (300 ng/ml) or CpG (5 μM). At 48 h, cells were labeled with anti-CD19 and anti-CD86. Shown are representative dot plots (left) and mean values (right) for n = 7. **(F)** PBMCs from HCL patients were incubated with medium alone (Ctrl) or in the presence of PAM3 (300 ng/ml) or CpG (5 μM) for 24 h. Then venetoclax (0.1 or 1uM) or 0.01% DMSO as vehicle was added for additional 24h and HCL cell death was analyzed as described above. Shown are the results for the cohort studied (n=7). For all the group graphics, each symbol represents a single patient and paired one-way ANOVA followed by multiple comparisons were performed unless otherwise indicated (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns, not significant).

cells for years. For us, all these characteristics resulted in little available material to increase the size of the cohort of study, vary concentrations and exposures to the drug or to further into the mechanisms of action and deepen into the role of Bcl-2 inhibition in this leukemia.

CONCLUSION

We have observed that venetoclax is able to induce a clear pro-apoptotic effect in primary HCL cells. This effect does not appear to operate differently when studying samples derived from the classic, variant, or VH4-34 forms of the disease. HCL cells can be activated by co-culture with stromal cells and by TLR triggering, as already shown for T-cell mediated stimulation. Activation of HCL leads to resistance to venetoclax-induced cell death. The signals provided by the accessory cells that compose the HCL microenvironment in the bone marrow or spleen of patients could mimic such activation *in vivo* in the human disease. We propose that venetoclax could be considered for HCL therapy, and that combinatory strategies with drugs that target the microenvironment may improve its efficacy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PM, MG, MB, and RG designed the research. PM and MG wrote the manuscript. AV, PM, MG, AC, and EG performed experiments and analyzed data. EM and JP collaborated in the design and data presentation. RB, HF, GC, MC, J-HF, and GB provided patient samples and advice and contributed in the interpretation of the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.598319/full#supplementary-material

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