NEW INSIGHTS INTO THE COMPLEXITY OF TUMOR IMMUNOLOGY IN B-CELL MALIGNANCIES: DISEASE BIOLOGY AND SIGNALING

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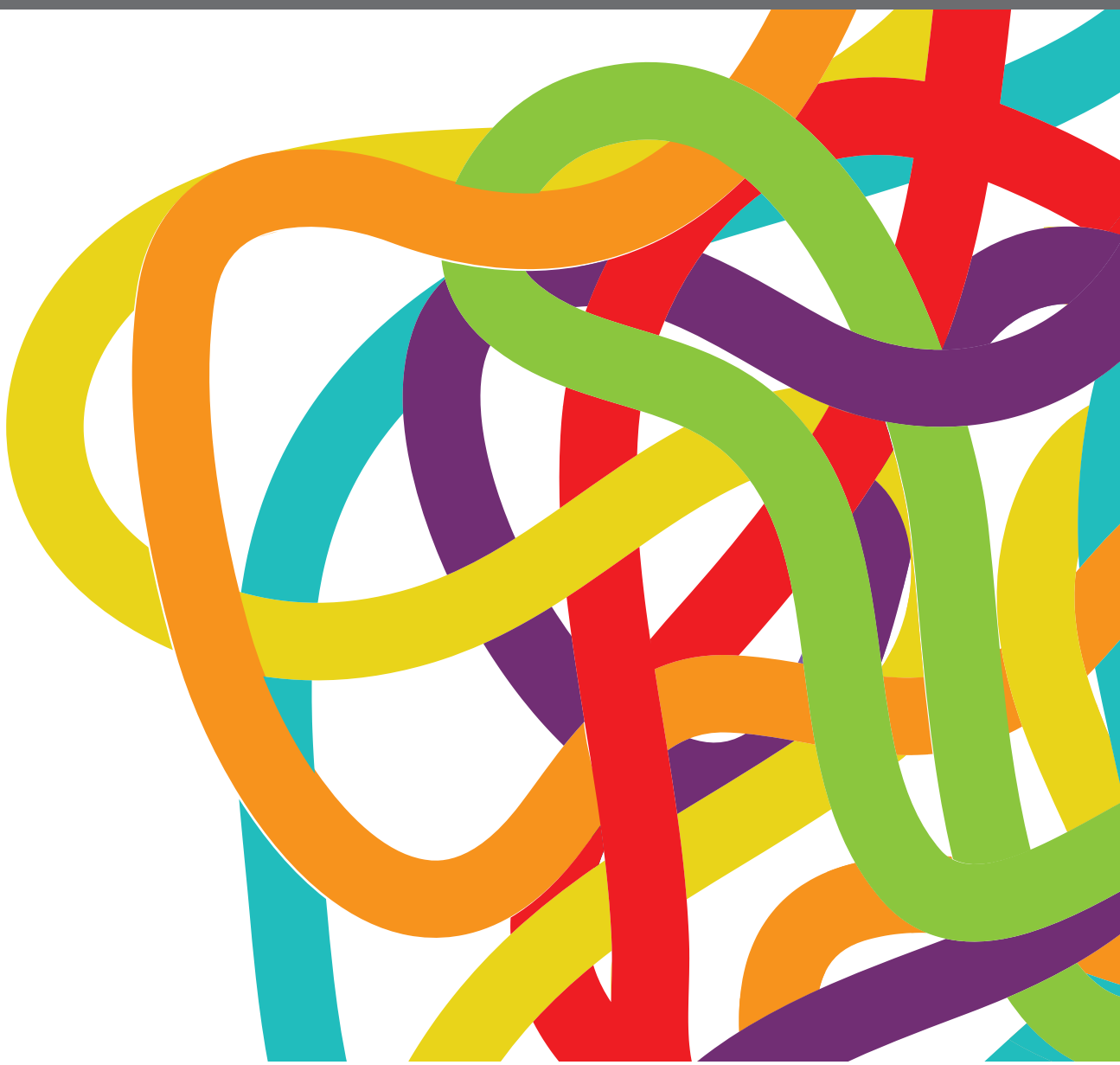

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NEW INSIGHTS INTO THE COMPLEXITY OF TUMOR IMMUNOLOGY IN B-CELL MALIGNANCIES: DISEASE BIOLOGY AND SIGNALING

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EDITORIAL
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Editorial: New Insights Into the Complexity of Tumor Immunology in B-Cell Malignancies: Disease Biology and Signaling

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Editorial on the Research Topic

New Insights Into the Complexity of Tumor Immunology in B-Cell Malignancies: Disease Biology and Signaling

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Over the last decade, the interactions between neoplastic lymphocytes and the tumor microenvironment, especially the immune system and stromal cells, have proven to be key in the pathogenesis of B-cell neoplasms, including chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia, Hodgkin and Non-Hodgkin lymphomas (NHL), and multiple myeloma. In these malignancies, tumor cells in the bone marrow, lymph nodes, spleen or in peripheral blood are by nature surrounded by immune cells such as B and T lymphocytes, regulatory T cells, monocytes, macrophages, and neutrophils, which were shown to harbor tumor-supportive and immunosuppressive features. Hence, understanding the complex crosstalk between malignant and non-malignant cells in lymphoid malignancies is crucial to design innovative therapeutic strategies, including immunotherapy. To this end, an in-depth characterization of tumor-associated immune cells and other accessory cells and their complex interactions with malignant cells within the tumor microenvironment will be crucial to identify new therapeutic targets and to develop successful combination treatment strategies including immunomodulatory drugs. In this first volume, 13 articles explore disease biology and signaling in different B-cell malignancies and their connection to the microenvironment.

CLL cells are mainly quiescent in the peripheral blood but a small proliferative pool exists in the lymph nodes. In a timely review (1), Haselager, Kater and Eldering explain the different signals implicated in CLL proliferation and their connections to the lymph node microenvironment. They stress on the importance of the B cell receptor (BCR) activation in CLL cell growth and the therapeutic targeting of this pathway using inhibitors of the Bruton's tyrosine kinase (BTK). However, *in vitro* BCR triggering is unable to generate significant cell proliferation, highlighting the complexity of the *in vivo* situation and the need for better *in vitro* modelling to mimic the disease. This is the main focus of a mini review (2) by Scielzo and Ghia in which they highlight the current lack of a suitable culture model in B-cell malignancies compared to solid tumors. They detailed the current innovation regarding static and dynamic 3D culture in which accessory cells can be

introduced with the goal to better reflect the *in vivo* microenvironment. These models should be further developed and will become indispensable to evaluate the potency of novel drugs *in vitro*.

As stated above, BCR activation is a key component of B-cell malignancies. In a dedicated review (3), Thurner, Bewarder and colleagues discuss the current understanding of specific antigens recognized by the BCR of various lymphomas. These antigens can be exogenous from bacteria or autoantigens which are mostly the results of abnormal post-translational modifications. This knowledge could lead to the development of new therapies exploiting the recognition of lymphoma cells *via* these specific peptides.

CLL cells are highly dependent on their interactions with the microenvironment. This is illustrated by the fact that primary CLL cells undergo rapid and spontaneous apoptosis when cultured *in vitro*, but survival is promoted when stromal cells are added to the culture. In a very detailed review (4), Dubois, Stamatopoulos and colleagues explained the bidirectional crosstalk between stromal cells, mainly mesenchymal stem cells and follicular dendritic cells, and malignant cells which support leukemic cell survival, chemotaxis and homing through the activation of specific signaling pathways. Hence, targeted therapies disrupting this multifaceted crosstalk could abolish the stroma-supportive effects and favor elimination of the leukemic clone. Myeloid cells are also important players in the microenvironment of B-cell malignancies. In an original research article (5), Farnworth-McHugh, Gregory and colleagues demonstrated that in starry-sky B-cell lymphoma, tumor-associated macrophages accumulate and display phagocytic activity towards apoptotic lymphoma cells supporting their pro-oncogenic phenotype. The receptor tyrosine kinase MERTK is critical in this phenomenon, opening novel therapeutic opportunities with targeted kinase inhibitor.

Understanding the complex relationship between different lymphoma entities and their ecosystem, especially in the light of tumor heterogeneity, is fundamental to unravel specific vulnerabilities and design efficient (immuno) therapies. Ysebaert, Fournié and colleagues explain in an informative review (6), how single-cell transcriptomic technologies can be used to tackle these problematics. They also discuss how these novel technical approaches can be used in clinical trial to monitor treatment response and the development of resistance and toxicity with the aim to foster precision medicine.

Metabolism rewiring is an important feature of cancer development. Böttcher, Mougiakakos and colleagues described in a comprehensive mini review (7), the metabolic alterations found in CLL and different B-cell NHL, and how they can impact anti-lymphoma immunity. Indeed, they introduced a rather novel concept of immunometabolic regulation where lymphoma and immune cells communicate through metabolic reprogramming, creating novel dependencies that could be therapeutically exploited. In the future, efforts should be undertaken to decipher these metabolic pathways with the aim to target lymphoma cell vulnerabilities and at the same time reactivate immune cells with a more favorable microenvironment.

In a complementary review (8), Domka, Goral and Firczuk addressed the dual role of reactive oxygen species (ROS) in B-cell malignancies. Malignant B cells display an imbalanced redox homeostasis that require their metabolic adaptation which is in part supported by stromal cells. ROS have also detrimental effects on immune cells affecting the anti-tumor immune response. Therapies targeting the antioxidant system could therefore represent novel approaches in B-cell malignancies.

ZAP-70 is an important tyrosine kinase mainly implicated in T-cell receptor activation which can be aberrantly expressed in different B-cell malignancies, in particular in a subset of CLL patients associated with unfavorable outcome. In a focused review (9), Chen, Moore and Ringshausen explored the recent evidence demonstrating that beyond its intrinsic role in malignant B cells, ZAP-70 can also influence the crosstalk between tumor cells and their microenvironment, especially the immune cells. They also reviewed the function of ZAP-70 in T cells' and NK cells' anti-tumor immunity emphasizing the need of future studies to evaluate the benefit or harm of ZAP-70 therapeutic targeting in the frame of B-cell malignancies.

Another crucial enzyme in B cells is AID (activation-induced cytidine deaminase) which is implicated in immunoglobulin gene diversification. In a focused review (10), Oppezzo, Navarrete and Chiorrazi explained the detrimental role of AID in leukemogenesis and disease progression by its "off-target" mutagenic activity, and also how AID is regulated in the lymph node microenvironment. In a mini review (11), Munguia-Fuentes, Maqueda-Alfaro, Yam-Puc and colleagues described the microenvironment of lymphoid tissue germinal centers where B cells undergo antigen-driven somatic hypermutations, a mechanism that is associated with malignant transformation.

Sustained NF-κB activation is key in the pathogenesis of B-cell lymphomas. The adaptor protein MYD88 is an important player in the Toll-Like receptors signaling pathway which can be activated by the microenvironment and leads to NF-κB activation. In an original research article (12), Cardona-Gloria, Weber and colleagues identified different splice variants of MYD88 with variable signaling capacities in human B cells. Importantly, they demonstrated that malignant B cells favor splice variants that have NF-κB promoting activities. Contrary to myeloid cells, the negative feedback loop leading to the synthesis of variants with no signaling activities is absent in B cells, potentially explaining their susceptibility to lymphoma transformation.

Telomeres maintenance is also crucial for tumorigenesis. In a detailed review (13), Jebaraj and Stilgenbauer summarized the current knowledge on telomere length, telomerase activity, and associated proteins of the shelterin complex in CLL. All these components are dysregulated in CLL with a complex interconnection underlying their role in different phases of disease development. Nevertheless, studies should be conducted to evaluate the intrinsic and extrinsic signals leading to these modulations and the associated mechanisms in order to therapeutically target them.

With this Research Topic dealing with the microenvironment of B-cell malignancies, in particular its connection to disease biology along with the signaling pathways involved, we hope to

bring to the readers a timely and interesting overview of how the microenvironment shape the development and progression of these diseases independently of the genetic mutations of the tumor clone.

AUTHOR CONTRIBUTIONS

All authors edited the research topic. JP wrote the editorial. All authors contributed to the article and approved the submitted version.

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cROSsing the Line: Between Beneficial and Harmful Effects of Reactive Oxygen Species in B-Cell Malignancies

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B-cell malignancies are a heterogeneous group of hematological neoplasms derived from cells at different stages of B-cell development. Recent studies revealed that dysregulated redox metabolism is one of the factors contributing to the pathogenesis and progression of B-cell malignancies. Elevated levels of oxidative stress markers usually correlate with the advanced stage of various B-cell malignancies. In the complex tumor microenvironment, reactive oxygen species affect not only malignant cells but also bystander cells, including immune cells. Importantly, malignant cells, due to genetic dysregulation, are able to adapt to the increased demands for energy and reducing equivalents via metabolic reprogramming and upregulation of antioxidants. The immune cells, however, are more sensitive to oxidative imbalance. This may cause their dysfunction, leading to immune evasion and tumor progression. On the other hand, the already imbalanced redox homeostasis renders malignant B-cells particularly sensitive to further elevation of reactive oxygen species. Indeed, targeting antioxidant systems has already presented anti-leukemic efficacy in preclinical models. Moreover, the prooxidant treatment that triggers immunogenic cell death has been utilized to generate autologous anti-leukemic vaccines. In this article, we review novel research on the dual role of the reactive oxygen species in B-cell malignancies. We highlight the mechanisms of maintaining redox homeostasis by malignant B-cells along with the antioxidant shield provided by the microenvironment. We summarize current findings regarding therapeutic targeting of redox metabolism in B-cell malignancies. We also discuss how the oxidative stress affects antitumor immune response and how excessive reactive oxygens species influence anticancer prooxidant treatments and immunotherapies.

Keywords: oxidative stress, ROS, B-cell malignancies, immune evasion, CLL, B-ALL, lymphoma, leukemia

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INTRODUCTION

Oxidative metabolism is vital for all aerobic organisms. Reactive oxygen species (ROS) formed during the oxidative metabolism are important signaling molecules. However, to maintain redox homeostasis, the levels of ROS must be tightly controlled. Oxidative stress is defined as an imbalance between the generation or uptake of ROS and their scavenging. Exaggerated, prolonged oxidative stress leads to the oxidative macromolecule damage and cell death.

Dysregulated redox homeostasis is a hallmark of cancer and may lead to higher steady-state levels of ROS in cancer cells (1, 2). This is caused by the elevated production of ROS due to oncogene activation and intensified oxidative metabolism in cancer cells, as well as by ROS generated extrinsically, within the tumor microenvironment (TME). Although dysregulation of redox homeostasis has been mainly studied in solid tumors, it also occurs in hematological malignancies. Increased levels of ROS were demonstrated in many B-cell malignancies including B-cell acute lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL), and B-cell lymphomas (3–6). Noteworthy, B-cell-derived malignant cells are particularly sensitive to prooxidant treatments, which may be caused by their specific metabolic reprogramming (7, 8) or reliance on selected antioxidant pathways (9, 10). Recent studies revealed that also the TME shapes the redox homeostasis. Selected stromal cells of the TME, e.g., mesenchymal stem cells, aid malignant cells to alleviate oxidative stress, supporting their survival (11, 12). Other cells, such as macrophages or polymorphonuclear subset of myeloid-derived suppressor cells (PMN-MDSCs), generate high amounts of ROS, contributing to elevation of ROS levels in the TME (13, 14). This may negatively affect the function of effector immune cells, causing impairment of antitumor immune response.

Here we review novel findings of the manifold effects of steady-state and therapy-induced ROS on B-cell malignancies, including their direct influence on malignant and normal cells' survival and/or function. We also discuss the possible consequences of increased ROS levels on the effectiveness of anticancer therapies, mainly prooxidant treatments and immunotherapies. The multifaceted effects of ROS on B-cell malignancies are presented in **Figure 1**.

INTRINSIC AND EXTRINSIC SOURCES OF ROS IN B-CELLS MALIGNANCIES

Elevated ROS levels and subsequent oxidative stress are features of various B-cell malignancies, which in majority of cases correlate with the clinical stage of the disease and patients' survival. The accumulation of oxidation products of phospholipids and/or DNA in serum as well as elevated levels of intracellular ROS in malignant B cells were observed in patients suffering from CLL (4, 15, 16). Recently, the increased levels of oxidative stress biomarkers were also found in B-ALL (6) and different types of non-Hodgkin lymphoma (NHL) (3).

ROS come from a number of sources and are generated both by malignant cells (intrinsic) as well as the cells of the TME, nutrients, pollutants or therapies (extrinsic). Intrinsic ROS are by-products of metabolic processes such as mitochondrial oxidative phosphorylation, fatty acid oxidation (FAO), and protein folding in the endoplasmic reticulum. In humans there are over 40 different ROS-generating enzymes including various NADPH oxidases (NOXs), cytochrome P450, superoxide dismutases (SOD), and others (17–19). In CLL, the elevated ROS have been linked to increased mitochondrial biogenesis and activity (4). Although NOXs are not responsible for ROS overproduction in CLL cells (4), NOX5 is specifically expressed in hairy cell leukemia and generates H_2O_2, which promotes oncogenic signaling (20). High amounts of ROS also come from FAO occurring in peroxisomes and mitochondria. The lipoprotein lipase along with peroxisome proliferator activated receptor (PPAR)α are elevated in CLL, drive FAO and reprogram malignant cells to use lipids as an alternative energy source (21, 22). Other types of B-cell malignancies also display enhanced FAO. For instance, a subtype of diffuse large B-cell lymphoma (DLBCL) depends on oxidative phosphorylation and FAO for its survival and growth (23).

In addition to enhanced ROS generation, the oxidative stress in B-cell malignancies results from diminished capacity of antioxidant systems (24). Indeed, decreased activity of SOD2 and catalase (CAT) has been reported in CLL and DLBCL patients compared to healthy individuals (16, 25). Reduced CAT expression along with increased amounts of mitochondria were found in less aggressive CLL subtype (26).

Apart from cancer cells, other cells of the TME, in particular myeloid cells, may contribute to ROS production, mainly via NOX-dependent manner (27, 28). The accumulation of selected populations of monocytes and their immunosuppressive reprogramming were reported in CLL patients (29, 30) as well as in the murine Eμ-TCL1 model of human CLL (31). Moreover, Manukyan and co-workers observed functional alterations in circulating neutrophils in CLL patients including phenotype change and elevated ROS production (32).

HOW MALIGNANT B-CELLS COPE WITH ELEVATED ROS LEVELS?

The effects of ROS on cancer cells vary depending on their concentrations (33). Low ROS levels may activate malignant B-cell proliferation through stimulation of B-Cell Receptor (BCR) and pre-BCR signaling (34), while higher levels induce apoptosis. To prevent oxidative stress and subsequent cell death due to elevated ROS levels, cells utilize numerous antioxidants. They include both small non-enzymatic molecules, such as glutathione (GSH), bilirubin, ferritin, NADPH, as well as antioxidant enzymes, e.g., thioredoxins (TXNs), thioredoxin reductases (TXNRDs), peroxiredoxins (PRDXs), CAT, SODs, heme oxygenase (HO-1) and a plethora of GSH-dependent enzymes (35, 36). The renewal of reduced GSH, TXN and other antioxidant molecules relies on cellular supplies of NADPH, which is primarily generated via pentose phosphate pathway (PPP).

Various transcription factors controlling redox balance are operating in selected B-cell malignancies. Signaling related to nuclear factor erythroid 2-related factor 2 (NRF2), a master regulator orchestrating antioxidant response, is enhanced in primary CLL (37) and mantle cell lymphoma (38) cells. Enzymes most frequently activated in B-cell malignancies belong to the TXN family. PRDX1 and PRDX2 are upregulated in Burkitt lymphoma cells (39), PRDX1 and PRDX3 in primary CLL cells (10, 40), PRDX1 and TXN1 in B-ALL cells (6), and TXN1 in the OXPHOS-DLBCL (41). The increase of antioxidant response

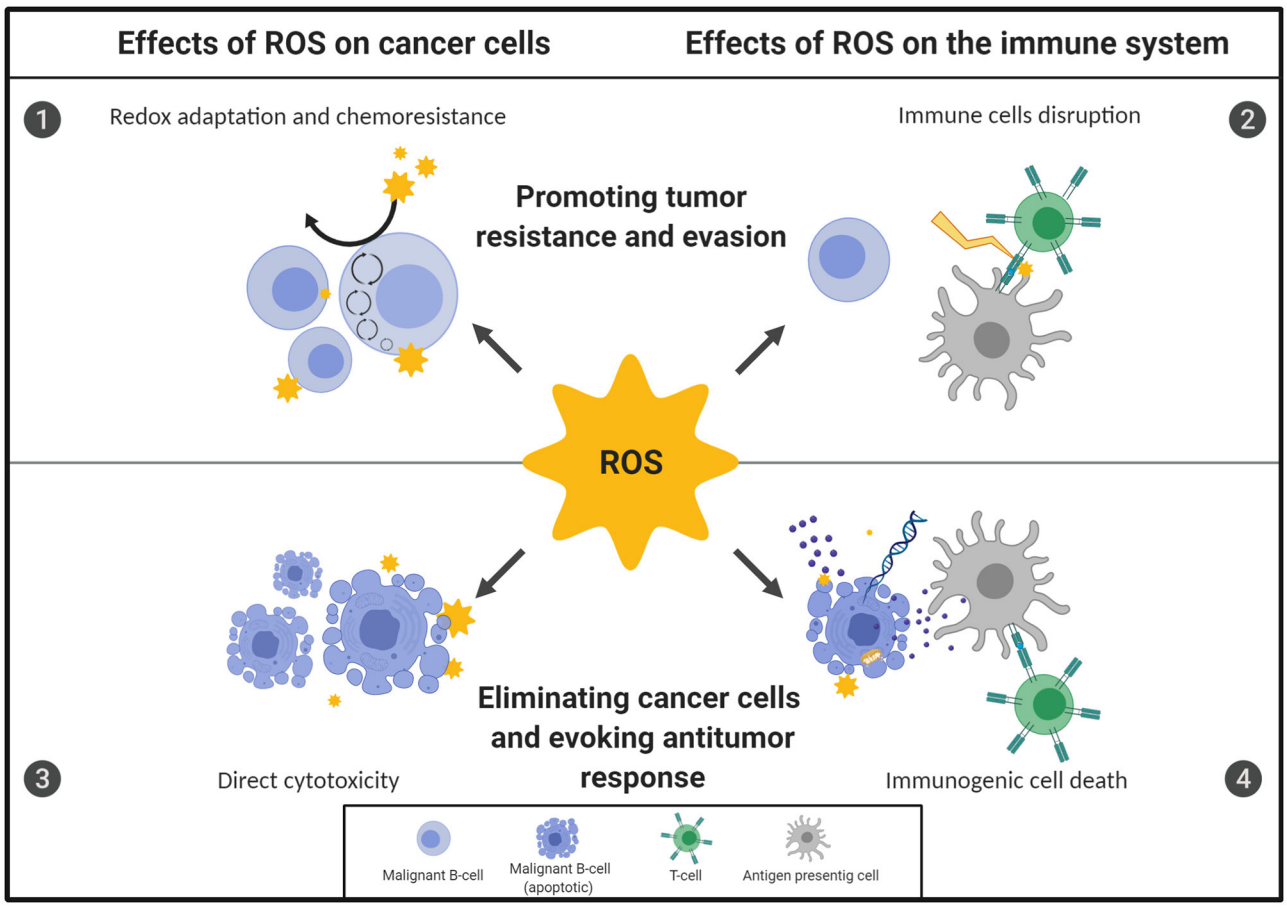

FIGURE 1 | Oxidative stress displays a dual role in the fate of B-cell malignancies. The ultimate outcome of redox imbalance on B-cell malignancies depends on the source, concentration, location of reactive oxygen species (ROS), as well as the duration of exposure. Importantly, the overall effect of ROS on the fate of B-cell neoplasms depends on the action of ROS both on malignant B-cells and on the cells of the TME, including immune cells. However, malignant cells adapt to the increased demands for energy and reducing equivalents by increasing their antioxidant capacity and rewiring their metabolic energy pathways, which renders them therapy-resistant [1]. At the same time, the highly oxidative microenvironment may negatively affect function of the effector immune cells, causing impairment of antitumor immune response, contributing to immune evasion and further tumor progression [2]. Conversely, targeting specific antioxidant pathways in malignant B cells or inhibiting the antioxidant support provided by the TME trigger direct, oxidative-stress mediated cytotoxicity and can effectively eradicate malignant B cells [3]. Moreover, some forms of oxidative stress-mediated cell death under certain conditions are immunogenic. This leads to the activation of immune cells and subsequent development of antitumor response, what further contributes to the efficacy of prooxidant treatments [4]. The figure was created using BioRender.com.

in B-ALL occurs also via serine/threonine-protein phosphatase 2A (PP2A), which negatively regulates protein kinase B (AKT) and thus activates other key transcription factors promoting antioxidant response—the Forkhead box proteins (FOXO1 and FOXO3a). The antioxidant capacity in the B-ALL cells is further supported by NADPH production via activation of PPP, which is also mediated by PP2A (8).

TME AND STROMAL SUPPORT IN MAINTAINING REDOX HOMEOSTASIS

Bone marrow niche plays a crucial role in survival and progression of B-cell malignancies. Primary CLL and B-ALL cells undergo apoptosis when grown *in vitro* without stromal support (40, 42). The co-cultures with stromal cell lines, primary mesenchymal stem cells (MSC) (6) or adipocytes (43), promote survival of primary CLL and B-ALL cells and increase their resistance to therapies (43, 44). Tumor-stroma interactions occur on many levels (45). Recent studies highlight the key role of stromal cells in alleviating oxidative stress in malignant B-cells (40). The stromal support can be delivered directly, by providing antioxidants, or indirectly, by inducing antioxidant response in malignant B-cells.

It has been found that TXN1 secreted by stromal cells in the CLL lymph nodes, promoted proliferation and survival of the primary CLL cells (12). In another study, the MSC in the bone marrow aided CLL cells by uptake of cystine via Xc- transporter and subsequent secretion of cysteine, which was then used by malignant cells to synthetize GSH and overcome oxidative stress conditions (11). The depletion of the external cysteine by recombinant cysteinase in the Eμ-TCL1

mice resulted in significantly prolonged median survival time of the mice, confirming the crucial role of the MSC-derived cysteine in leukemia progression (46). Similarly, a dependence on stromal cysteine support was also reported in B-ALL (47). The mechanisms of stromal redox support in lymphomas are less thoroughly documented, although there is some evidence that the DLBCL cells may be aided by GSH received from fibroblastic reticular cells (48).

Stromal cells can also reduce oxidative stress and protect from ROS-inducing chemotherapy by transfer of organelles to leukemic cells via tunneling nanotubes (TNTs). These cellular extensions act as bridges between cancer and stromal cells that enable intercellular transport (49, 50). Activated stromal cells transmitted mitochondria to B-ALL cells using TNT and protected B-ALL cells from cytarabine-induced apoptosis (44). However, the exact mechanism of this protection remains unclear. Presumably, it is associated with triggering of adaptive antioxidant signaling.

By comparing the transcriptomes of primary CLL cells grown in a monoculture or a co-culture with HS5 stromal cells, Yosifov et al. observed a significant differences in the expression of genes involved in ROS generation, ROS detoxification, and hypoxic signaling (40). Noteworthy, the CLL samples displaying the "co-culture-like" gene expression signature correlated with significantly worse patients' survival (40).

Alleviation of oxidative stress in the leukemic niche can also occur as a result of communication between malignant cells and stromal cells using extracellular vesicles. B-ALL cells metabolically reprogrammed stromal cells via secretion of extracellular vesicles, switching their main energy pathway from oxidative phosphorylation to aerobic glycolysis (51). Such alterations are likely to favor tumor survival by reducing oxidative stress in the microenvironment. A similar mechanism of exosome-driven metabolic reprogramming has also been discovered in CLL (52).

THERAPEUTIC TARGETING OF REDOX PATHWAYS IN B-CELL MALIGNANCIES

The dependence of malignant B-cells on antioxidants can be utilized in therapy. Treatments based on the generation of excessive ROS, so called "prooxidant," are selectively toxic to malignant B-cells and some of them exert antitumor effects *in vitro* and *in vivo*, mainly in preclinical models. Inhibition of the TXN system with auranofin or SK053 selectively killed malignant B-cells and displayed anti-leukemic activity in animal models of CLL (53) and B-ALL (6). Some efficacy against B-cell malignancies has been also demonstrated for agents interfering GSH and L-Cys (46, 54). Although blocking of a single antioxidant pathway may result in some toxicity to malignant B-cells, it is usually at best partially effective (6, 53). Inhibitors of the antioxidant systems more potently kill malignant B-cells when administered in combinations with other drugs, and particularly so in combination with ROS-generating agents such as L-ascorbate (10).

Another way to evoke excessive oxidative stress in malignant B-cells is to block the antioxidant shield provided by the microenvironment. Approaches which proved effective in preclinical models include inhibition of Xc- cystine transporter with sulfasalazine (55) as well as blocking of TNTs formation and transfer of mitochondria to B-ALL cells by inhibiting microtubule polymerization with vincristine (44). The recently presented, unbiased approach aimed to find agents capable of blocking stroma-mediated protection identified ouabain and emetine, drugs perturbing redox homeostasis. Along with the increase in mitochondrial ROS levels in CLL cells and depletion of cellular NADPH pool, emetine delayed CLL development in Eμ-TCL1 murine model (40).

The important feature of prooxidant approaches is their substantial B-lineage selectivity. Although the underlying mechanisms are not fully understood, some explanations were proposed. It was shown that thiol-reactive compounds activated NRF2-mediated signaling less extensively in malignant and normal B-cells as compared to other subpopulations of leukocytes and were more cytotoxic to CLL cells (37). The unique sensitivity of B-cells to dysregulation of redox homeostasis has been recently attributed to B-cell-specific metabolic reprograming. B-lymphoid transcription factors PAX5 and IKZF1 were shown to repress the key enzymes of the PPP, which resulted in insufficient generation of NADPH. To salvage oxidative stress, malignant B-cells upregulate PP2A, a key enzyme which redirects glucose metabolism to PPP. Accordingly, inhibition of PP2A delayed development of several different B-cell malignancies and had little effect on myeloid leukemia (8).

HOW OXIDATIVE STRESS AFFECTS IMMUNE RESPONSE?

The effects of ROS on immune system are at least two-sided. Prooxidant therapies are potent inducers of immunogenic cell death (ICD) in malignant cells. However, prolonged oxidative stress may also negatively affect the functions of selected populations of immune effector cells. The concept of ICD has altered the traditional view of non-immunogenic apoptosis and revealed that some forms of cell death better stimulate immune response than others. In the cancer field, dying cells undergoing ICD elicit robust, coordinated innate and adaptive immune response against tumor-specific antigens. ICD and the molecular processes that define immunogenicity are summarized here (56). A number of prooxidant treatments such as ionizing radiation, photodynamic therapy, ROS and endoplasmic reticulum stress-inducing chemotherapy or drugs interfering with cancer-specific energy metabolism, have been tested for their ability to induce ICD and stimulate antitumor immune response against different types of cancers [for further reading see (57–59)]. Only some of these ROS-inducing treatment modalities have been investigated in B-cell malignancies. In CLL cells, the PPARα antagonists increased TNFα and decreased IL-10 release, as well as stimulated T cell proliferation in allogenic mixed lymphocytic reaction (22). However, the most extensively tested in the context of triggering

immunogenicity of B-cell malignancies is ionizing radiation. As demonstrated in murine models of B-cell lymphoma, the time of local irradiation delivery has significant impact on the immunogenicity and the overall treatment efficacy. Only the irradiation delivered in an accelerated mode triggered ICD, increased infiltration of $CD4^+$ and $CD8^+$T cells, dendritic cells (DC), decreased the number of regulatory T cells (Tregs) in the TME and resulted in tumor regression (60). Ionizing radiation has been also used to generate antitumor vaccines. In NHL cell lines, ionizing radiation combined with heat shock, promoted expression of genes associated with antigen processing and presentation. The treatment was used to generate DC-based autologous vaccines against relapsed NHL. Although the clinical response was observed only in one third of patients, it correlated with the ability of malignant cells to undergo ICD (61). Similar approach has been already tested in CLL. Partial response, manifested by enhanced T cell proliferation and increase in a co-stimulatory molecule CD80 in CLL cells, was detected in one third of patients and correlated with lack of disease progression (62). Overall, the observed response to oxidized vaccines was relatively weak and the follow-up clinical trials have not been undertaken.

Apart from the direct cytotoxic effects on cancer cells and a positive impact on the immune response by inducing ICD, ROS can affect the course of the disease and therapy outcome by directly interacting with immune cells. Numerous findings accumulated over years (summarized in **Table 1**) have identified various effects of high ROS levels on different populations of immune cells. The chronic oxidative stress observed in B-cell malignancies may contribute to the immunosuppressive microenvironment and be a component of the immune evasion by malignant B-cells. Furthermore, it may negatively affect the efficacy of immunotherapies involving immune cell populations amenable to oxidative stress-mediated dysfunctions (e.g., therapeutic monoclonal antibodies, cellular therapies with autologous cytotoxic immune cells). The effects of oxidative stress on immune cells were not comprehensively studied in B-cell malignancies so far and the investigations are mostly limited to CLL. NK cells are the population of innate immune cells with remarkable sensitivity to negative ROS effects (73). CLL patients with higher ROS levels had elevated proportion of $CD56^{bright}CD16^{dim}$ NK cells in their circulation (4), the subpopulation which is characterized by significantly smaller cytotoxic activity (81). Alterations in conventional T lymphocytes were also observed in CLL patients with high ROS levels. Both $CD4^+$ and $CD8^+$T cells had reduced expression of CD3ζ, a key subunit of T cell receptor. Additionally, $CD4^+$T cells' activation markers (CD69, HLA-DR, and CD137) were decreased. When $CD4^+$T cells were *in vitro* stimulated for proliferation and activation in the presence of primary CLL cells, the addition of a ROS scavenger, N-acetylcysteine, significantly increased the expression of the activation markers and IFNy production in the T cells (4).

The oxidative imbalance also entails changes in other T cell subpopulations. In CLL patients it is associated with reduced number of memory T cells and a shift toward Th9 cells. The Th9 cells are a subpopulation of $CD4^+$T cells, characterized by the secretion of IL-9, the cytokine linked with leukemogenesis (82). Tregs are another $CD4^+$T cell subpopulation which is enriched in B-cell malignancies (83, 84). In CLL, the increase in Tregs correlated with disease progression (85). It was shown that Tregs survive better in the highly oxidative TME than conventional $CD4^+$ T cells (80), yet it remains to be elucidated whether the accumulation of Tregs in B-cell malignancies is related to increased oxidative stress.

TABLE 1 | Effects of excessive ROS levels on different populations of immune cells.

Immune cell subset	ROS effects
Macrophages	M2 polarization (63–65) Induction of immunosuppressive phenotype (66) Release of immunosuppressive chemokines (66)
MDSC	Maintaining undifferentiated, immunosuppressive phenotype (67–69)
Dendritic cells	Impaired antigen presentation by DCs (70)
NK Cells	Impaired activation and degranulation (71) Decreased cytotoxicity (72) Induction of apoptosis (73, 74)
Cytotoxic T-Cells	Promoting mitochondrial exhaustion of $CD8^+$T-Cells (75) Suppression of T-cell responses (76) Induction of apoptosis (77)
Regulatory T-Cells	Treg accumulation in the tumor microenvironment (78) Inducing adenosine-mediated immunosuppression (79) Better survival under oxidative stress (80)

Another important question is how treatment-related oxidative stress, which is stronger but more transient, affects antitumor immune response. It was demonstrated that anti-CD20 therapeutic monoclonal antibodies, rituximab and ofatumumab, induced potent ROS release from human monocytes cultured *in vitro* in the presence of primary CLL cells (72). Importantly, the mAbs triggered NK cell apoptosis when the NK cells were co-cultured with ROS-producing monocytes, reducing the efficacy of antibody-dependent cellular cytotoxicity (ADCC) and antitumor efficacy (72). These results suggest that the immunosuppressive ROS may also be generated in CLL patients during mAbs immunotherapy and may diminish its efficacy. Indeed, accumulation of immunosuppressive myeloid cell populations were detected both in murine model of CLL as well as in CLL patients (30).

CONCLUDING REMARKS

In recent years, a growing number of studies reports dysregulated redox homeostasis in various B-cell malignancies and the unique vulnerability of malignant B-cells to undergo oxidative stress-mediated apoptosis. We observe significant progress in our understanding of key antioxidant pathways in specific subtypes of B-cell malignancies. As exaggerated oxidative stress leads to apoptosis, this may be therapeutically utilized, as it renders

malignant B cells susceptible to antioxidant system inhibitors and other prooxidant treatments.

Although prooxidant therapeutic approaches have already proved effective in preclinical models of B-cell malignancies, their effective use in patients requires further research. This is mainly caused by the multifaceted effects of ROS on the immune system. On one hand, ROS trigger ICD of malignant B cells, which has been utilized in the production of autologous vaccines against CLL and lymphomas. ICD may also contribute to the overall effectiveness of prooxidant therapies used *in vivo*, due to the stimulation of the antitumor immune response. However, prolonged exposure to high ROS levels may negatively affect the viability and function of immune effector cells, which diminishes antitumor immune response and undermines the effects of therapy. To date, in B-cell malignancies only a few studies confirm that ROS may impair viability and effector functions of NK and T cell. However, it is still poorly understood how oxidative stress contributes to the immunosuppression observed in advanced B-cell malignancies, and even more so, how oxidative stress influences the development of an antitumor immune response as a result of ROS-inducing therapies. Further studies are needed to investigate the effects of ROS elicited by prooxidant therapies on antitumor immune response in B-cell malignancies. These studies should include both immunocompetent and immunodeficient animal models, as well as monitoring the function of immune cells during clinical studies testing the effects of prooxidant therapies in patients with B-cell malignancies.

AUTHOR CONTRIBUTIONS

MF developed the idea for the topic of the article. MF, AG, and KD contributed to the final conception, literature search, writing, editing, and critical revision of the manuscript. KD prepared a figure and a table. All authors contributed to the article and approved the submitted version.

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REVIEW
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Importance of Crosstalk Between Chronic Lymphocytic Leukemia Cells and the Stromal Microenvironment: Direct Contact, Soluble Factors, and Extracellular Vesicles

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Chronic lymphocytic leukemia (CLL) is caused by the accumulation of malignant B cells due to a defect in apoptosis and the presence of small population of proliferating cells principally in the lymph nodes. The abnormal survival of CLL B cells is explained by a plethora of supportive stimuli produced by the surrounding cells of the microenvironment, including follicular dendritic cells (FDCs), and mesenchymal stromal cells (MSCs). This crosstalk between malignant cells and normal cells can take place directly by cell-to-cell contact (assisted by adhesion molecules such as VLA-4 or CD100), indirectly by soluble factors (chemokines such as CXCL12, CXCL13, or CCL2) interacting with their receptors or by the exchange of material (protein, microRNAs or long non-coding RNAs) via extracellular vesicles. These different communication methods lead to different activation pathways (including BCR and NFκB pathways), gene expression modifications (chemokines, antiapoptotic protein increase, prognostic biomarkers), chemotaxis, homing in lymphoid tissues and survival of leukemic cells. In addition, these interactions are bidirectional, and CLL cells can manipulate the normal surrounding stromal cells in different ways to establish a supportive microenvironment. Here, we review this complex crosstalk between CLL cells and stromal cells, focusing on the different types of interactions, activated pathways, treatment strategies to disrupt this bidirectional communication, and the prognostic impact of these induced modifications.

Keywords: chronic lymphocytic leukemia, microenvironment, mesenchymal stromal cells, extracellular vesicles, prognostic factor

INTRODUCTION

During the last 20 years, the number of reports dealing with the interaction between chronic lymphocytic leukemia (CLL) and the surrounding cells constituting its microenvironment has increased exponentially. Focus has been placed on one particular actor in the bone marrow microenvironment that is also present in several lymphoid tissues: mesenchymal stromal cells (MSCs). The first evidence that the microenvironment is crucial for leukemic cell survival comes from CLL cells undergoing rapid apoptosis when cultured alone but are rescued when cultured

in direct contact with stroma. In the nineties, Panayitidis et al. (1) and our group (2) were among the first to highlight that this dependency plays a critical role in the pathophysiology of CLL. In addition to direct contact via adhesion molecules, CLL cells, as well as MSCs, communicate via the secretion of soluble factors (including chemokines, cytokines, and growth factors) that influence leukemic cell trafficking and homing within bone marrow niches. These niches are sanctuaries providing different survival signals and protect CLL cells from spontaneous and drug-induced apoptosis. Parallelly, in secondary lymphoid tissues such as lymph nodes, CLL cells interact with another actor of the stromal microenvironment: the follicular dendritic cell (FDC). FDCs are involved in the homing, the survival (3) and the proliferation (4) of CLL B cells within the germinal centers by producing several cytokines and chemokines (5, 6), by expressing several adhesion molecules (7–9) for CLL cells or by direct cell contact (3, 10). Recently, a new communication method via the production of extracellular vesicles (EVs) also adds complexity to these bidirectional CLL/MSC interactions. Using different interactions, the leukemic clone is able to manipulate the surrounding cells to recruit or to be recruited, re-educate and literally transform the surrounding cells into a protective microenvironment. In the present review, we summarize all the available data describing the crucial role of the stromal microenvironment in CLL, different manners of communication, activated pathways and how they can be targeted and, finally, the impact of this crosstalk on CLL patient prognosis.

CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in Western countries, accounting for 37% of cases with an incidence of 4.9 per 100 000, a male/female ratio of 1.9, a median age at diagnosis of 70 years old and more than 20 000 new cases diagnosed in 2019 in the United States (11). The disease is caused by the clonal expansion of leukemic B cells and is characterized by the expression of CD5, CD19, and CD23 (12), a defect in apoptosis (13, 14), and for the majority of CLL cells, a resting state (15, 16). However, proliferation centers have been described (15, 17) and using a deuterium oxide *in vivo* labeling method in which patients consumed deuterated water (^2H_2O), the lymph node has been identified as the anatomical site harboring the largest fraction of newly born cells with a calculated birth rate up to 3.3% of the clone per day (18). Another characteristic of CLL is its clinical heterogeneity (19). Some patients have an indolent course and live decades without any treatment, while others have a more aggressive disease, need early treatment and have a shortened survival. This heterogeneity can be predicted by a plethora of prognostic markers. The mutation status of the immunoglobulin heavy chain region (IgHV) (20), some cytogenetic abnormalities based on the Döhner classification [del(17p), del(11q), trisomy 12, del(13q)] (21), the expression of zeta–associated protein 70 (ZAP70) (22), lipoprotein lipase (LPL) (23), CD38 (24), CD49d (25), CD69 (26), some microRNAs [miR-29c and miR-223 (27), miR-34a (28), miR-150 (29)], and the presence of point mutations (tumor protein 53–TP53) (30).

While the cell origin of the disease is still under debate, the scientific community agrees that B cell receptor (BCR) pathways are crucial for the selection, development, proliferation, and survival of CLL clones (31–33). The BCR is composed of a surface immunoglobulin (Ig) made of 2 heavy and 2 light chains that are non-covalently associated with the heterodimer Ig-α/Ig-β (also known as CD79a/CD79b). External antigens from the microenvironment (34) as well as intra-BCR self-antigens (35) trigger BCR signaling, leading to the recruitment of tyrosine kinases [spleen tyrosine kinases (SYKs) and Lck/Yes novel tyrosine kinase (LYN)] that phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) of Ig-α/Ig-β (36). This induces a cascade of downstream events, including activation of Bruton's tyrosine kinase (BTK) (37), phosphoinositide 3-kinase (PI3K) (38), protein kinase C (PKC) and ras-dependent extracellular signal-regulated kinase (ERK) (39), that ultimately lead to the upregulation of nuclear factor kappa B (NFκB) (40). This signaling cascade promotes CLL B cell survival (41, 42) and has therefore been considered a very potent therapeutic target that we will discuss in this review (43, 44).

MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells (MSCs) are among the first actors in the CLL microenvironment that have been studied, even if, at that time, they were not called MSCs (1). These cells were discovered in 1968 by Friedenstein et al., who were the first to report an adherent fibroblastic-like cell population that was able to differentiate into osteoblasts, chondrocytes or adipocytes (45). In 1991, these cells were named "mesenchymal stem cells" by Caplan et al. (46), and from then, the term "MSC" has been popular. The

Abbreviations: AKT, AKT serine/threonine kinase 1; ATM, ataxia telangiectasia mutated; BAFF:B cell-activating factor of tumor necrosis factor family; BCL2, B-cell lymphoma 2; BCL-XL, B-cell lymphoma-extra-large; BCMA, B-cell maturation antigen; BCR, B-cell receptor; bFGF, basic fibroblast growth factor; BTK, Bruton's tyrosine kinase; CAF, cancer-associated fibroblast; CCL, (C-C motif) chemokine ligand; CCR, (C-C motif) chemokine receptor; CLL, chronic lymphocytic leukemia; CR, complement receptor; CREB, cyclic AMP response element binding protein; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; Del, deletion; ERK, extracellular regulated kinases; EVs, extracellular vesicles; FDC, follicular dendritic cells; H3K27me3, trimethylation of lysine 27 on histone H3 protein subunit; IC, immune complex; ICAM1, intercellular adhesion molecule 1; Ig, immunoglobulin; IgHV, immunoglobulin heavy variable region chain; IL, interleukin; ISCT, International Society for Cellular Therapy; ITAM, immunoreceptor tyrosine-based activation motifs; ITGB, integrin beta; JAK, Janus kinase; LFA-1, lymphocyte function-associated antigen 1; LPL, lipoprotein lipase; LYN, Lck/Yes novel tyrosine kinase; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid leukemia cell differentiation protein 1; miR, microRNA; MSC, mesenchymal stromal cells; NFκB, nuclear factor kappa B; NLC, nurse-like cells; Notch2, neurogenic locus notch homolog protein 2; OS, overall survival; PDGF, platelet-derived growth factor; PFS, progression free survival; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKD, protein kinase D; SDF1, stromal cell derived factor 1; STAT3, signal transducer and activator of transcription 3; SYK, spleen tyrosine kinase; TACI, transmembrane activator and calcium modulator and cyclophilin ligand-interactor; TGFβ1, transforming growth factor β1; TNFα, tumor necrosis factor α; TP53, tumor protein 53; TSP-1, thrombonspondin-1; TTFT, predict time to first treatment; VCAM-1, the vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VLA-4, very late antigen-4; Wnt, wingless integration site; ZAP70, zeta-associated protein 70.

first source of MSCs was found in bone marrow, but several other sources have been described (adipose tissue, Wharton's jelly of the umbilical cord, dental pulp, skin, etc.) in numerous organs in which cell renewal is needed (47). MSCs are generally recovered by simple plastic adhesion, resulting in a heterogeneous cell population with different stemness potentials. Therefore, to avoid any controversies, the term "stem" in "mesenchymal stem cell" has been replaced by "stromal," referring to a bulk population with secretory, immunomodulatory, and differentiation potential and homing properties (48). MSCs are heterogeneous cells and cannot be defined by a single marker. Therefore, in 2006, the International Society for Cellular Therapy (ISCT) proposed a set of minimal criteria to define human multipotent MSCs (49): [1] MSCs must adhere to plastic when maintained in culture; [2] MSCs should express (≥95%) CD105, CD73, and CD90, as measured by flow cytometry but should not express (≤2%) hematopoietic markers (CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II); and [3] finally, MSCs should be able to differentiate into osteoblasts, adipocytes and chondroblasts. The number of MSCs in bone marrow aspirate represents ∼0.01–0.001% of the total population of nucleated cells; therefore, in the majority of cases, MSCs require *ex vivo* expansion to obtain a reasonable number of cells to establish a feeder layer. Although these *ex vivo* expanded MSCs could be different from native MSCs, some studies have already shown that MSCs obtained from CLL patients present different properties compared to healthy MSCs. In 1995, our group observed increased production of transforming growth factor β1 (TGFβ1) by CLL patient-derived stromal cells compared to that of healthy stroma (50, 51). Pontikoglou et al. demonstrated that CLL patient-derived MSCs exhibit a similar phenotype compared to healthy MSC, as well as a similar differentiation potential and a CLL apoptosis protection. On the other hand, they also showed that these cells have defective cellular growth due to increased apoptotic cell death and exhibit aberrant production of stromal cell derived factor 1 [SDF1, also named C-X-C motif chemokine ligand 12 (CXCL12)] or TGFβ1, two important cytokines that are crucial for the survival of leukemic cells (52). Janel et al. confirmed this low proliferative capacity (53). In addition, they also observed increased culture failure, a polygonal aspect and an increased proportion of senescence-associated β-galactosidase-positive cells in CLL patient-derived MSCs compared to those of healthy MSCs (53). These two *ex vivo* reports suggest that CLL patient derived MSCs are probably already dependent on leukemic clones, at least for their long-term survival, as the leukemic clones are dependent on MSCs for their own survival. While this phenomenon has been reported for other malignancies such as multiple myeloma (54), the dependency of MSCs from CLL cells and their low proliferation rate have never been directly demonstrated *in vivo*. In addition, it should be noted that several reported differences between CLL-MSCs and age-matched healthy individual MSCs are known as senescence-associated [reviewed in (55)] and are therefore not necessarily linked to the disease itself. This is an important limitation of *in vitro* studies with MSC: their *ex vivo* expansion does not allow the study of "native" MSCs under physiological situation.

FOLLICULAR DENDRITIC CELLS

Follicular dendritic cells (FDCs) are accessory cells located in the central region of primary follicles and in the light zone of normal germinal centers (56). Based on their dendritic appearance, FDCs were mistakenly considered as a subset of conventional dendritic cells. However, FDCs are from stromal origin and emerge from perivascular precursors (57), unlike conventional dendritic cells which are of hematopoietic origin. They also have different functions: one of the most important features of FDCs is their ability to capture antigen–antibody complexes (called "immune complexes"—IC) on their cellular surface through the involvement of complement receptors 1 (CR1 or CD35) and 2 (CR2 or CD21) (58), and present unprocessed antigen to the B cells. This was observed for the first time in 1965 using high-resolution electron microscopic autoradiographs and radioactively labeled microbial antigens (59). In the following years, several different names such as dendritic macrophages (60) or dendritic reticular (61) cells were used for these cells. In 1978, Chen et al. finally introduced the name FDCs (62) but admitted later that this name was not ideal. However, even if it was demonstrated that FDCs did not express class II MHC like conventional dendritic cells, the name FDCs still remains. Because of their ability to bind IC, FDCs are indispensable for secondary and tertiary lymphoid organ development and maintenance. FDCs are normally localized in secondary lymphoid organs such as the spleen or the lymph node (10) however, in CLL patients, FDCs have also been observed in nodular bone marrow infiltrates (63). Because of their cytokine secretion, the adhesion molecules they carry, their ability to activate BCR signaling, and their protective effect on the survival of CLL B cells, FDCs represent another important player of the stromal microenvironment, particularly in secondary lymphoid organs. A schematic representation of the different CLL/FDC interactions is shown in **Figure 1** and is discussed below.

DIRECT CONTACT: CLL/STROMA COCULTURE, HOMING, AND ADHESION MOLECULES

When CLL B cells are removed from the human body and plated alone in culture, they rapidly undergo apoptosis (13, 14). Co-culture of CLL B cells with MSCs prevents this cell death (1, 2). However, efficient protection is achieved when the two cell types are in contact, since separation with a filter prevents the protection from apoptosis (2, 66–69). In addition to protection from spontaneous apoptosis, this effect can be extended to drug-induced apoptosis (67, 70, 71). Since the first reports with primary MSCs, apoptosis protection has been confirmed with different stromal cell lines, such as HS5 (human) or M210B4 (mouse) (67). During co-culture, CLL B cells migrate spontaneously to and beneath the feeder layer of MSCs. This phenomenon is called pseudoemperipolesis to distinguish it from true emperipolesis, which involves penetration of living cells by other cells (66, 70, 71). This migration is induced by chemoattractants produced by MSCs, especially CXCL12, which

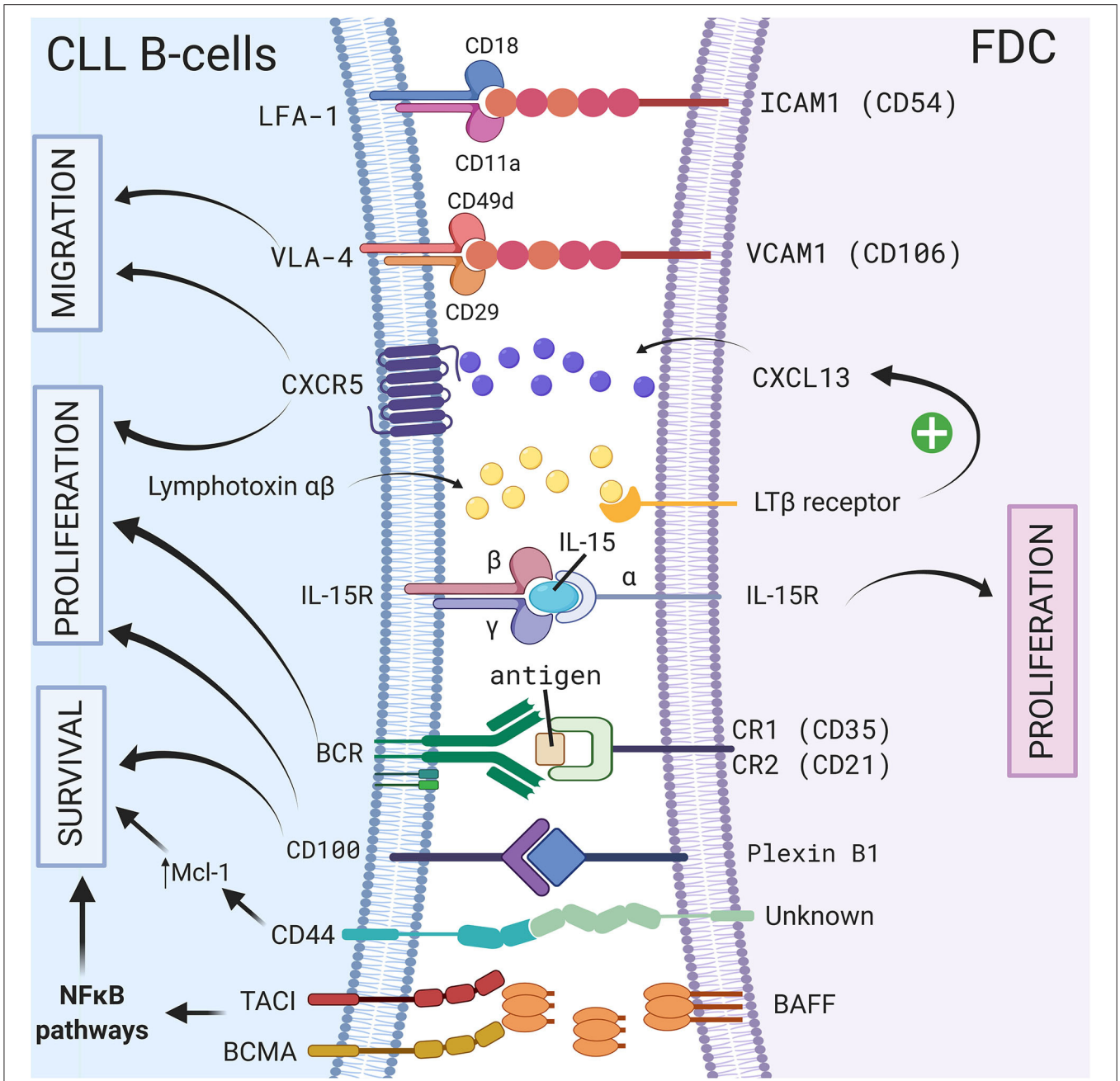

FIGURE 1 | Crosstalk between CLL B-cells and FDCs via direct contact or soluble factors. In secondary lymphoid organs, CLL B cells interact with FDCs via different molecules, receptor/ligand couples including ICAM1/LFA-1 (8), VCAM1-VLA-4 (8), CD100/Plexin-B1 (9), immune complex/BCR (64), CXCR5/CXCL13 (10), BCMA/BAFF or TACI/BAFF (65), or by transpresentation of IL-15 from FDCs to germinal center B cells (6). These interactions could lead to a leukemic cell survival via a CD44-dependent mechanism involving up-regulation of Mcl-1 in CLL B cells (3), the activation NF-κB pathway (65), the migration, or the proliferation of leukemic cells. Figure created with BioRender.com.

will be discussed in the next chapter. Similarly, *in vitro* coculture of CLL B cells with HK cells, a follicular dendritic cell line, rescues CLL cells from spontaneous and drug induced apoptosis (3). Interestingly, this protection is at least partially mediated by a CD44-dependent mechanism involving up-regulation of Mcl-1 in CLL B cells (3).

The binding of CLL B cells to stromal cells requires simultaneous action of β1 integrin (ITGB1, also known as CD29) and β2 integrin (ITGB2, also known as CD18) (72, 73). The activation of these integrins occurs by heterodimerization, creating a conformational change, increased affinity and redistribution on the plasma membrane (74). These results were

consistent with a more recent report that very late antigen-4 (VLA-4), which is composed of a CD49d and CD29 dimer, is an important integrin for retention of CLL cells in the microenvironment by interacting with its ligand vascular cell adhesion molecule 1 (VCAM-1 or CD106) on stromal cells (75). In addition, Brachtl et al. demonstrated a prominent role of CD49d in the homing of CLL cells to bone marrow niches and in human bone marrow infiltration (76). Interestingly, activation of VLA-4 and lymphocyte function-associated antigen 1 (LFA-1, which is composed of CD11a and CD18—also known as αLβ2 integrin) is triggered by CXCL12 produced by MSCs (74, 77), notably via the Janus kinase 2 (JAK2) pathway (78) but also by BCR stimulation, reinforcing the adhesive capacities of CLL B cells (79). While LFA-1 is involved in chemokine-mediated migration of CLL cells from patients with lymphadenopathies (80), in cells from the majority of CLL patients, there is a defect in chemokine-induced inside-out activation of LFA-1 (81). Interestingly, this defect can be overcome by the engagement of vascular endothelial growth factor (VEGF) receptor(s) and VLA-4 by their respective ligands (81). In addition to this activation defect, Hartmann et al. also showed that CLL cells expressed significantly reduced LFA-1 due to low β2 integrin transcripts compared to healthy B cells (77). For some authors, LFA-1 is not involved in the adhesion of CLL cells to bone marrow MSCs (73) but is important for their adhesion to FDCs that express high level of ICAM1 (7). Indeed, FDCs expressed ICAM1 and VCAM1 allowing, the interaction with LFA-1 and VLA-4, respectively, on CLL B cell surface (8). Granziero et al. also highlighted the interaction between CD100 (present on CLL B cell surface) and Plexin-B1 (present on bone marrow MSCs and FDCs) and showed that it extends CLL B cell viability and enhances proliferation (9).

Coculture of leukemic cells with MSCs is associated with actin polymerization (71, 82) and consequent cytoskeletal remodeling in CLL cells. For other leukemias such as chronic myeloid leukemia (83, 84) and acute lymphoblastic leukemia (85–87), tunneling nanotubes have been identified as a novel mode of intercellular crosstalk. Tunneling nanotubes are long and thin membranous structures that allow the exchange of material [such as mitochondria (85), vesicles or proteins (83)] between leukemic cells and stromal cells. While it has never been described in CLL, it is not impossible that tunneling nanotubes between CLL cells and MSCs could be a new way of communication allowing the transfer of mitochondria or proteins to CLL cells, as reported for other leukemias. However, we should be cautious when interpreting these results since the majority of data available today for CLL/microenvironment interactions represent a two-dimensional view of an *in vitro* coculture experiment. Therefore, new 3D culture systems have been proposed to study leukemia cells (88–90) and 3D coculture systems for studying specifically CLL/microenvironment interactions are under investigation, as presented in recent hematology congresses (91–93).

The contact between CLL B cells and MSCs induces dramatic gene expression modifications (94, 95), including an increase in antiapoptotic molecules such as B cell lymphoma 2 (BCL2) (96, 97), B cell lymphoma-extra large (BCL-XL) (96, 98), myeloid leukemia cell differentiation protein 1 (MCL1) (67, 71, 98), and β-catenin (95), as well as soluble factors that will be discussed below. Caveolin was also suggested to play a role in CLL survival in coculture: indeed, caveolin is increased in cocultures of CLL B cells with the NK. Tert stromal cell line (99) and play a role in CLL development in the Eμ-TCL1 mouse model (100). Numerous pathways are consequently activated within CLL B cells, including Toll-like receptor (94) and BCR (29). How BCR signaling is activated by MSCs is still unclear, but Binder et al. suggested that a BCR with a common stereotyped heavy chain complementarity-determining region 3 [from "subset1" (33)] recognizes vimentin and calreticulin, which are highly expressed on stromal cells (34). Interestingly, blocking vimentin by recombinant soluble CLL BCR reduces stromal-mediated apoptosis protection (34). In secondary lymphoid organ context, FDCs are able to stimulate BCR and activate normal B cells via FDC-bound antigen associated with CR1/2 (64). Heinig et al. identified follicular stromal networks that locally interact with leukemia cells isolated from Eμ-Tcl1, a CLL mouse model (10). In addition, these authors showed that the majority of leukemic cells in contact with FDCs expressed proliferation markers, suggesting that FDCs could participate to the stimulation and proliferation of CLL B cells in germinal centers (10).

Other authors highlight the possible epigenetic modifications induced by stromal cells. Xu et al. observed that CLL B cell protection in the presence of the murine stromal cell line HESS-5 is associated with hypomethylation of the trimethylation of lysine 27 on histone H3 protein subunit (H3K27me3) (101). Coculture with different stromal cell lines showed increased oxidative phosphorylation in CLL, which probably helps to increase their metabolism, allowing these cells to meet the energy demands for transcription and translation (102).

Direct contact between CLL B cells and stromal cells also induces modifications in the stromal cells. Mangolini et al. showed that in a coculture system, neurogenic locus notch homolog protein 2 (Notch2) is activated in MSCs and regulates genes involved in inflammation and extracellular matrix formation, which are both important components of the CLL microenvironment (95). In addition, these authors demonstrated that coculture stabilizes β-catenin in CLL, activating the wingless integration site (Wnt) pathway. A schematic representation of the different cell-to-cell contact interactions is shown in **Figure 2**.

SOLUBLE FACTORS: CYTOKINES, CHEMOKINES, AND GROWTH FACTORS

Soluble factors play a key role in CLL B cell trafficking and homing (103). Leukemic cells could travel in the body from the peripheral blood to the bone marrow, where they receive survival signals. Numerous cytokines are produced by bone marrow MSCs, but one of the most studied cytokines is CXCL12, which interacts with its receptor, C-X-C motif chemokine receptor 4 (CXCR4, also known as CD184), on leukemic cells (66, 69, 75). Möhle et al. showed that CLL B cells overexpress CXCR4 compared to normal B cells, making them more able to respond to CXCL12 (104). This was confirmed by *in vivo* studies showing that higher CXCR4 levels increase the risk for lymphoid organ

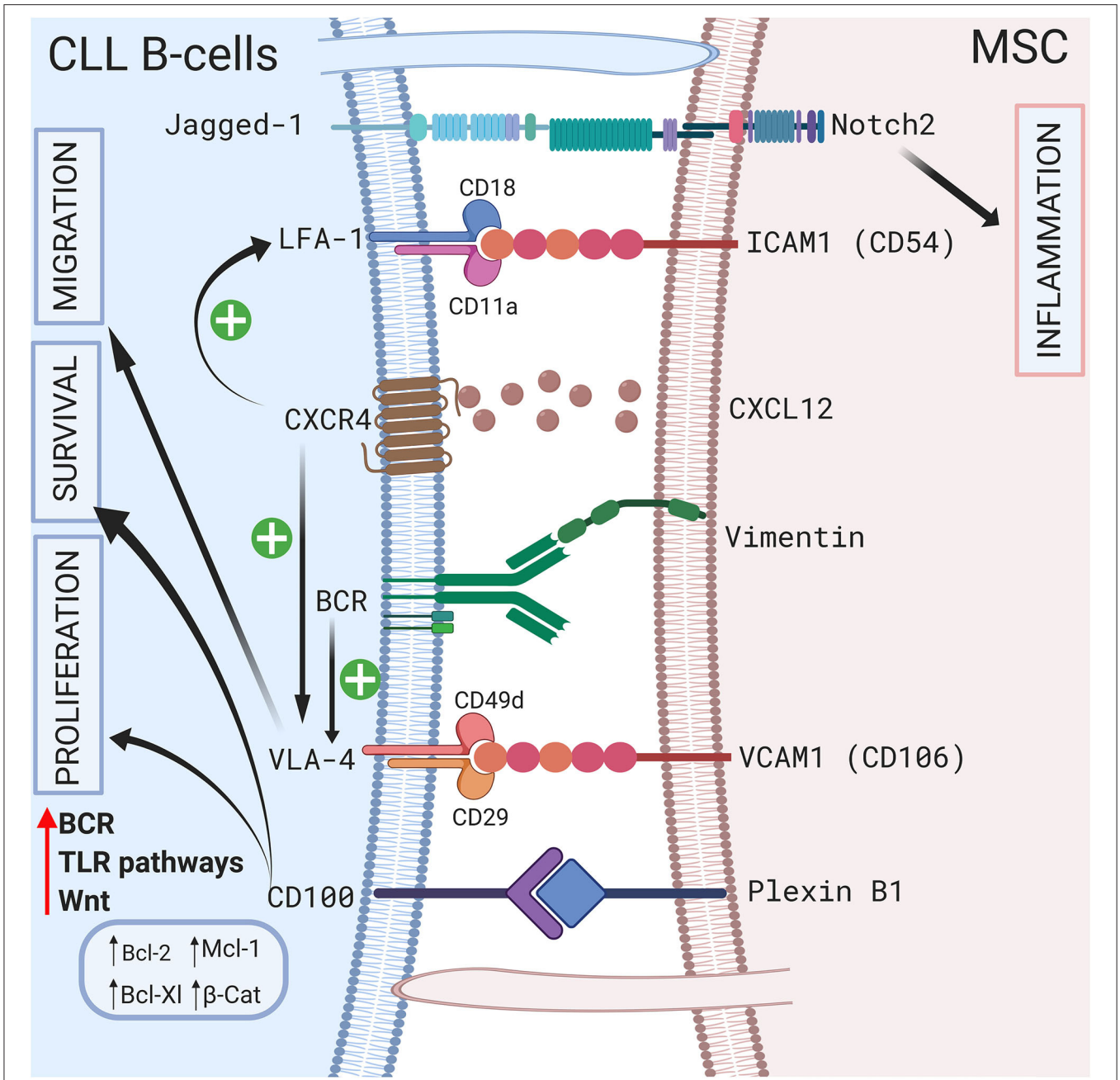

FIGURE 2 | Crosstalk between CLL B-cells and MSCs via direct contact. CLL B-cells interact with MSCs by direct contact by different ways. First via pseudopods that increase cell surface contact. Second by different receptors and ligands including Jagged1/Notch2 (95), LFA-1/VCAM1 (72, 73), VLA4/VCAM1 (75), CD100/Plexin B1 (9), BCR/vimentin (34). Interestingly the CXCR4/CXCL12 axis plays a central role by triggering VLA-4 and LFA-1 axis (74, 77). BCR stimulation also increase VLA-4 (79). All these interactions lead to the proliferation, the migration and/or the survival of leukemic cells by inducing the upregulation of several anti-apoptotic protein including Bcl-2 (96), Mcl-1 (67, 71, 98), or Bcl-XL (96, 98). Figure created with BioRender.com.

infiltration (105). Interestingly, CXCR4 expression is dynamically regulated on CLL cell surface. Using CXCR4 and CD5 staining on deuterium-labeled cells, Callissano et al. indeed showed that $CXCR4^{dim}CD5^{bright}$ cell fraction is enriched in young, vital and proliferating cells while $CXCR4^{bright}CD5^{dim}$ fraction is composed of older, less robust and resting cells (105). Based on these data, these authors hypothesized lifecycle of CLL B cells: on a stroma, CLL cells could be stimulated and activated. Then, they begin to divide, to upregulate CD5 and to downregulate CXCR4, detaching from the stroma, and are released in the circulation ($CXCR4^{dim}CD5^{bright}$ phenotype). Over time, cells begin to re-express CXCR4 ($CXCR4^{bright}CD5^{dim}$ phenotype). These cells have the greatest chance of detecting and following a CXCL12 gradient, thereby reentering lymphoid solid tissue

and receiving prosurvival stimuli. The binding of CXCL12 to CXCR4 induces actin polymerization, cytoskeletal remodeling, transendothelial migration and tissue homing of leukemic cells (66). In addition to its chemoattractant effects, CXCL12 has also been shown to be a survival factor (71, 106). Following CXCL12 binding, CXCR4 is downregulated on the CLL B cell surface by endocytosis, making the cells less responsive to CXCL12 and allowing their recirculation in the peripheral blood (66). Interestingly, Saint-Georges et al. showed that BCR stimulation also downregulated CXCR4 via protein kinase D (PKD) phosphorylation (107). Consistent with these reports, Ghobrial et al. showed that CXCR4 expression is decreased in bone marrow or lymph nodes (108). This downregulation of CXCR4 (coupled to the high expression of CD5) is therefore used to identify cells that recently emigrated from tissue into the blood circulation (105). CXCR4 activation triggers numerous intracellular pathways, including the PI3K (82), signal transducer and activator of transcription 3 (STAT3) (66), and p44/42 mitogen-activated protein kinase (MAPK) (106) pathways, leading to BTK (109), ERK (110), and AKT serine/threonine kinase 1 (AKT) (111) activation as well as calcium released (66). Interestingly, BTK is rapidly activated by CXCL12 in leukemic cells, indicating once more that the CXCL12/CXCR4 axis is interconnected with the BCR pathway (109). Similar to the CXCR4/CXCL12 axis, the CXCR5/CXCL13 axis also plays a role in CLL homing and trafficking, since CXCR5 is overexpressed on the CLL B cell surface and CXCL13 is secreted by stromal cells in B cell areas of secondary lymphoid tissues (112): FDCs produce CXCL13 which directs B lymphocytes to the "light zone" of the germinal center (5). Using the Eμ-Tcl1 mouse model of CLL, Heinig et al. demonstrated that CXCR5 depletion reduces Eμ-Tcl1 leukemogenesis, CLL proliferation and that this chemokine is indispensable for the recruitment of CLL cells into the germinal center since CXCR5-defective cells localized in the marginal zone of the B-cell follicle (10). In addition, these authors observed that lymphotoxin α and β produced by CLL B cells stimulated FDCs to produce CXCL13, suggesting that CLL/FDC reciprocal interactions leads to stromal compartment remodeling (10).

The levels of several cytokines produced by leukemic cells are dysregulated compared to those of healthy donors (113), but the basal level can also be influenced by coculture with MSCs, as demonstrated by Trimarco et al., who found that coculture induced an increase in the production of interleukin 8 (IL-8), (C-C motif) chemokine ligand 4 (CCL4), CCL11, and CXCL10 in the supernatant (114). However, based on mRNA expression, Plander et al. showed that the increases in IL-6 and IL-8 were due to MSCs (115). Other authors suggested that the major IL-6 source in a coculture system is the leukemic compartment (116). In addition, MSCs in coculture produce IL-1β, while CLL B cells produce tumor necrosis factor α (TNFα), suggesting that coculture creates an inflammatory environment (115). Interestingly, IL-8 induces prolonged survival of CLL B cells *in vitro* in an autocrine manner (117). The increase in CXCL10 was specific to CLL/MSC coculture and was not observed with normal B cells, suggesting a potential role in CLL pathophysiology (114). Moreover, CXCR3, the CXCL10 receptor, is expressed on the CLL B cell surface and mediates chemotaxis (118). CCL4 and CCL3 have also been reported to be increased in CLL B cells after coculture with nurse-like cells (NLCs) or after BCR stimulation (42). It is believed that CCL4 and CCL3 attract (C-C motif) chemokine receptor (CCR5)-positive regulatory T cells (119) or monocytes/macrophages *in vivo* in conditions that could confer survival signals to CLL B cells (42, 120–122). Using CLL coculture with the human stromal cell line HS-5, Schulz et al. similarly observed an increase in CCL2 secretion by stromal cells that was involved in the recruitment of macrophages (94). Other cytokines that can rescue primary CLL cells from apoptosis, such as IL-1α and IL-15, are also produced by MSCs after CLL contact by inducing PKC-β in stromal cells (123). IL-15 is also produced by human FDCs *in vivo* and by an FDC cell line *in vitro* (6), and has a paracrine and autocrine effect. Indeed, IL-15 is captured by IL-15Rα on the surface of FDC/HK cells and this membrane-bound form could, by transpresentation from FDCs to germinal center B cells via cell-cell contact, trigger IL-15 signaling in B cells (6) but also enhance human primary FDCs proliferation and regulate their cytokine secretion (4). In line with these observations in normal B cells, the addition of IL-15 in CLL/FDC coculture enhances CLL proliferation (10).

In secondary lymphoid organs, similarly to NLCs, FDCs produce the B cell-activating factor of tumor necrosis factor family (BAFF), an essential factor for B cell homeostasis (124, 125) but also for the survival of CLL cells (126). Endo et al. demonstrated that BAFF supports CLL B cell survival through the activation of the canonical NF-κB pathway after binding to the B-cell maturation antigen (BCMA) or the transmembrane activator and calcium modulator and cyclophilin ligand-interactor (TACI), two BAFF receptors (65).

Growth factors also sustain CLL B cell survival in a coculture model. Gehrke et al. observed that VEGF produced by HS-5 stromal cells but not CLL B cells is essential for their coculture-mediated survival (127). Our group also suggested that compared to healthy MSCs, the increased secretion of TGFβ1 by CLL-derived MSCs play a crucial pathogenic role in CLL (50). In addition, Kay et al. observed an increase in VEGF, thrombospondin-1 (TSP-1) and basic fibroblast growth factor (bFGF) in the supernatant of a coculture system of primary CLL B cells and CLL patient-derived MSCs (69). These authors suggested that this increased secretion was due to MSCs (69). In 2009, Ding et al. observed activation of the AKT pathway in MSCs in coculture with leukemic cells, supporting the existence of bidirectional interactions (68). One year later, the same authors demonstrated that the platelet-derived growth factor (PDGF) produced by CLL B cells is responsible for this activation and induces VEGF production in MSCs in a PI3K-dependent manner (128). A schematic representation of the different interactions between CLL B cells and MSCs via the production of soluble factors is shown in **Figure 3**.

EXTRACELLULAR VESICLES: A NEW WAY OF CROSSTALK

In recent years, a new method of intercellular communication via the exchange of extracellular vesicles (EVs) has been

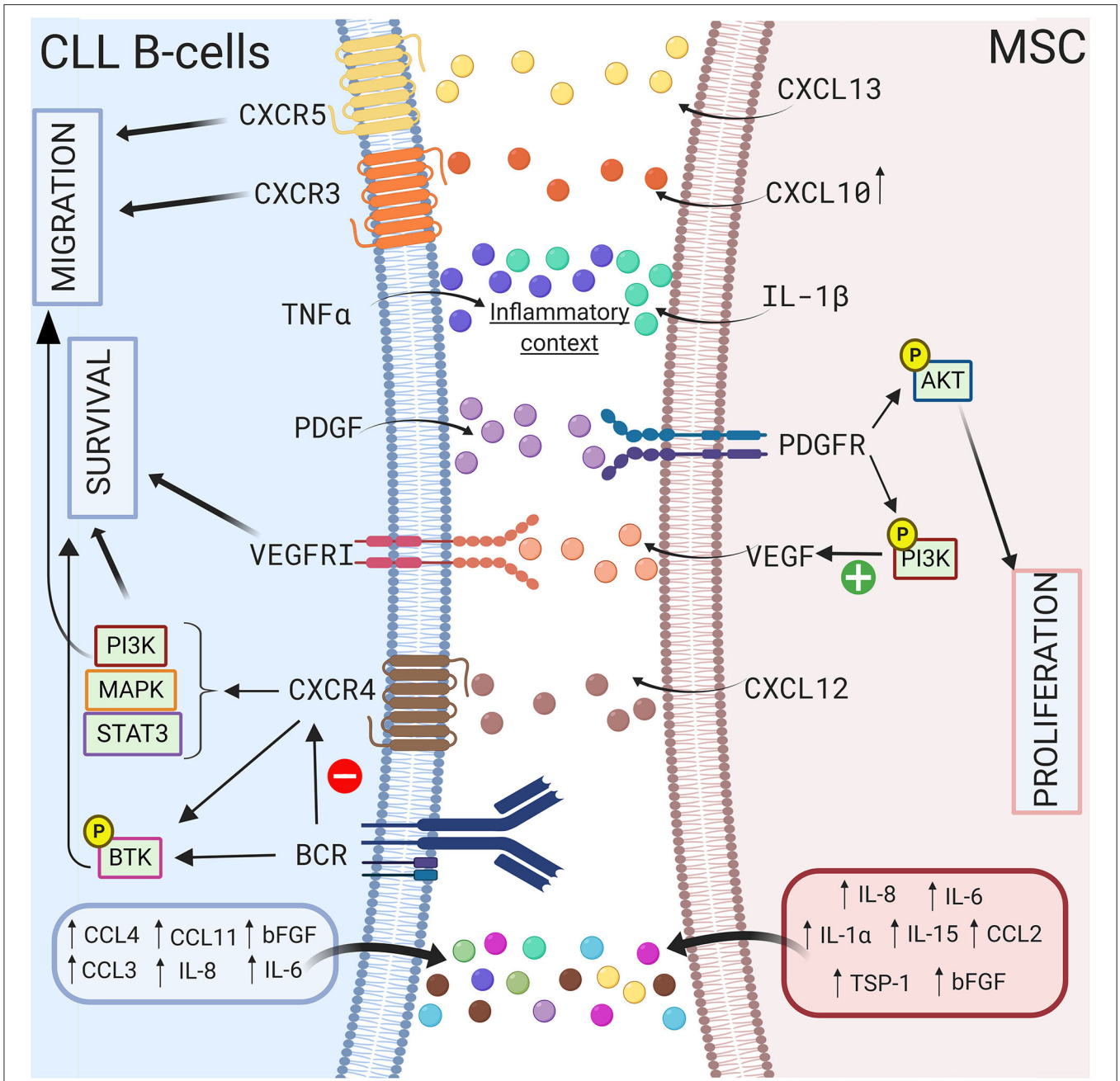

FIGURE 3 | Crosstalk between CLL B-cells and MSCs via soluble factors. CLL B-cells interact with MSCs by several soluble factors including cytokines (115, 117), chemokines (42, 114), and growth factors (69, 127, 128). MSCs can produce CXCL10, CXCL13, CXCL12 that binds to their respective receptor on CLL B-cells CXCR3 (118), CXCR5 (112), CXCR4 (66, 69, 75). The triggering of CXCR4/CXCL12 axis lead to the activation of several pathways including PI3K (82), MAPK (106), or STAT3 (66) leading to the survival and the migration of the leukemic cells. Interestingly, BCR stimulation induces the downregulation of CXCR4 (107), the activation of BTK (109), and the increased secretion of some cytokines. MSCs in coculture also produce IL-1β while CLL B-cells produce TNFα suggesting that coculture creates an inflammatory context (115). Figure created with BioRender.com.

described. Observed 50 years ago as "platelet dust" (129), EVs were long considered cellular debris, but today, they are known to play important roles in several pathophysiological processes, including immune responses, tissue regeneration, blood coagulation (130), and crosstalk between normal/cancer cells (131). EVs can be divided into 2 different groups based on their origin: exosomes and microvesicles (also known as ectosomes). Exosomes are released from multivesicular bodies (late endosomes) at the plasma membrane and generally have a size ranging from 30 to 150 nm, while

microvesicles result directly from plasma membrane budding and pinching and have a size between 100 nm and 1 μm (132, 133). EVs can shuttle and transfer their content from one cell to another. Several reports have described that EVs carry DNA fragments, different species of RNA (mRNA, Y RNA, and microRNA), proteins, peptides or lipids (134–137).

To date, few reports have described the role of EVs in CLL B cells and MSC communication. Crompot et al. were the first to highlight the impact of bone marrow MSC EVs on CLL B cells *in vitro* (138). These authors showed that MSC-derived EVs are rapidly incorporated in CLL B cells and that they increase CLL cell migration, suggesting that these EVs could give CLL cells survival advantages *in vivo*. In addition, MSC-derived EVs protect leukemic cells from spontaneous and drug-induced apoptosis, as well as induce gene expression modifications. Overlap of gene signatures induced by EVs with other microenvironmental stimuli [such as BCR stimulation (139) or NLC coculture (42)] suggested that a substantial part of cell-to-cell communication is mediated by EVs. Finally, several (but not all) effects of MSC-derived EVs mimic BCR stimulation, which has been described as crucial in CLL B cell survival (139, 140).

On the other hand, CLL B cells also release EVs that can modulate their microenvironment. Paggetti et al. demonstrated that exosomes can be taken up by stromal cells and transfer microRNA-150 (29), microRNA-155 (141) and microRNA-146a (142), which have been described in CLL B cells (135). Moreover, leukemic exosomes induce an inflammatory phenotype in stromal cells by increasing AKT and cyclic AMP response element binding protein (CREB) phosphorylation via the NF-κB pathway, resulting in a cancer-associated fibroblast (CAF)-phenotype (135). Interestingly, this phenomenon is coupled to the increase in VEGF, CXCL10, CCL2, IL6, intercellular adhesion molecule 1 (ICAM1) or CXCL12, which are important molecules involved in the homing of CLL cells that were previously discussed (135, 143). Ghosh et al. reported that CLL microvesicles carry Axl protein that could be transferred to MSCs, leading to an increase in AKT phosphorylation (143). Farahani et al. showed that CLL exosomes encapsulate an abundant amount of microRNA-202-3p that, once integrated in stromal HS-5 cell lines, enhanced their proliferation and decreased apoptosis by inducing the expression of genes such as c-fos and ataxia telangiectasia mutated (ATM) (144). Interestingly, several authors observed an enrichment in specific microRNAs in exosomes compared to that of the cell compartment (135, 144, 145). As explained previously, BCR stimulation is crucial for CLL B cell survival, and multiple microenvironmental stimuli, such as MSC or NLC coculture, could trigger BCR signaling. In this context, Yeh et al. observed that BCR stimulation increases exosome production by CLL B cells but also modifies their microRNA-150 and microRNA-155 content (145). These data suggest that different microenvironmental stimuli could be amplified via EVs. A schematic representation of the influence of EVs in CLL B cell/MSC crosstalk is shown in **Figure 4**.

TARGETING CLL/MSC CROSSTALK

As explained previously, CLL B cells migrate from the peripheral blood to tissues in response to different chemokines. When leukemic cells reach the stromal microenvironment, they enter a protective niche against drug-induced apoptosis. Therefore, mobilizing these cells out of these niches to increase their chemosensitivity has been a proposed strategy. The first studies tried to inhibit CLL/MSC crosstalk by acting extracellularly on the CXCR4/CXCL12 axis. We showed that AMD3100 (also known as plerixafor), a bicyclam molecule and specific antagonist of the CXCR4 receptor (146), prevents the binding of CXCL12 and results in a decrease in pseudoemperipolesis and an increase in chemosensitivity to different drugs (71). Recently, a clinical trial combining plerixafor and rituximab (an anti-CD20 antibody) confirmed an increase in cell mobilization in peripheral blood but an overall response rate of 38% (147). Other authors tried to target this axis using a CXCR4 antibody (148) or by decreasing the expression of CXCR4 using a histone deacetylase inhibitor (70, 149). Another proposed strategy was to inhibit the ligand CXCL12 and not the receptor. NOX-A12, an RNA oligonucleotide in the L-configuration that binds and neutralizes CXCL12, has been shown to decrease CLL B cell migration and increase chemosensitivity but surprisingly increases pseudoemperipolesis (150). All authors agree that inhibition of the CXCR4/CXCL12 axis is only an adjuvant and therefore should always be coupled with a cytotoxic drug.

Since the CXCR4/CXCL12 and BCR pathways are interconnected, another way to interfere with the migration and homing of leukemic cells into a protective microenvironment is to target the intracellular pathways using specific drugs. One of the more potent and recently discovered drugs is ibrutinib (previously called PCI-32765), a Bruton's tyrosine kinase inhibitor (37). This small molecule acts by covalently binding cysteine 481 in the active site of BTK and consequently inhibits downstream events such as MAPK, PI3K, or NF-κB activation (151). This inhibition therefore results in a drastic reduction in migration and adhesion of CLL B cells in the lymphoid tissue (particularly the lymph node) and their mobilization in peripheral blood (152, 153). In this context, Tissino et al. reported a relationship between ibrutinib exposure and impaired CLL cell adhesion on VCAM-1 substrates *in vitro* and a progressive reduction of constitutive VLA-4 activation during *in vivo* ibrutinib treatment (79). However, decrease of VLA-4 activation by ibrutinib is still under debate since BTK inhibition could be bypassed by triggering the CXCR4/CXCL12 axis (154) or by an exogenous BCR stimulation in a BTK-independent manner involving PI3K (79). In contrast, other authors observed that BTK inhibition prevents CXCL12-induced triggering of LFA-1 and VLA-4 integrins (109). In addition, ibrutinib also reduces the surface level of CXCR4 by inhibiting cycling from and to the membrane (153). Not surprisingly, other drugs targeting BCR pathways, such as a more specific BTK inhibitor (acalabrutinib; previously named APC-196) (155), SYK inhibitor (fostamatinib) (156) or PI3K inhibitor (idelalisib, duvelisib) (157, 158), will have very similar effects on migration, homing and mobilization of leukemic

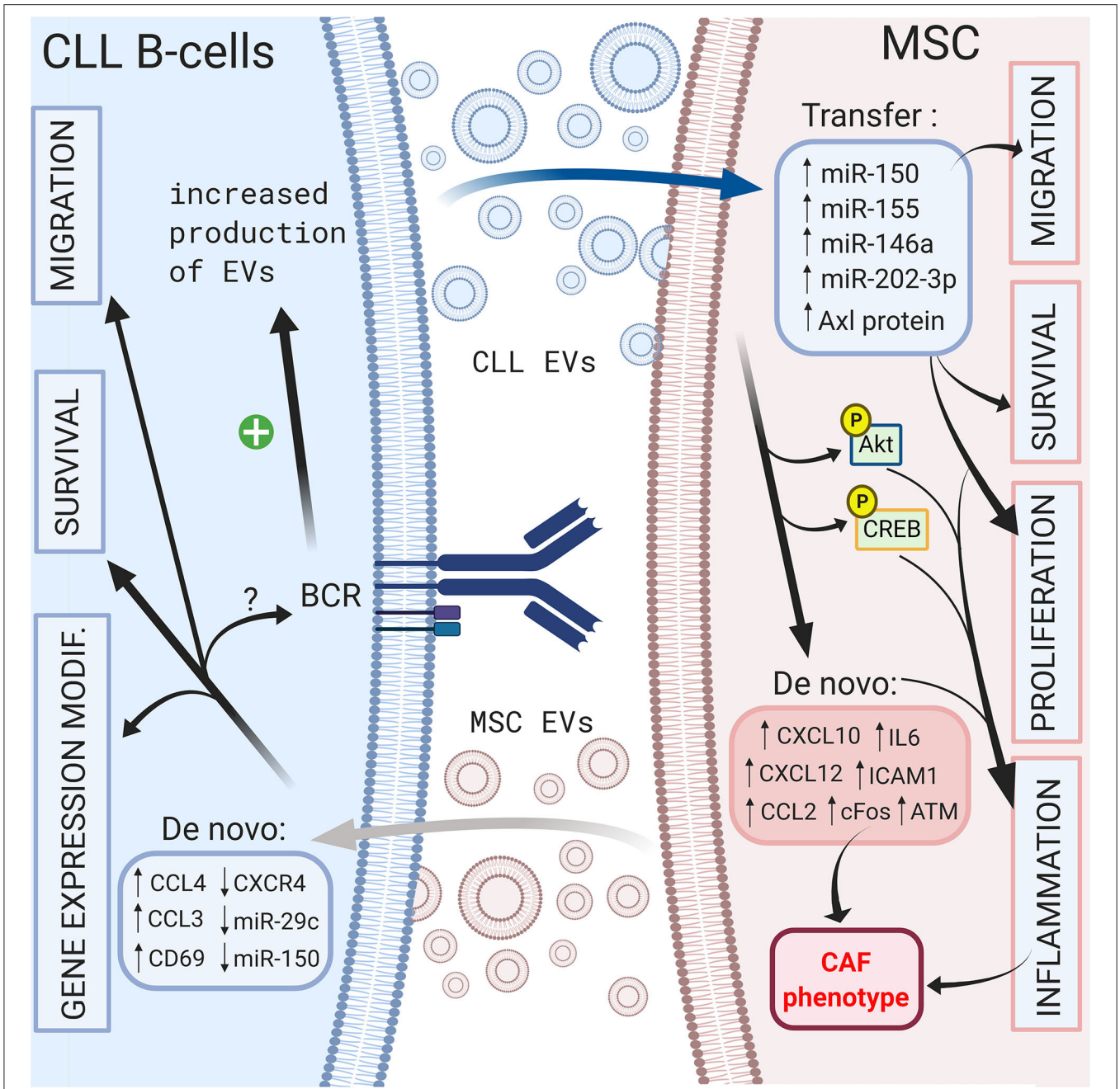

FIGURE 4 | Crosstalk between CLL B-cells and MSCs via extracellular vesicles. Bi-directional communication exists between CLL B-cells and MSCs via the production of extracellular vesicles by both cell types. MSC EVs increase the migration, the survival of CLL B-cells and change their gene expression profile (138). CLL B-cells derived EVs can transfer microRNA (135, 144, 145) or protein (143) leading to the migration, the survival and the proliferation of MSCs (135). In addition, they induce an inflammatory phenotype in stromal cells resulting into a cancer-associated fibroblast (CAF)-phenotype (135). Interestingly BCR stimulation increases the production of CLL EVs (145). Figure created with BioRender.com.

cells in the circulation by inhibiting chemotaxis in response to CXCL12 and CXCL13 and reducing adhesion to VCAM1 and fibronectin (159, 160). Complementarily, idelalisib or duvelisib also significantly reduced the ability of stromal cells to support CLL migration and adhesion (161). Another way to disrupt CLL/MSC crosstalk and overcoming drug resistance in CLL patients is to directly target PKC-β signaling pathway in MSC: indeed, Park et al. showed that small-molecule PKC-β inhibitors antagonize prosurvival signals from stromal cells and sensitize tumor cells to targeted and non-targeted chemotherapy, resulting in enhanced cytotoxicity (162). In addition, they also showed that stromal PKC-β controls the expression of

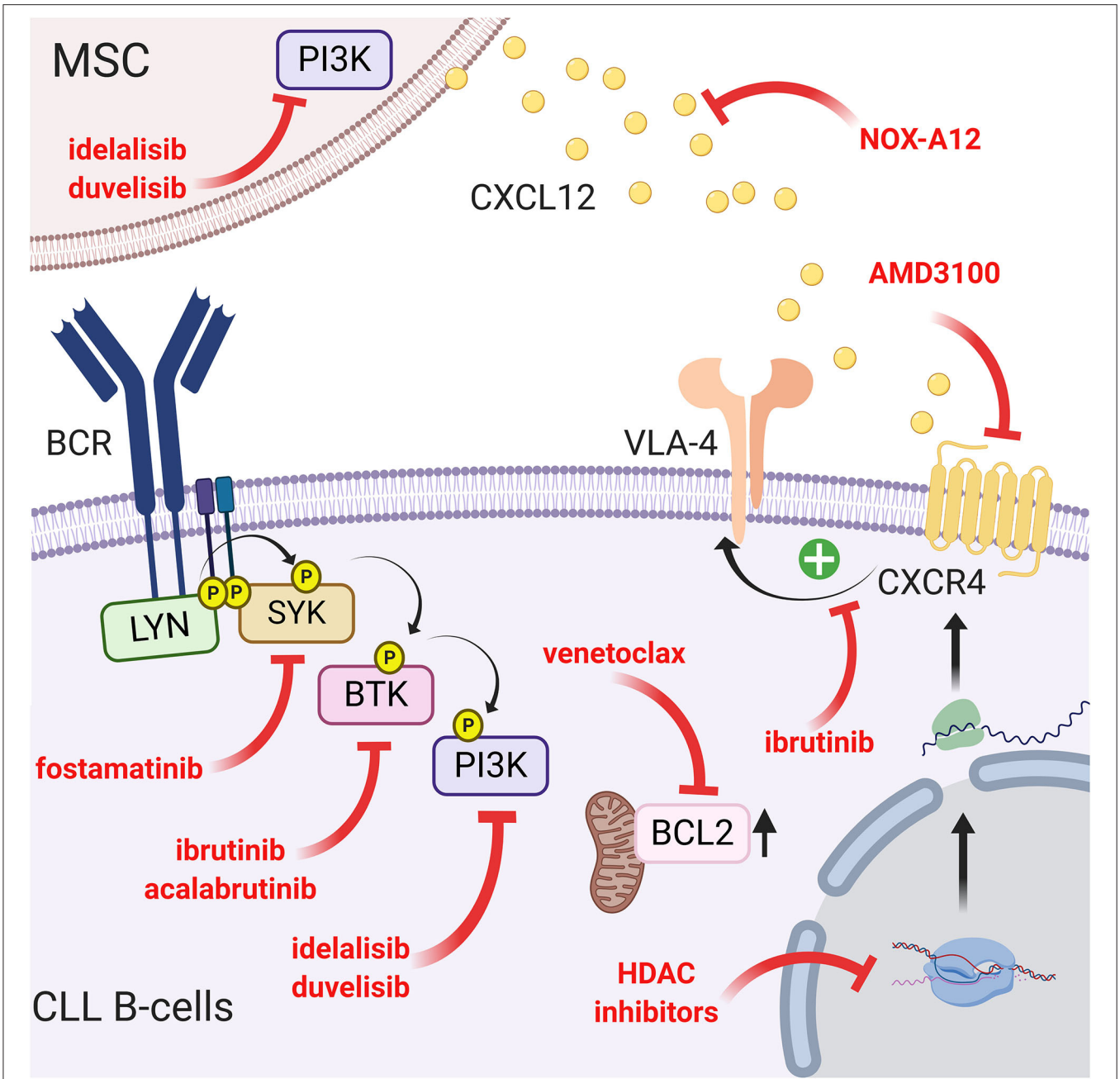

FIGURE 5 | Targeting the CLL/MSC crosstalk using CXCR4/CXCL12 and BCR signaling inhibitors. Several therapeutic strategies have been proposed to inhibit CLL/MSC interactions. (a) blocking of CXCR4 using AMD3100 (71) or using a CXCR4 antibody (148); (b) blocking CXCL12 using NOX-A12 (150); (c) CXCR4/CXCL12 and BCR pathways are interconnected, another way to interfere is to target BCR pathway using different inhibitors: ibrutinib that inhibits BTK and downstream events such as MAPK, PI3K, or NF-κB activation (151) but also reduces VLA-4 activation induced by CXCL12 binding (109) and cell adhesion (79). Acalabrutinib (155) that similarly acts on the migration, the homing and the mobilization of leukemic cells in the circulation. Idelalisib or duvelisib also reduced the ability of stromal cells to support CLL migration and adhesion (161); (d) finally, targeting the over-expression of BCL2 partially induced by the microenvironment has also been proposed (163, 164). Figure created with BioRender.com.

adhesion and matrix proteins, required for activation of PI3Ks and ERK-mediated stabilization of BCL-XL in tumor cells (162). Microenvironment stimuli provided during CLL/MSC coculture lead to the increase of BCL2 through Notch-1, Notch-2, Notch-4 signaling (97). This could partially explain the high level of BCL2 expression in CLL, real hallmark of leukemic cells. Therefore, targeting BCL2 overexpression has been proposed using venetoclax (or ABT-199), an efficient and selective small-molecule inhibitor for BCL2 (163, 164). A schematic representation of the different targeting strategies to

TABLE 1 | Summary of CLL prognostic markers linked to microenvironment interactions.

Prognostic factor	Correlation	References	Poor prognosis	Link with microenvironment	References
IgHV	TFS, OS	(20)	Unmutated (UM)	UM are more prone to apoptosis, more dependent to microenvironment stimulus	(168)
		(24)		UM are associated with an ability to respond to BCR	(169)
				Stimulation by downregulation CXCR4 and CD62L	(170)
ZAP-70	TFS, OS	(22)	High expression	ZAP70+ CLL B-cells have better migration capacities	(172, 173)
		(171)		Gene signature linked to migration, homing or CXCR4/CXCL12 pathways	(166)
CXCR4/ CXCR3	Leucocyte counts, TTFT	(174–176)	Low expression	Decrease in coculture, after BCR stimulation	(137, 166)
CD69	PFS, OS	(26)	Positive	Increase on CLL cells in coculture with MSCs	(68, 166)
				Increase on CLL cells after treatment with MSC EVs	(138)
CD38	TFS, OS	(24, 178)	Positive	Increase on CLL B-cells after 2 weeks in coculture with stromal cells	(68)
LPL	TFS, OS	(171, 180, 181)	High expression	Increase in CLL B-cells after a BCR stimulation	(179)
CCL3/ CCL4	TFS	(121)	High plasma level	Increase after coculture with NLCs	(42)
				Increase after coculture with MSCs	(114)
				Increase after treatment with MCS-derived EVs	(138)
IL-8	Other markers, OS	(182)	High plasma level	Increase in the supernatant of CLL/MSC coculture	(114, 115)
PDGF	ZAP-70, CD38, need of therapy	(128)	High plasma level	PRGF receptor were selectively activated in MSCs by CLL conditioned medium PDGF is were detected in CLL conditioned medium	(128)
VEGF	ZAP-70, CD38, need of therapy	(128)	High plasma level	VEGF is detected in CLL conditioned medium PDGF induced MSC VEGF production	(127)
miR-29c	TFS, OS	(27)	Low expression	Decrease after BCR stimulation	(138)
CD49d	TFS, OS	(184–186)	Positive	Increase on CLL B-cells in coculture with MSCs	(183)

overcome the protection of the microenvironment is shown in **Figure 5**.

THE MICROENVIRONMENT AND PROGNOSTIC FACTORS

For almost 20 years, a plethora of prognostic markers have been described to stratify CLL patients (165). Many of these markers are linked to the capacities of CLL B cells to interact with their microenvironment (166). The gold standard prognostic marker is the mutation status of IgHV (167). Coscia et al. observed that cells obtained from IgHV normal patients are more prone than those with mutated IgHV cells to undergo spontaneous apoptosis *in vitro*, suggesting that unmutated cells are more dependent on survival stimuli from the microenvironment (168). In addition, patients with unmutated IgHV have been shown to be more responsive to external stimuli such as the BCR stimulation by downregulating CXCR4 and CD62L (169, 170). These *in vitro* results suggest that in unmutated IgHV CLL patients, leukemic cells would be more likely to respond to MSC stimuli and be more "BCR activable" in an *in vivo* situation leading to cell survival. Several surrogate markers for IgHV mutation status have also been described (165). Of these, ZAP70 expression has been strongly associated with prognosis (22, 171). Interestingly, numerous authors have shown that ZAP70+ CLL B cells have better migratory capacities and that they are characterized by a gene signature linked to migration, homing or CXCR4/CXCL12 pathways (166, 172, 173). Again, based on these *in vitro* migration capacities, we could speculate that in ZAP70+ patients, cells could have more opportunities to interact with MSCs but also other protecting cells in the *in vivo* bone marrow and lymph node microenvironment. We also observed CXCR4 downregulation on CLL B cells from patients with a poor prognosis when they were co-cultured with MSCs, as well as an increase in CD69 surface expression (166). The CXCR4 level was correlated with leukocyte count (174), and when combined with CXCR3 expression, it has been proposed as a prognostic marker to predict the time to first treatment (TTFT) (175, 176). The lower expression of CXCR4 has also been observed *in vivo* on CLL cells isolated from bone marrow or lymph node compared to peripheral blood (177). Therefore, it is believed that CXCR4 downregulation in the tissues allows a recirculation of leukemic cells in peripheral blood creating by this way their shuttling between the different body compartments (18, 66, 105). Based on this hypothesis, $CXCR4^{high}$ CLL cells would be more efficiently attracted to bone marrow MSCs *in vivo* and, after contact, will downregulate CXCR4. The increase in CD69 on leukemic cells in coculture with MSCs (68, 166) or after treatment with MCS-derived EVs (138) is also linked to prognosis, since CD69 positivity is associated with shorter progression-free survival (PFS) and overall survival (OS) (26). The higher expression of CD69 has also been observed *in vivo* on CLL cells isolated from bone marrow or lymph node compared to peripheral

blood (147) and is the reflection of the CLL activation status. Another example is the high expression of CD38, which has been described as an independent prognostic marker in CLL (24, 178) and is upregulated on CLL B cells after 2 weeks in coculture with stromal cells (68). However, it should be noted that this marker can change during disease course, suggesting that MSC/CLL *in vivo* interactions can also vary during disease. As described above, leukemic cells can also receive BCR stimulation from the microenvironment that induces dramatic changes in their gene expression and secretion (139). Pallasch et al. demonstrated that lipoprotein lipase (LPL) is increased in CLL B cells by BCR stimulation (179). LPL has been described as a strong prognostic marker (171, 180, 181). The release of CCL3 and CCL4 after coculture with NLCs (42) or MSCs (114) or after treatment with MCS-derived EVs (138) is another example showing that the consequences of microenvironmental interactions could be used as prognostic factors, since CCL3 and CCL4 levels in the plasma of CLL patients are associated with the time from diagnosis to initial therapy (121). Herishanu et al. observed that CLL3 and CCL4 expression is increased in CLL cells from bone marrow and even more from lymph node (177) suggesting that CLL/MSC and CLL/NLC interaction probably also occurs *in vivo*. A similar conclusion could be drawn for the increased secretion of IL-8 (114, 115), PDGF (128), and VEGF (127) by MSCs in co-culture and the association of elevated plasma levels of these factors with high-risk factors and more advanced stage in CLL patients (128, 182). The decrease in microRNA-29c after BCR stimulation (138) could also explain its prognostic power (27). CLL B cells in coculture with MSCs significantly upregulate the expression of CD49d (183), which is also a very strong prognostic marker (184–186). Taken together, these data suggest that several prognostic markers are the direct consequence of leukemic cell interactions with the microenvironment while others which do not change along time (such as unmutated IgHV) define patients whose cells are more able to respond to microenvironmental stimuli *in vivo*. **Table 1** summarizes the different prognostic markers, their prognostic power in CLL and their link with the microenvironment.

CONCLUSIONS

Over the last two decades, many reports have demonstrated the different ways CLL B cells and stromal cells in the bone marrow and lymph node communicate. These cell interactions are bidirectional, inducing many changes in both cell types: dysregulation of adhesion molecules, abnormal secretion of cytokines, chemokines and growth factors and modification of normal trafficking and homing. CLL cells are able to crosstalk with close surrounding cells by direct cell-to-cell contact and can communicate with distant cells via the production of extracellular vesicles. CLL cells can modify healthy cells in different ways, altering them from their physiological functions. All these different interactions make it difficult to study this topic exhaustively, but recent studies have highlighted crucial and targetable pathways. Targeting BCR signaling has been shown to mobilize leukemic cells out of their protective microenvironment. Some new small molecules have already demonstrated their efficacy in CLL patients, improving their overall survival. Our knowledge of how leukemic cells are able to interact also brings out a plethora of prognostic markers that are only a reflection of how efficient this crosstalk is. Despite some indirect data (such as serum level of some cytokines or gene expression from CLL cells isolated from different body compartments), the vast majority of the data we have access to today about CLL/MSC interactions derive from *ex vivo* studies. The *ex vivo* expansion of MSCs requiring multiple passages, the use of stromal cell lines, the isolation of CLL cells from patient blood or the use of leukemic cell lines could not reflect all the aspects of the *in vivo* situation. Therefore, further studies are needed to extensively understand the "true" *in vivo* CLL/MSC biology. However, understanding CLL/microenvironment communication has already helped us discover new treatment strategies, but further functional characterizations will open new ways to avoid patient relapse in the future.

AUTHOR CONTRIBUTIONS

ND and BS: conception, design, and writing of the manuscript. ND, EC, NM, DB, LL, and BS: review, and/or revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Potential Oncogenic Effect of the MERTK-Dependent Apoptotic-Cell Clearance Pathway in Starry-Sky B-Cell Lymphoma

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The histological architecture of certain aggressive B-cell lymphomas (prototypically Burkitt's lymphoma, BL) is characterized by a "starry-sky" (SS) appearance. This is caused by tumor-associated macrophages (TAMs), which appear in standard histological preparations as "stars" in a darkly stained "sky" of lymphoma cells. SS-TAMs accumulate in response to constitutive apoptosis in these tumors and are activated by the apoptotic tumor cells to a pro-oncogenic phenotype. The extent to which SS-TAMs contribute to lymphoma growth through responses generated by interactions with apoptotic tumor cells is unknown. Here, we demonstrate a role for the receptor tyrosine kinase, MERTK, in the oncogenic activity of SS-TAMs. We show that MERTK expression is largely restricted to the macrophages of human BL and of murine models of SS B-cell lymphoma and that it is upregulated in SS-TAMs as compared to the germinal center or paracortical macrophages of normal lymph nodes. Our results further demonstrate that MERTK is active in the phagocytosis of apoptotic lymphoma cells by macrophages and, most significantly, that SS lymphoma growth is markedly inhibited in *Mertk*$^{-/-}$ mice. These results point toward the MERTK apoptotic-cell clearance/response pathway playing a key role in growth of aggressive B-cell lymphoma and identifies MERTK as a novel potential antilymphoma target.

Keywords: non-Hodgkin lymphoma, apoptosis, TYRO3-AXL-MERTK, receptor tyrosine kinase, macrophage, phagocytosis

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INTRODUCTION

Constitutive tumor-cell apoptosis is high in aggressive cancers, including non-Hodgkin lymphoma (1). Emerging evidence indicates that responses to apoptosis in the tumor microenvironment can promote cancer growth, not only in primary tumors, but also in post-therapeutic relapse (2–7). In SS lymphomas, apoptosis is highly prominent in standard biopsy preparations, and a key response to apoptotic tumor cells is the accumulation of TAMs, together with their activation to a pro-oncogenic phenotype (5). It remains unclear however, how apoptotic tumor cells activate TAMs to help promote net tumor growth. Here, we focus on MERTK, a member of the TYRO3/AXL/MERTK family of receptor tyrosine kinases, which regulate tissue development and homeostasis via two mechanistically related immunosuppressive functions: the clearance of cells undergoing apoptosis and anti-inflammatory signaling (8–10).

It is becoming increasingly evident that the TYRO3/AXL/MERTK signaling axis has oncogenic properties in a wide range of cancers. In hematopoietic malignancies, although individual components of this axis are expressed by tumor cells and/or TAMs (11–14), their role(s) in the pathogenesis of these cancers have not been defined. MERTK is a well-established phagocyte receptor for clearance of apoptotic cells (8–10) operating via its ligands, GAS6, and Protein S (PROS1), which bridge the receptor to phosphatidylserine (PS) exposed at apoptotic cell surfaces. Inhibition of MERTK leads to persistence of apoptotic cells, especially in the germinal centers of lymphoid follicles and to the associated emergence of autoimmune disease symptoms (10, 15, 16). Preferential expression of *MERTK* and *GAS6* are associated with reparatory, M2-like macrophage polarization (5, 17), which is typical of wound-healing macrophages and of TAMs (18). In the present brief investigation, we tested the hypothesis that MERTK is involved in the clearance of apoptotic lymphoma cells by SS-TAMs and that it is important for the growth of aggressive, SS lymphoma.

MATERIALS AND METHODS

Cell Lines and Animal Models

The BL cell line, BL2 was derived from a sporadic, Epstein-Barr virus–negative case of BL (19). The THP-1 line was established from a patient with monocytic leukemia (20). Both lines were cultured *in vitro* as we have previously described (21). BL2 cells were xenografted subcutaneously to SCID mice according to our established methods (5) and formed aggressive, starry-sky tumors. We previously derived the MycEd1 cell line (5) from an aggressive starry-sky B-cell lymphoma of a male C57BL/6 mouse carrying the λ-*MYC* transgene (22). MycEd1 cells were cultured *in vitro* and also used in transplantation experiments using our previously established protocols (5) in wild-type (WT) and *Mertk*$^{-/-}$ C57BL/6 mice (23) (kindly provided by Dr. Greg Lemke, The Salk Institute for Biological Studies). MycEd1 tumor growth was assessed following subcutaneous injection of 5×10^6 viable Myc-Ed1 cells into 6- to 12-week-old male WT or *Mertk*$^{-/-}$ mice. Mice were observed daily, and growth of tumors was monitored using calipers. In all experiments, mice were humanely sacrificed either (a) when tumors reached maximal dimensions according to the UK Animals (Scientific Procedures) Act 1986 regulations or (b) day 20 post-injection, whichever was the sooner.

Gene Expression Analysis

"*In situ* transcriptomics" of SS-TAMs from BL (BL2) xenografts, lymph node germinal center (GC), and paracortical macrophages was performed following laser capture microdissection of macrophages exactly as described (5). λ-MYC [MycEd1 cell line, see (5, 24)] gene expression was carried out on Affymetrix Mouse Gene 2.1 GeneChip arrays. Gene expression analysis of BL2 and primary human dendritic cells [DC, myeloid positive control cells, prepared as we have described previously (25)] was carried out on Affymetrix Human Genome U133 Plus 2.0 arrays. Data were processed in R and normalized with RMA. Real time RT-PCR was carried out as follows: RNA was isolated using RNeasy Mini Kit (Qiagen), DNAse-treated and reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies). Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems). qPCR was performed using an ABI 7900 Real Time PCR Machine with ABI SDS (Sequence Detection System) software.

Immunocytochemistry and Immunohistochemistry

Human cells (viable or apoptotic BL2 or THP1) were labeled with antihuman MERTK-PE (clone 125518) R&D FAB8912P (isotype mouse IgG2b, κ) or mouse PE-conjugated isotype control (clone 11711) R&D IC002P and mouse cells (MycEd1 or bone marrow–derived macrophages, BMDMs) with antimouse MERTK-PE (clone 108928) R&D FAB5912P (isotype Rat IgG2A) or Rat IgG2A PE-conjugated isotype control (clone 54447) R&D IC006P prior to flow cytometric analysis.

Formalin-fixed, paraffin-embedded tissues were sectioned and stained with standard hematoxylin and eosin or were used in immunohistochemistry (IHC) as described (5). Sections were labeled with monoclonal mouse antihuman CD68 clone PG-M1 (Dako M0876) or polyclonal goat antihuman MERTK (R&D AF891), and subsequently with either goat antimouse IgG, biotinylated (Vector #BA-9200) or horse antigoat IgG, biotinylated (Vector #BA-9500). Following incubation with Vectastain Elite ABC Reagent and DAB (Vector SK-4100), samples were counterstained in hematoxylin.

Immunoblotting

WT murine BMDMs treated with or without 200 nM dexamethasone for 24 h, MycEd1 cells, undifferentiated THP1, THP1 cells differentiated with PMA (100 ng/ml) for 2 days, and BL2 cells were lysed in reducing cell lysis buffer, 5 μg lysate were loaded per well on a 4–12% Bis-Tris NuPAGE gel, run under reducing conditions then transferred to Hybond-P. Membrane was blocked in 5% BSA then incubated in 1:1,000 goat antimouse MERTK (R&D #AF591) or 1:1,000 goat antihuman MERTK (R&D #AF891), for mouse and human lysates, respectively, in 5% BSA overnight at 4°C. Membranes were then incubated in 1:5,000 donkey antigoat IgG-peroxidase (Jackson ImmunoResearch #705-035-003) in 5% milk for 1 h at room temperature and then developed using ECL.

Apoptosis, Ligand Binding, and Phagocytic Clearance of Apoptotic Cells

Human monocyte-derived macrophages (HMDMs) and murine BMDMs were prepared as described (24, 26). In brief, BMDMs were prepared from the femurs of 8- to 12-week-old mice and cultured for 7–8 days with 100 ng/ml rhM-CSF (R&D Systems, Abingdon, UK) on bacteriological-grade Petri dishes. HMDMs were prepared from peripheral blood monocytes enriched using the pan monocyte isolation kit, human (MACS Miltenyi #130-096-537) and cultured in 2% human AB serum for 7 days. Apoptosis was induced in lymphoma cells by UV-treatment and

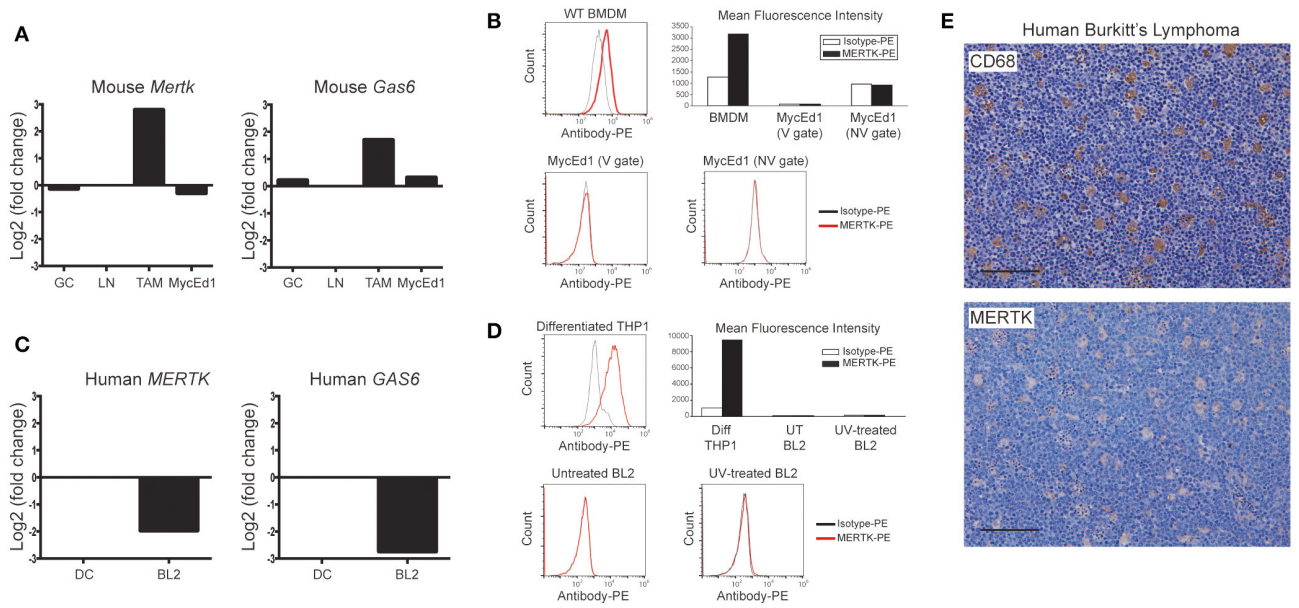

FIGURE 1 | Preferential expression of MERTK by macrophages in SS-NHL. **(A)** *Mertk* and *Gas6* expression are low/absent in murine SS-NHL cells (MycEd1) relative to SS-TAMs. Mouse gene expression data for *Mertk* and *Gas6* are shown as $\log_2$ fold-change relative to paracortical lymph node macrophages (LN). Expression is also shown for tingible body macrophages of lymph node germinal centers (GC). **(B)** Absence of mouse MERTK protein expression from murine MycEd1 lymphoma cells. Untreated MycEd1 cells were analyzed by flow cytometry (MycEd1 cultures have a high level of constitutive apoptosis). WT BMDMs were used as positive control cells. **(C)** Human *MER*TK and *GAS6* gene expression data in BL2 cells shown relative to dendritic cells (DC). **(D)** Human MERTK protein as analyzed by flow cytometry is absent from BL2 tumor cells; differentiated THP-1 cells were used as positive controls. BL2 cells were either left untreated or were UV-treated (300 mJ/cm^2) and incubated for 3 h at 37°C to become apoptotic (cells were 60% apoptotic by AxV/Sytox blue). Representative flow cytometric analyses are shown; gating strategies, including those delineating viable (V) and nonviable (NV) cells, are shown in **Supplementary Figure 1D**. **(E)** SS-TAMs in BL are CD68 and MERTK positive. Representative micrographs, scale bar = 100 μm.

assessed by flow cytometry following annexin V (AxV)/Sytox Blue staining (5).

Ligand binding: GAS6 and PROS1 proteins were coupled using the Cy5 Antibody Labeling Kit (GE Healthcare, Buckinghamshire, UK). Briefly, protein to be labeled (at 1.0–1.2 mg/ml) was exchanged into 100 mM sodium hydrogen carbonate buffer (pH 8.3) and incubated with Cy5 mono-reactive dye pack for 30 min in the dark at room temperature as recommended by the manufacturer (www.GELifesciences.com). The reaction was terminated by addition of glycine to a final concentration of 50 mM, and then protein was buffer-exchanged into PBS. The degree of protein labeling was estimated from measurement of absorbance at 280 and 650 nm (Cy5) and was routinely found to be between 2.5 and 3.5 moles of dye/mole of protein. Functionality of labeled protein was assessed by testing the potential to induce MERTK phosphorylation and the capacity to confer MERTK-dependent phagocytosis of apoptotic cells (data not shown). Ligand binding to lymphoma cells (±UV treatment) was carried out along with Annexin-V-488 and Sytox Blue labeling (Life Technologies) in 20 mM HEPES buffer containing 140 mM NaCl, 0.1% BSA and 2 mM $CaCl_2$ followed by flow cytometry. To control for nonspecific binding of the fluorescent proteins, binding was carried out using buffer in which the $CaCl_2$ was substituted by 2.5 mM EDTA (specific binding of Annexin V, GAS6, and PROS1 all require Ca^{2+}). Phagocytosis of apoptotic cells was assayed objectively by flow cytometry using well-characterized, established methods (27). In these assays, apoptotic cells were stained with 1 μM pHrodo (Life Technologies #P36600) and macrophages were labeled with 0.5 μg/mL CellTrace Far Red (Life Technologies #C34564). Phagocytic macrophages exhibit enhanced pHrodo fluorescence following internalization of labeled apoptotic lymphoma cells into the acidic environment of phagosomes. GAS6 and gla-less GAS6 were kindly provided by Dr. Erin Lew, The Salk Institute, and the gla-less PROS1 was kindly provided by Mary Jo Heeb (Scripps Research Institute) (28); PROS1 (HPS 4590AL) was purchased from Enzyme Research Laboratories (South Bend, IN, USA).

RESULTS

MERTK Is Expressed Preferentially by Macrophages in SS-Lymphoma

Throughout these investigations, we used well-characterized models of murine and human SS-lymphoma (MycEd1 and BL2, respectively). Through *in situ* transcriptomics of SS-TAMs of BL xenografts (5), we initially noted increased expression by TAMs of several members of the TYRO3/AXL/MERTK axis, including *Mertk* and *Gas6*, as compared with tingible

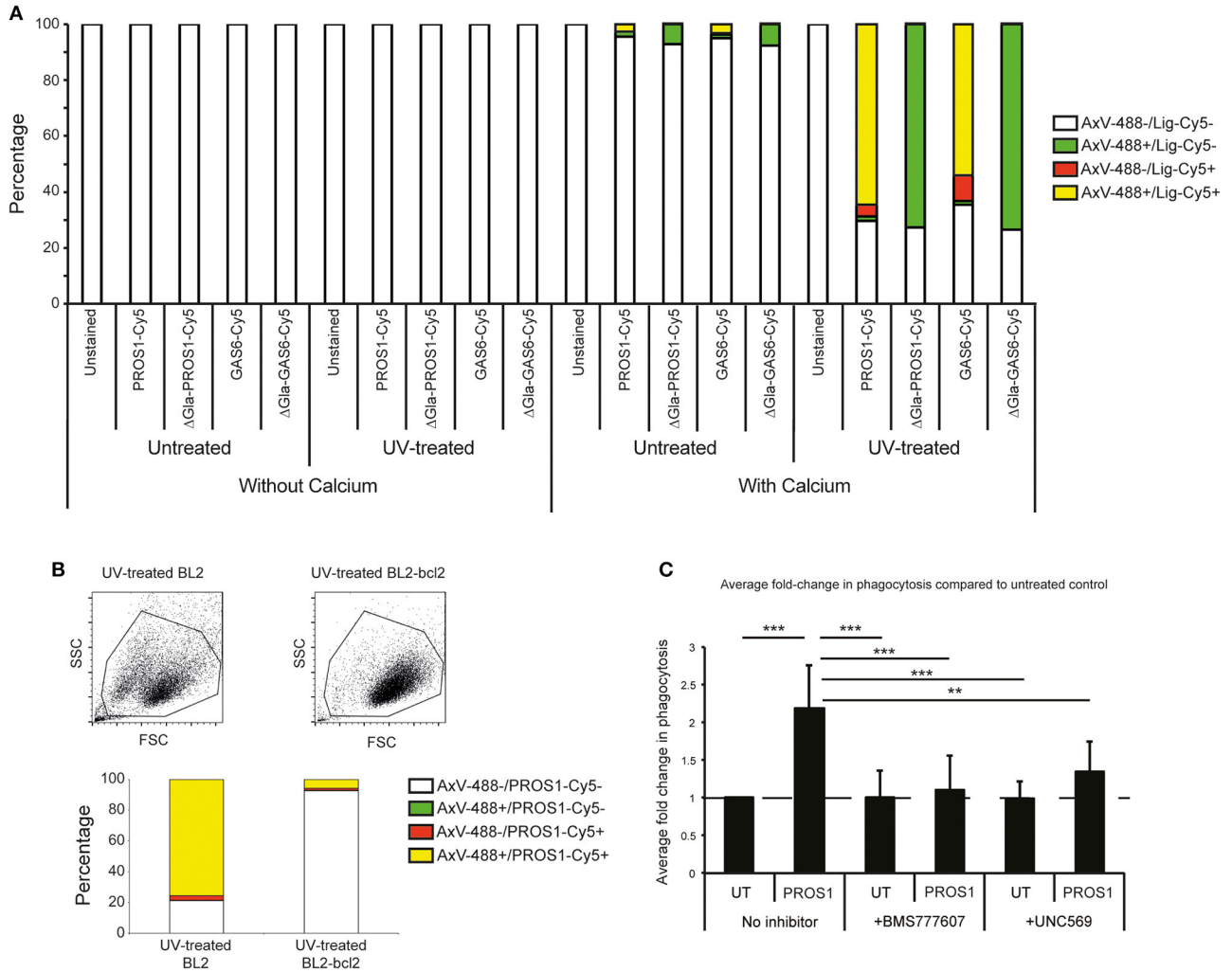

FIGURE 2 | Requirement for MERTK and its ligands in macrophage clearance of apoptotic human lymphoma cells. **(A)** MERTK ligands PROS1 and GAS6 preferentially bind apoptotic BL2 cells in a Ca^{2+} and gla-domain-dependent manner. Flow cytometric analyses of untreated (low levels of constitutive apoptosis) or UV-treated (high levels of apoptosis) BL2 cells incubated with Cy5-conjugated ligands (PROS1-Cy5, PROS1-Gla-less-Cy5, GAS6-Cy5, and GAS6-Gla-less-Cy5) in the presence of 2 mM $CaCl_2$ or 2.5 mM EDTA ("without calcium") and with AxV-488 for dual staining. The without-calcium samples were included to control for nonspecific binding of the fluorescent proteins (see section Materials and Methods). **(B)** MERTK ligand, PROS1 preferentially binds apoptotic BL2 cells as compared with apoptosis-resistant BL2-bcl2 cells. BL2 cells were UV-treated and incubated for 5 h at 37°C to become apoptotic. BL2-bcl2 cells undergoing the same procedure were used as a viable control. Cells were washed and resuspended in buffer with 2 mM $CaCl_2$. Cells were labeled with AxV-488, PROS1-Cy5 or both. Note that nonirradiated control BL2-bcl-2 cells bound neither AxV, nor MERTK ligands (not shown). **(C)** Phagocytosis of apoptotic BL2 cells by HMDMs is enhanced by PROS1 and inhibited with MERTK inhibitors (BMS 777607 and UNC569). Apoptotic BL2 cells were stained with pHrodo and HMDMs were labeled with CellTrace Far Red. Stained HMDMs were pretreated with either 500 nM BMS 77760 or 2.5 μM UNC569 for 40 min prior to phagocytosis assay. Apoptotic cells were coincubated with HMDMs at a ratio of 7:1 ± 25 nM PROS1. Inhibitors were diluted during the coculture to 100 nM and 0.5 μM for BMS 77760 and UNC569, respectively. BL2 and HMDMs were cocultured for 40 min prior to lifting the HMDMs using trypsin/EDTA and analysis by flow cytometry as described (27). Data shown are mean fold changes of phagocytic macrophages ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ One-way ANOVA with Bonferroni post-test ($n = 6$–8).

body macrophages from germinal centers or paracortical lymph node macrophages. By contrast, expression of mouse *Mertk* and *Gas6* by murine lymphoma cells (MycEd1) was low or absent (**Figure 1A**). Absence of MERTK protein expression by murine lymphoma cells was confirmed by flow cytometry (**Figure 1B**), by immunoblotting (**Supplementary Figure 1A**) and by real-time RT-PCR (**Supplementary Figure 1B**). Focusing on human BL, we found that *MERTK* and *GAS6* gene expression was low or absent from BL2 cells, relative to DCs (**Figure 1C**) and that MERTK protein expression was absent from BL2 cells *in vitro* (**Figure 1D**) and largely restricted to SS-TAMs *in vivo* (**Figure 1E**), confirming the transcriptomics data. Furthermore, TAMs engulfing apoptotic BL cells *in situ* tended to be $MERTK^{+}$ (**Figure 1E**). Absence of MERTK expression by BL2 cells was also confirmed by immunoblotting (**Supplementary Figure 1C**).

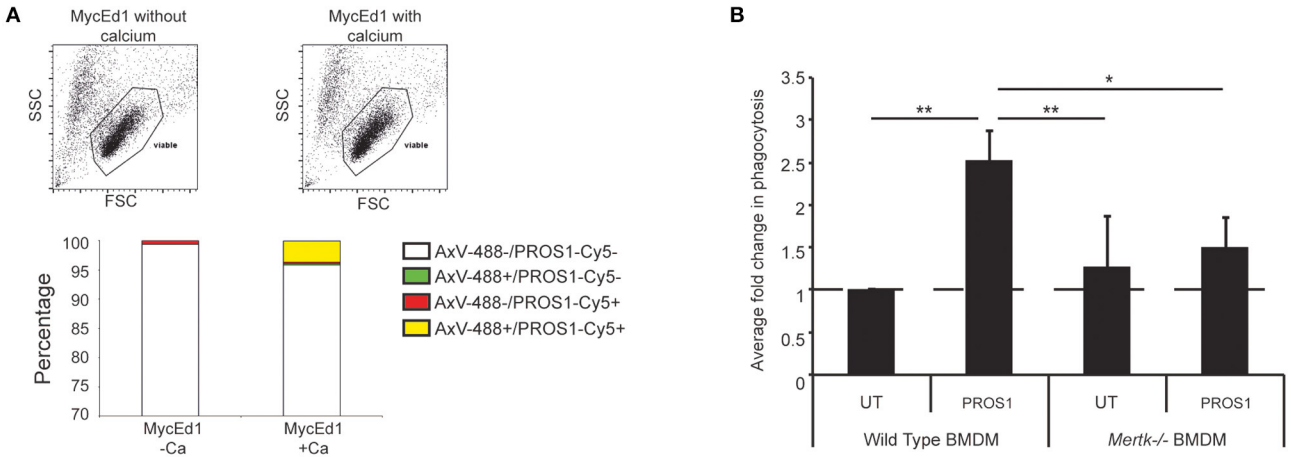

FIGURE 3 | Requirement for MERTK in macrophage clearance of apoptotic murine lymphoma cells. **(A)** Flow cytometric analyses demonstrating that MERTK ligand, PROS1 binds apoptotic murine lymphoma cells (MycEd1) in a Ca^{2+}-dependent manner. MycEd1 cells ± UV treatment were incubated with PROS1-Cy5 in the presence of 2 mM $CaCl_2$ ($+Ca^{2+}$) or 2.5 mM EDTA ($-Ca^{2+}$) and with AxV-488 for dual staining. The "viable" scatter gate is a well-established gate used to reliably detect apoptotic lymphoma cells prior to plasma membrane permeabilization (29). Nonirradiated control MycEd1 cells bound neither AxV nor PROS1 (not shown). **(B)** PROS1 enhances phagocytosis of apoptotic MycEd1 cells by wild-type but not *Mertk*$^{-/-}$ BMDMs. BMDMs from wild-type and *Mertk*$^{-/-}$ mice were coincubated with pHrodo-labeled UV-treated MycEd1 cells in the presence or absence of 25nM PROS1 for 45 min at 37°C. Data shown are mean fold changes of phagocytic macrophages ± SEM. *$p < 0.05$, **$p < 0.01$ One-way ANOVA with Bonferroni post-test ($n = 3$–4).

MERTK and Its Ligands Are Required for Efficient Clearance of Apoptotic Human Lymphoma Cells by Macrophages

To determine the potential role of MERTK and its ligands in phagocytic clearance of apoptotic BL2 cells, we next tested whether the ligands GAS6 and PROS1 are capable of opsonizing these cells. As shown in **Figures 2A,B**, both murine recombinant Gas6 (which is known to be a good ligand for human MERTK) and PROS1, purified from human plasma, bound strongly to apoptotic BL2 cells in a manner dependent on the PS-binding Gla domains of the ligands. Furthermore, we used PROS1 to demonstrate increased phagocytosis of apoptotic BL2 cells by HMDMs *in vitro* (**Figure 2C**). The PROS1-enhanced phagocytosis was suppressed by the MERTK kinase inhibitor UNC569 and by the c-MET inhibitor BMS777607, which, at the concentration used, also has MERTK inhibitory activity (**Figure 2C**). These results confirm that MERTK signaling supports phagocytic clearance of apoptotic BL2 cells by macrophages and, together with the *in situ* expression analyses, are consistent with the notion that engulfment of these cells by SS-TAMs is MERTK-dependent.

Murine Macrophage MERTK Is Required for Clearance of Apoptotic Lymphoma Cells and for SS-Lymphoma Growth *in vivo*

Because MERTK activation by apoptotic lymphoma cells may provide anti-inflammatory and immunosuppressive signals that promote tumor growth, we next tested the requirement for MERTK in an aggressive, preclinical transgenic murine SS lymphoma model, λ-*MYC* (22), which we have used previously using our derived MycEd1 line (5). We found that, just as in human BL, MycEd1 cells became MERTK ligand binding when they underwent apoptosis (**Figure 3A**), and phagocytosis by macrophages was demonstrably MERTK-dependent (**Figure 3B**). Similar to BL2 xenografts in mice (**Figure 4A**, upper panel), immunohistochemical expression of murine MERTK in MycEd1 tumors was mainly by stromal cells, notably SS-TAMs, rather than by the tumor cells themselves (**Figure 4A**, lower panel). This reflected expression profiling and flow cytometric analyses of MycEd1 cells, which indicated little or no expression of *Mertk* RNA or MERTK protein, respectively (**Figures 1A,B** and **Supplementary Figures 1A,B**). Strikingly, growth of MycEd1 tumors *in vivo* was found to be very strongly dependent on *Mertk* (**Figures 4B,C**).

DISCUSSION

These results demonstrate a positive, causative link between MERTK expression and growth capacity of SS NHL, at least in the exemplar models studied here. In the context of the proven ability of apoptotic lymphoma cells ultimately to facilitate SS tumor growth, we propose that inhibition of MERTK may be helpful in combination with apoptosis-inducing antilymphoma therapeutics. The present study does not elucidate the detailed mechanism(s) by which MERTK controls SS lymphoma growth. Based on the evidence presented, taken together with published activity of MERTK in immunosuppressive signaling following engagement of appropriately opsonized apoptotic cells (via GAS6 and PROS1), we suggest that interactions between apoptotic lymphoma cells and MERTK-expressing stromal/immune cells of the tumor lie at the root of the mechanism. These may stimulate suppression of antitumor

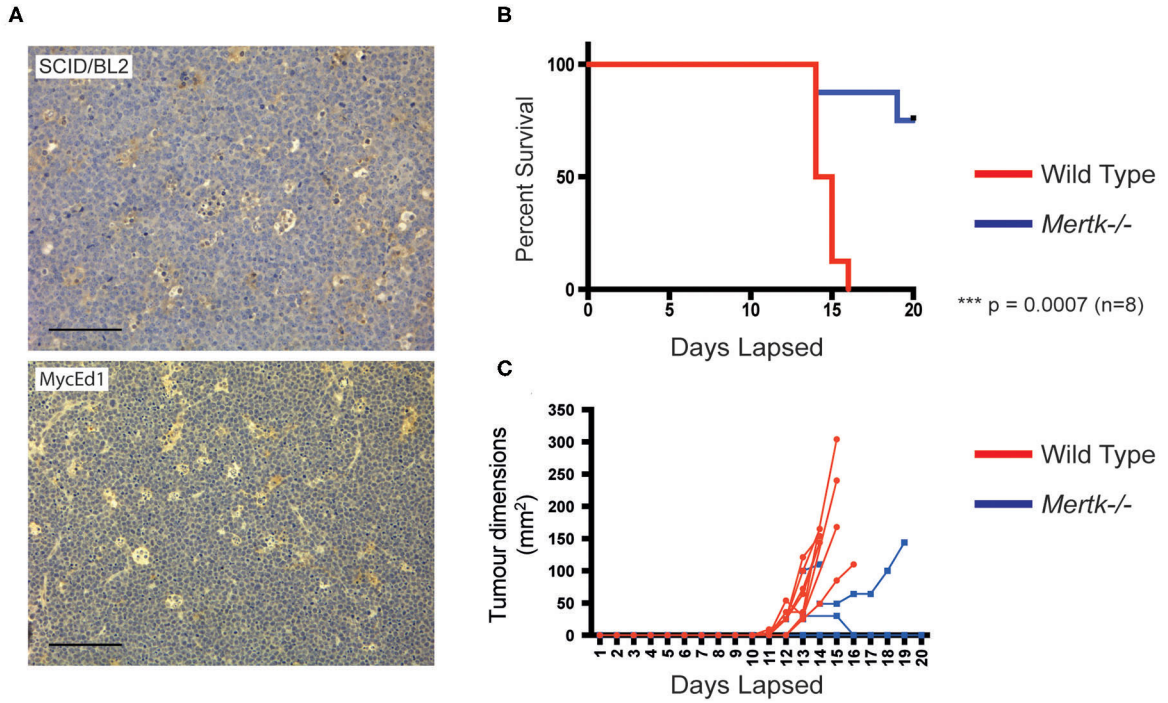

FIGURE 4 | Growth of aggressive SS lymphoma requires Mertk. **(A)** SS-TAMs in mouse model lymphomas are Mertk-positive. Immunohistochemistry of mouse MycEd1 tumors or BL2 xenograft tumors labeled with antimouse Mertk. Scale bar = 100 μm. Representative images. Survival is enhanced **(B)** in parallel with inhibition of MycEd1 tumor growth **(C)** in *Mertk*$^{-/-}$ mice. Male *Mertk*$^{-/-}$ and aged-matched WT littermate control C57BL/6 mice were injected subcutaneously with 5 × 10^5 MycEd1 lymphoma cells. Mice were observed daily and growth of tumors was monitored using calipers. $P < 0.001$ Mantel-Cox log rank test.

immunity or alternative, trophic responses, such as growth factor production or angiogenesis. In these contexts, it is noteworthy that *Gas6* is upregulated in SS-TAMs, at least in BL2 xenografts (**Figure 1A**), suggesting that these macrophages of the lymphoma microenvironment are armed with both receptor and ligand for such responses.

Intriguingly, amelioration of λ-*MYC* SS lymphoma growth in the *Mertk*$^{-/-}$ mice phenocopies mice deficient in *Gals3* [galectin-3, see (24)], suggesting that MERTK and galectin-3 could provide different components in a common, pro-oncogenic mechanism. This possibility is supported by evidence that galectin-3 has been implicated both in apoptotic cell clearance by macrophages (30, 31) and as a ligand for MERTK (32). However, we have been unable to demonstrate the latter capacity in relation to apoptotic lymphoma cells (our unpublished observations). Given the capability of galectin-3 to support M2-like activation of macrophages, including TAMs (30, 33), our results are consistent with MERTK and galectin-3 each being required for critical, possibly independent pathways in pro-oncogenic TAM activation. In order to understand in further detail the possible roles of MERTK in the stromal/immune microenvironment of SS-lymphoma, several aspects of the work reported here merit further investigation. These include extended studies of the function of MERTK in polarizing TAM activation (especially including genes like *MRC1*, *MSR1*, and *LRP1*) that we have previously found to be upregulated in SS-TAMs engaged in engulfment of apoptotic cells as well as investigations into the importance of MERTK expression and activity in other immune/stromal cells. Furthermore, other TYRO3/AXL/MERTK family members, notably AXL, may be important in this regard.

In conclusion, the work presented here provides a strong rationale for the TYRO3/AXL/MERTK axis, notably MERTK, to be targeted in antilymphoma therapy. Further studies in additional lymphoma models will be required to prove the generality of these results and to elucidate in detail the underlying mechanisms that support MERTK-dependent NHL growth.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO database, the accession number for datasets analyzed in this study is GSE64366.

ETHICS STATEMENT

The animal studies were approved by the University of Edinburgh Animal Welfare and Ethical Review Body and under the UK Animals (Scientific Procedures) Act 1986 Project Licence number PPL 70/8139.

AUTHOR CONTRIBUTIONS

SF-M, NB, LM, MP, CL, and PH performed research and analyzed data. CG and ID designed the research and analyzed data. CG, ID, and SF-M wrote the manuscript with input from other authors. 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/fimmu.2020.01759/full#supplementary-material

Supplementary Figure 1 | Untreated MycEd1 cells were analyzed by **(A)** Western blotting for MERTK protein or **(B)** real-time PCR for *Mertk* message. WT BMDMs with or without 24 h 200 nM dexamethasone treatment were used as positive control cells for Western blotting. WT and *Mertk*$^{-/-}$ BMDMs were used as positive and negative controls, respectively, for real-time PCR. **(C)** BL2 cells were analyzed for MERTK by Western blotting. Differentiated THP1 cells were used as positive controls. **(D,E)** Scatter plots showing the gating used in **Figures 1B,D**, respectively.

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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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REVIEW
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Proliferative Signals in Chronic Lymphocytic Leukemia; What Are We Missing?

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Chronic lymphocytic leukemia (CLL) cells cycle between lymphoid tissue sites where they actively proliferate, and the peripheral blood (PB) where they become quiescent. Strong evidence exists for a crucial role of B cell receptor (BCR) triggering, either by (self-)antigen or by receptor auto-engagement in the lymph node (LN) to drive CLL proliferation and provide adhesion. The clinical success of Bruton's tyrosine kinase (BTK) inhibitors is widely accepted to be based on blockade of the BCR signal. Additional signals in the LN that support CLL survival derive from surrounding cells, such as CD40L-presenting T helper cells, myeloid and stromal cells. It is not quite clear if and to what extent these non-BCR signals contribute to proliferation in situ. *In vitro* BCR triggering, in contrast, leads to low-level activation and does not result in cell division. Various combinations of non-BCR signals delivered via co-stimulatory receptors, Toll-like receptors (TLRs), and/or soluble cytokines are applied, leading to comparatively modest and short-lived CLL proliferation *in vitro*. Thus, an unresolved gap exists between the condition in the patient as we now understand it and applicable knowledge that can be harnessed in the laboratory for future therapeutic applications. Even in this era of targeted drugs, CLL remains largely incurable with frequent relapses and emergence of resistance. Therefore, we require better insight into all aspects of CLL growth and potential rewiring of signaling pathways. We aim here to provide an overview of *in vivo* versus *in vitro* signals involved in CLL proliferation, point out areas of missing knowledge and suggest future directions for research.

Keywords: chronic lymphocytic leukemia, proliferation, micro-environment, CD40, toll-like receptor, crosstalk

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INTRODUCTION

CLL is the most frequent hematologic cancer and is characterized by the clonal expansion of $CD5^+CD19^+$ malignant B cells (1). CLL patients can be distinguished into 2 categories with distinct clinical outcome, based on the presence or absence of somatic hypermutation in the immunoglobulin heavy chain variable region (IGHV) genes of the clonotypic B cell receptor (BCR). Patients with low IGHV mutation levels (<2% change from the germline sequence), referred

to as unmutated (um-CLL), experience a significantly more aggressive disease than those with mutations, referred to as mutated (m-CLL). IGHV mutation status remains one of the most robust prognostic markers in CLL, yet it does not entirely reflect the heterogeneity of the disease (2).

In addition, CLL is a prime example of a B cell malignancy that is crucially dependent on signals from the microenvironment. CLL cells cycle between lymphoid tissue sites and peripheral blood (PB). CLL cells accumulating in the PB become quiescent, whereas active CLL cells at lymphoid tissue sites are provided with signals from surrounding cells, such as CD40L-presenting T helper cells, myeloid, and stromal cells (3). Since CLL cells are strictly microenvironment-dependent, the crosstalk with the surrounding microenvironment in promoting CLL survival and proliferation has been a focus of intense research.

In order not to perish by neglect, CLL cells need to return to the proliferation sites in lymphoid tissues. This notion is supported by BCR kinase inhibitors that have entered the clinic, foremost the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib, which was found to induce lymphocytosis in patients due to the release of activated CLL cells from lymphoid tissue sites into the PB, preventing migration back into the lymphoid tissue sites and thereby halting disease progression. However, stopping ibrutinib treatment reverses remission and some patients may relapse even on ibrutinib treatment, highlighting the need for greater understanding of the mechanisms that promote CLL proliferation (4).

Strong evidence exists for a crucial role of BCR signaling to drive CLL disease progression, especially the success of inhibitors targeting BCR-associated kinases (5). However, *in vitro* BCR triggering only leads to low-level activation without induction of proliferation, suggesting that additional factors that play a role *in vivo* are missing (6). Several other receptors are known to mediate interactions between CLL cells and the microenvironment, such as CD40 or Toll-like receptor (TLRs), in combination with cytokine receptors which have been shown to induce proliferation upon *in vitro* stimulation (7, 8). It is not quite clear if and to what extent these non-BCR signals contribute to proliferation *in vivo*. Perhaps a combination of stimuli is what may really drive CLL proliferation *in vivo*, or makes CLL develop into more aggressive disease.

In this review, we aim to provide an overview of *in vivo* versus *in vitro* signals involved in CLL proliferation. With focus on BCR, CD40 and TLR signaling, we will attempt to separately describe *in vivo* and *in vitro* data and, in each case, discuss how these receptor-mediated signaling modes may drive CLL. By integrating multiple facets of the CLL microenvironment, we aim to bridge the gap between *in vivo* and vitro studies, point out areas of missing knowledge and suggest future directions for research.

IN VIVO CLL PROLIFERATION

The conceptual framework of CLL biology has changed over the past decades. The traditional view was that CLL is a disease deriving from an inherent defect in apoptosis, in which slowly proliferating CLL cells accumulate due to diminished cell death. In this view, CLL cells continue to accumulate until they reached a level that is detrimental to the patient (9). However, *in vivo* labeling studies using deuterated water have changed this view by documenting the dynamic cellular kinetics of CLL cells. These studies showed that CLL is a dynamic condition, comprising of CLL cells that multiply and die at variable rates (9). Proliferation rates in patients with stable white blood cell count (WBC) indicated that CLL cells are continually dying and replenishing. Therefore, fast clonal birth is not necessarily associated with high WBC increase, suggesting that WBC does not reflect underlying cellular dynamics but rather the net effect of clonal turnover between cell birth and death rates (10). These studies consistently estimated that between 0.07–1.75% of CLL cells circulating in the PB are added to the population each day (9–13). Importantly, patients with birth rates >0.35% were much more likely to exhibit active or progressive disease than patients with lower birth rates (10). Also in patients with recently diagnosed disease, high CLL birth rate was a strong predictor of the need for earlier initial treatment, reinforcing the concept that enhanced cell proliferation is an important driver in the biology of disease progression (13, 14).

Interestingly, both birth rates and death rates of CLL cells were lower than those of healthy B cells, suggesting that CLL cells divide slower and have a lower turnover than their normal counterpart (11, 12). In addition, telomere lengths of CLL cells were shorter than those of healthy B cells in age-matched healthy donors (HDs), showing that CLL cells completed more rounds of proliferation than healthy B cells (15). These observations indicate that CLL progression appears to be the consequence of an imbalance of decreased cell turnover combined with excess proliferation, resulting in a longer replicative history of CLL cells (11).

The observed *in vivo* proliferation rates of CLL cells promote the acquisition of genetic mutations (16). Combined with the fact that CLL cells are less susceptible to apoptosis, CLL cells are able to obtain a more extensive replicative history, suggesting that disease progression is not a result of accumulation but rather of stochastic generation of subclones. Over time, more pathological subclones could be selected which may further affect CLL birth and death rates (9). Importantly, the accumulation of genetic changes may eventually result in subclones that may prevail of microenvironmental control at later stages (17). The insight that CLL is a dynamic disease with both substantial proliferation and death rates is important, since this allows novel clonal variants to expand more quickly to a substantial level (10). Clonal evolution with outgrowth of novel variants harboring genetic alterations has been well described in CLL and has significant impact on clinical outcome (18). However, *in vivo* labeling studies have not managed to link such genetic aberrations to increased proliferation rates (13), suggesting that they are a consequence rather than the cause of increased proliferation in CLL.

CLL Proliferation Occurs at LN Sites

In aforementioned *in vivo* labeling studies, the fraction of labeled CLL cells in many patients continued to increase for many weeks after the end of the labeling phase, implying they had to have remained in a separate compartment for some time prior to

being released into the PB (10). Subsequent *in vivo* labeling studies identified the LN as the anatomical site harboring the largest fraction of newly born CLL cells, with birth rates as high as 3.3% of CLL cells circulating in the PB per day. In contrast, the BM did not seem to be a major proliferation site (12). Gene expression profiling and Ki-67 staining support that active proliferation occurs in the LN from which newborn cells enter the PB (14, 19). Finally, immunohistochemical studies have demonstrated the presence of proliferating CLL cells within specific structures in the LN, resembling proliferation centers, otherwise known as pseudofollicles (20, 21).

Extensive immunophenotyping and intraclonal analyses suggest a spectrum of circulating CLL cells with at one end the proliferative fraction, enriched in recently divided cells that have recently emigrated from the LNs ($CXCR4^{low}/CD5^{high}$), and at the other end the resting fraction, enriched in older, less vital cells that need to either immigrate back to the LN or die ($CXCR4^{high}/CD5^{low}$) **(Figure 1)** (14, 22). Moreover, gene expression analysis indicated higher levels of pro-proliferation and anti-apoptotic genes in the proliferative $CXCR4^{low}/CD5^{high}$ fraction (22).

Thus, LN tissues are the preferred site for CLL cell proliferation, possibly due to accessory cells within the microenvironment that promote proliferation, propagated through diverse receptors such as the BCR, CD40, and TLRs (19, 23, 24). Characterization of BCR, CD40, and TLR signaling in primary CLL cells of the proliferative fraction may pinpoint the importance of each of these individual modes of stimulation and is of interest for understanding the process by which CLL cells residing in these proliferative niches are contributing to disease progression. Aside from BCR, CD40, and TLR stimulation, various other *in vitro* stimulations and culture conditions have been applied in the context of CLL proliferation, including factors like BAFF and APRIL, as well as co-culture with stromal cells, follicular dendritic cells or nurse-like cells (1). These and other candidates are certainly of interest, yet the interaction with feeder cells combined with their secretion of cytokines makes the identification of essential factors difficult (6). In addition, our previous efforts have not managed to show a direct role for BAFF or APRIL in human CLL proliferation (25, 26). Therefore, we take a restricted approach and in the first part of the review we will provide an overview of the *in vivo* evidence of BCR, CD40, and TLR signaling in CLL proliferation, and in the second part of the review we will cover the *in vitro* data that support the role of BCR, CD40, and TLR signaling in the proliferation of CLL.

In Vivo BCR Signaling

Signaling through the BCR pathway is a key functional step of all normal and malignant B cells and is also a critical component in CLL (27). BCR signaling activity is elevated in CLL cells compared to healthy B cells, and deregulated BCR signaling is considered a driving mechanism leading to CLL development, progression, and relapse (28). BCR triggering leads to the activation of downstream signaling pathways, including the MAPK/ERK, PI3K/AKT/mTOR, and NF-κB pathways, which play a role in CLL survival and proliferation **(Figure 2)** (5, 29–31).

Critical evidence of the involvement of BCR stimulation in driving CLL is the expression of BCR-associated genes in LN CLL cells (19). In addition, LN CLL cells had higher pSYK levels compared to CLL cells from PB or BM, supporting BCR-dependent activation of CLL cells *in vivo* and suggesting that the LN is the crucial site for proliferation (19). Mouse models exhibiting spontaneous CLL development also show an important role for BCR signaling in the onset and development of CLL. These studies showed that overexpression of BTK leads to accelerated CLL onset (32). The IgH.ETμ mouse model shows that BTK expression is a prerequisite for CLL development (32) whereas the TCL1 mouse model showed that mice with ablation of BTK significantly delayed CLL development but still developed leukemia at rates similar to wild type TCL1 mice treated with ibrutinib (33–35).

In patients, CLL cells have elevated BTK expression (36) and pBTK levels compared to healthy B cells (28), as well as lower expression of surface IgM (sIgM) (37) which is additional evidence for BCR stimulation *in vivo*, as sIgM expression in CLL cells is downregulated after antigen stimulation which is reversed during circulation in the PB (38). This observation suggests endocytosis of sIgM *in vivo* which can only be due to interaction of the BCR with a ligand able to bind (27, 39). In CLL cells isolated from the PB, subgroups of cells could be distinguished with increasing sIgM and CXCR4 expression, likely regulating the ability to migrate to the LN and engage antigen in situ (38).

However, this raises questions in the context of the two CLL subsets stratified by IGHV gene mutations. Specifically, as um-CLL cells have polyreactive BCRs that may respond to a wide spectrum of epitopes whereas m-CLL cells have undergone somatic hypermutation and thus express BCRs specific to a certain epitope, and may therefore be less responsive to external signals (5, 40). Indeed, *in vivo* labeling studies measured higher CLL growth rates in the LNs of um-CLL patients compared to m-CLL patients (14). Consistent is the finding that um-CLL cells had much shorter telomeres than m-CLL cells, implying a more extensive replicative history (15).

Furthermore, LN CLL cells showed increased BCR signaling in um-CLL compared to m-CLL (19). This is consistent with findings in PB CLL cells, where even though BCR signaling was significantly lower, a preferential expression of BCR-regulated genes was found in um-CLL as compared to m-CLL, which most likely reflects BCR activation in the LN as cells carry a temporary imprint of their prior stimulation (19, 41). However, even in m-CLL patients, BCR signaling in LN CLL cells was significantly higher compared to PB, indicating that BCR signaling is also involved in this subset of CLL (4). ZAP-70 expression, which is one of the most prominent genes distinguishing um-CLL from m-CLL, may reinforce BCR responsiveness (42). ZAP-70 is structurally similar to the BCR-associated kinase SYK (5) and its expression was associated with greater BCR signaling capacity, implicating a role for the BCR in CLL proliferation (43). Therefore, this suggests that the ability to respond to antigen stimulation coupled to signal reinforcement may underlie the differences

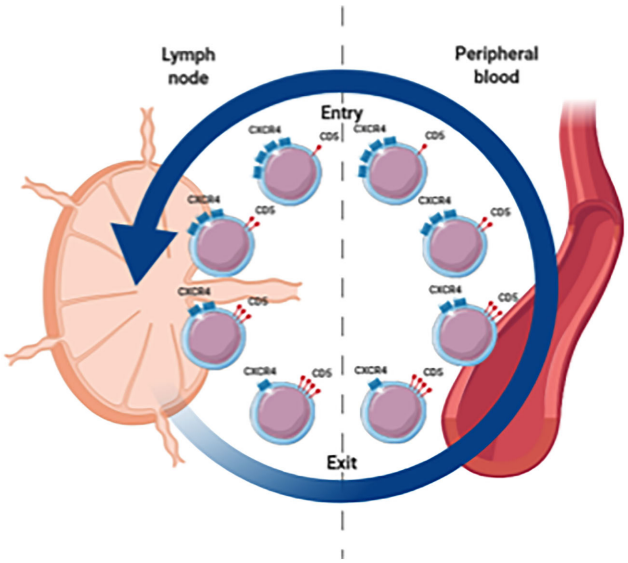

FIGURE 1 | Spectrum of circulating CLL cells illustrating the proliferative fraction enriched in LN emigrants versus the resting fraction enriched in LN immigrants. CXCR4high/CD5low cells are in a resting state. When these cells are activated in the LN, CD5 is upregulated and CXCR4 is internalized. These CXCR4low/CD5high cells are released from the LN and migrate into the PB. Eventually these cells become more quiescent leading to downregulation of CD5 and renewed surface expression of CXCR4 which increases the likelihood of a return to the LN.

in disease activity between the two prognostic subsets (29, 39, 44–46).

Antigen-Dependent BCR Signaling

BCR signaling can be broadly divided into two main types: one that is mediated by antigen and another that is independent of antigen, referred to as tonic BCR signalling (1, 47, 48). Antigen-dependent and antigen-independent BCR stimulation are two fundamentally different mechanisms of BCR signaling which exist in B cell lymphomas. However, it is not quite clear to what extent tonic or antigen-dependent BCR signaling play a role in driving CLL. The absence of mutations in BCR signaling components leading to antigen-independent pathway activation in CLL favors a dominant role for antigen-dependent BCR signalling (5). The fact that CLL proliferation only takes place in lymphoid tissues may suggest that relevant antigens are localized to discrete anatomic compartments, but more likely this indicates the lack of additional signals outside of these compartments that trigger CLL proliferation, such as T cell-derived signals in the LN (16).

Strong molecular evidence for antigen-dependent BCR signaling in CLL is the presence of stereotyped BCRs, which support the idea of a common selecting antigenic epitope (49, 50). A study using antigen-specific TCL1 mice showed that neither acute nor chronic exposure to specific antigen influenced disease progression. Rather, CLL clones preferentially selected light chains paired with the antigen-specific heavy chains that conferred CLL cells the ability to interact with a broad range of autoantigens (51). These results suggest that pathogens may drive CLL by selecting and expanding pathogen-specific B cells that cross-react with one or more self-antigens, indicating that the BCR may in fact shape CLL progression *in vivo*. The specific antigens recognized by these stereotyped CLL BCRs are not well described, especially in the case of m-CLL. Whereas m-CLL clones exhibit more restricted autoreactivity (9, 40), the majority of um-CLL clones express low-affinity BCRs that are polyreactive recognizing self-antigens such as DNA, insulin and the cytoskeletal proteins myosin and vimentin, as well as foreign antigens such as bacterial DNA and lipopolysaccharides in addition to a spectrum of molecular motifs exposed on apoptotic cells (40, 52–57). One study showed and identified specificity to an autoantigenic target in one-fourth of CLL cases independent of IGHV mutation status (58). All identified BCR targets were cytoplasmic proteins. The translocation of cytoplasmic antigens to surface membrane blebs and apoptotic bodies would enable binding to the surface BCR of CLL cells (52). Importantly, the same study showed that BCRs belonging to the same stereotyped subset target identical antigens, but surprisingly, BCRs from individual CLL patients were specific for different epitopes of the same antigens. Finally, binding of the autoantigen to the respective CLL cells induced specific activation and proliferation, suggesting that autoreactivity of CLL cells *via* the BCR may be a general mechanism for driving CLL. Other stereotypic subsets of m-CLL have been described showing specificities to the cytomegalovirus phosphoprotein pUL32, the Fc-tail of IgG, as well as specificity to β-(1,6)-glucan, an abundant component in yeast and filamentous fungi (50, 59–61). However, it should be noted that there have been no recent reports of new subsets exhibiting specificity to exogenous antigens, illustrating that perhaps this represents a unique attribute of only a few CLL subsets. A study with TCL1 mice expressing transgenic BCRs with different antigen specificities showed that chronic interactions with low-affinity can induce CLL *in vivo*, whereas interactions with high-affinity antigens cannot (62). Additionally, the authors showed that low-affinity BCRs are positively selected, whereas high-affinity BCRs are not. Consequently, m-CLL clones remain more stable overall and expand at a slower rate, likely due to high-affinity binding to restricted sets of antigenic epitopes that either occur infrequently because they are on foreign antigens or because they induce anergy due to high-affinity binding (5).

Anergy is one of the mechanisms of the immune system to silence autoreactive B cells upon recognition of self-antigens. A state of BCR desensitization is induced by chronic binding of antigen *in vivo*, resulting in unresponsiveness when cells are stimulated with antigen *in vitro*. The fact that m-CLL is associated with a favorable disease course and bind antigen more specifically with higher affinity than um-CLL, suggests that exposure to antigen *in vivo* may lead to anergy of CLL cells (63). In mouse models, anergic B cells showed features of low levels of sIgM as the result of constant BCR internalization, increased basal intracellular calcium concentrations and

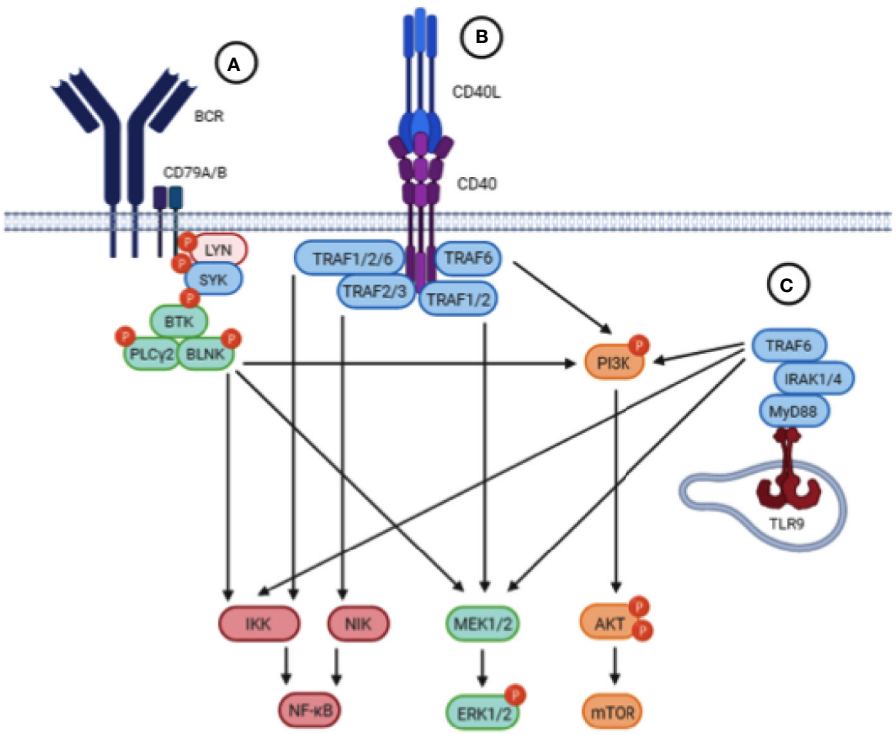

FIGURE 2 | Schematic representation of the BCR, CD40 and TLR signaling pathways. Upstream triggering of BCR, CD40, or TLR signaling lead to activation of similar downstream pathways, including the NF-kB, MEK/ERK, and PI3K/AKT/mTOR pathways. **(A)** The BCR is composed of an antigen-specific surface membrane immunoglobulin paired with the signal transduction component consisting of the CD79A/B heterodimer. Engagement of the BCR by antigen results in aggregation of BCR components that leads to the phosphorylation of ITAMs in the cytoplasmic tails of CD79A/B which triggers the recruitment and activation of the proximal tyrosine kinases LYN and SYK. Subsequently, BTK is activated which will activate PLCγ2 and BLNK, resulting in activation of downstream signaling pathways. **(B)** Upon binding of CD40L, the CD40 receptor on CLL cells trimerizes leading to the recruitment of TRAFs to the cytoplasmic domain of CD40. TRAFs may then cooperate in order to activate different signaling pathways downstream. **(C)** Upon TLR9 activation in the endosomal compartment of CLL cells, the TIR domain of TLR9 engages the TIR domain of the adaptor protein MyD88. MyD88 contains an IRAK1 domain which activates TRAF6, leading to the activation of downstream pathways.

constitutive activation of ERK1/2. This biochemical program is reversible and lasts as long as B cells are exposed to the antigen (64). Notably, repetitive BCR stimulation in healthy B cells resulted in anergy and CD5 expression, which is a feature of CLL (65). In addition, mouse models have shown that CD5 is necessary to maintain anergy in B cells as knocking out CD5 in these mice resulted in a loss of self-tolerance (66). In vitro studies analyzing responses to BCR triggering have identified a subset of CLL patients exhibiting indolent disease with CLL cells containing anergic features (67), including a lack of BCR signaling capacity and constitutive activation of ERK1/2 (64). Additionally, the BCR-associated kinase LYN initiates a negative feedback loop *via* recruitment of the phosphatase SHP-1 which inhibits BCR signaling and is overexpressed in CLL, further illustrating an anergic phenotype (68). Several studies have shown that the anergic phenotype of CLL cells, including sIgM expression and signaling capacity, reverses spontaneously after culture *in vitro* or following capping and endocytosis (39). This shows that downstream signaling pathways appear to be intact and that anergy can thus be attributed to surface immunoglobulins (sIgs), and this also provides direct evidence for engagement of putative antigen *in vivo* (39, 64).

Antigen-Independent BCR Signaling

The lack of antigen reactivity in m-CLL may indicate a role for antigen-independent BCR signaling, which is supported by the observation of phosphorylated LYN, SYK and canonical NF-κB in unstimulated CLL cells (5). Ligand-independent signaling is frequent in other malignancies where proliferation is subverted by the acquisition of genetic mutations in signaling components mimicking physiological stimulation of receptors (69). Such mutations in BCR signaling components are absent in CLL (5).

A special type of antigen-independent BCR activation has been described in CLL, which involves the binding of auto-epitopes existing on adjacent or in the same sIgs. Reactivity with sIg auto-epitopes could occur on a continuous basis. This also suggests a mutual BCR recognition on CLL cells which was confirmed by the binding of secreted CLL-derived BCRs to the surface of cell lines expressing either CLL or HD BCRs, but not to cells that did not express a BCR (70). Likewise, serum immunoglobulins from HD plasma were not able to bind HD B cells whereas serum immunoglobulins from CLL plasma were (71). It was shown that the binding was mediated by a conserved epitope in the second framework region of VH domains of the CLL BCR, as point mutations inside this motif abolished

autonomous signaling. This motif was located outside of the antigen-recognition site, indicating that the induced signaling is not mediated by external antigens. Consistently, VH domain point mutations of antigen-specific BCRs in m-CLL were still able to signal upon recognition of antigen, indicating that this type of tonic signaling is antigen-independent but does not rule out the involvement of extrinsic antigens in the pathogenesis of CLL (70). Consistent with these observations are imaging data showing that HD BCRs exist predominantly as monomers and dimers in the plasma membranes of resting B cells and form oligomeric clusters upon stimulation with antigen. In contrast, CLL BCRs form dimers and oligomers in the absence of a stimulus, reflecting an antigen-independent tonic activity of the BCR (72). A single amino acid exchange reverted the organization to monomers and thus prevented auto-aggregation of CLL BCRs (72).

As tonic signaling would essentially result in constitutive BCR signaling, this would thus lead to a tolerogenic signal that should result in anergy (5). Consistently, a lack of external BCR stimulation does not lead to spontaneous CLL proliferation *in vitro* (5). Therefore it can be hypothesized that binding of extrinsic cognate antigens is essential to overcome anergy of CLL cells and is thus required for CLL proliferation (50, 73). A study using the TCL1 transgenic mouse model showed that this unique autonomous signaling capacity is a prerequisite for CLL development. Moreover, the capacity of CLL cells to respond to antigen inversely correlated with time to leukemia development, suggesting that signals induced by external antigens contribute to the aggressiveness of the disease (62).

In summary, both antigen-dependent and antigen-independent BCR signaling have been described in CLL, and CLL cells can receive both continuous and intermittent BCR signals that may facilitate proliferation (5). Yet, ligand-dependent and tonic BCR signaling may not be mutually exclusive. CLL clones could originate as antigen-dependent, but evolve to become more autonomous if the critical BCR regions are mutated. Substantiating this possibility would require comparison of BCR sequences in a cohort containing early MBL and later CLL stages of the same patient, which to our knowledge has not been performed. Alternatively, these separate mechanisms could reflect different routes for clonal expansion after initial transformation (69).

BCR Inhibitors

The BCR signaling pathway has emerged as an important therapeutic target for B cell malignancies, including CLL (74). Several BCR-targeted agents, including inhibitors of BTK (ibrutinib), PI3Kδ (idelalisib), and SYK (fostamatinib) have demonstrated clinical efficacy, which led to FDA approval of idelalisib and ibrutinib (28, 75–78). Especially the introduction of ibrutinib has dramatically changed the management of CLL, allowing for treatment without chemotherapy (74). Ibrutinib inhibits the activation of BTK, which plays a role in proliferation, survival, migration and tissue adhesion of CLL cells (4, 79, 80). Treatment of patients with ibrutinib leads to lymphocytosis due to efflux of CLL cells from the proliferative LN compartment into the PB (28, 81), thereby depriving CLL cells from microenvironmental signals and halting disease progression (17).

The impact of ibrutinib treatment on *in vivo* CLL kinetics of CLL cells showed that no newly divided CLL cells entered the PB upon ibrutinib treatment. The average pretreatment birth rate decreased upon ibrutinib treatment whereas death rates increased (81). In addition, ibrutinib treatment resulted in a reduction of the proliferative $CXCR4^{low}/CD5^{high}$ fraction (82). Even though ibrutinib targets a key pathogenic pillar of CLL by depriving cells from antigen and interactions with the lymphoid microenvironment, it is not sufficient to eradicate disease, as stopping treatment reverses remission (17). The fact that the BCR components BTK and PLCG2 are specifically mutated in ibrutinib-resistant CLL underlines that therapeutic success depends critically on inhibition of this pathway (83).

Importantly, it was shown that maximum inhibition of BCR signaling *in vivo* was already achieved after one administration of the drug whereas maximum inhibition of downstream NF-κB signaling required repeated dosing (4). This indicates that aside from direct effects, continued treatment of ibrutinib leads to changes in the microenvironment that have indirect effects on CLL cells, highlighting the role of accessory cells mediating signaling *via* alternative receptors such as CD40 and TLRs. Next, we will discuss the existing evidence for *in vivo* CD40 and TLR signaling and their roles in CLL proliferation.

EVIDENCE FOR NON-BCR SIGNALS IN VIVO

In Vivo CD40 Signaling

CD40 expressed on CLL cells can be stimulated by its physiological ligand CD154 (CD40L) expressed on, for instance, activated CD4 T cells and follicular T helper cells (84, 85). Interaction of CD40 and CD40L stimulates the proliferation and differentiation of healthy B cells (85). CD40 stimulation on CLL cells activates downstream signaling pathways including MAPK/ERK, PI3K/AKT/mTOR, and NF-κB (86), thus largely overlapping with downstream BCR signaling pathways (**Figure 2**). As a result, CLL cells are activated and provided with strong survival signals rendering them highly resistant to a wide variety of therapeutics (7, 85). In addition, CD40 stimulation propels both CLL cells and healthy B cells in a proliferative state (7, 87). Interestingly, a few studies have reported the expression of CD40L on CLL cells as well, suggesting a mechanism in which activated CLL cells may directly stimulate CD40 on resting bystander CLL cells in a paracrine manner (88, 89).

The earliest observation for a role of CD40 signaling in CLL was the infiltration of CD4 T cells that express CD40L in CLL pseudofollicles that co-localize with $Ki\text{-}67^{+}$ CLL cells in these proliferation centers (23, 90). This is suggested to be a mechanism to regulate CLL proliferation, which was supported by *in vitro* stimulation of PB CLL cells with CD40L, inducing expression of CCL22, which serves as an attractant for CD4 T cells which in turn express CD40L (23). Moreover, co-culture of CLL cells with activated autologous T cells results in proliferation

of CLL cells (7, 91). Importantly, interference with CD40 signaling collapses LN germinal centers necessary for B cell development, differentiation and somatic hypermutation (92).

Interestingly, in lymphocytes isolated from PB of CLL patients, a fraction of proliferating T helper cells were observed in the presence, but not in the absence of CLL cells. Moreover, these CLL-associated T helper cells induce HLA class II-dependent activation and proliferation of CLL cells *in vitro*, suggesting that CLL is a disease driven by immune responses *via* a process in which T helper cells engage CLL cells in response to antigen presented on the CLL cells' own HLA class II molecules (93). It would be worthwhile if these intriguing findings can be confirmed by other studies.

Even though direct evidence for T cell-dependent CLL proliferation in patients is lacking, several mouse models have provided more insight. CLL cells xenografted in NOD-SCID mice require activated autologous T cells in order for the CLL cells to proliferate. Moreover, depletion of CD4 T cells inhibited proliferation whereas depletion of CD8 T cells did not (94). Also, LMP1/CD40 mice express a chimeric protein containing part of the Epstein-Barr viral Latent Membrane Protein 1, mimicking constitutively active CD40 triggering (95). These mice showed an increase of B cells in secondary lymphoid organs with an activated phenotype, increased proliferation and prolonged survival. In addition, they showed significantly impaired T cell-dependent immune responses, thus resembling CLL in many aspects (96). Moreover, LMP1/CD40 cells proliferated spontaneously *in vitro* in a CD40-dependent manner (95).

Combined, these observations indicate that CD40 may in fact be an important mediator in CLL proliferation, which is currently less widely recognized compared to the contribution of BCR signaling.

In Vivo TLR Signaling

Recurrent mutations in CLL include MYD88, a gene which encodes a downstream component of TLR signaling (69). TLRs are part of the innate immune system and respond to specific molecular patterns found in various microorganisms, including bacteria (43). These receptors are expressed in CLL cells and biologically active, suggesting an additional route of stimulation besides BCR signaling (69). TLR signaling leads to the activation of several downstream signaling pathways, including MAPK/ERK, PI3K/AKT/mTOR, and NF-κB (97), resulting in the activation and proliferation of CLL cells (43) **(Figure 2)**.

The most evident observations for a role of TLR signaling in CLL are increased expression of TLR9 in CLL cells compared to healthy B cells (98, 99), TLR pathway activation in LN CLL cells as shown by gene array studies (19, 24), as well as *in situ* proximity ligation assay experiments that showed the interaction of pIκBα with TLR9 and MYD88 in LN CLL cells (24). CLL BCR specificity for DNA or antigens physically linked to DNA further suggest a role for TLR signaling in driving CLL (8). Moreover, apoptosis-associated antigens bound by sIgs can also be recognized by TLRs after entrance to the endosomal compartment *via* sIgs (52). Interestingly, the observation that um-CLL cells are more responsive to BCR activation than m-CLL cells is mirrored by TLR activation *in vivo* and *in vitro* (24). A possible explanation is that the antigen-specific BCRs in m-CLL make them less likely to internalize antigen-linked TLR ligands compared to um-CLL, whose BCRs are polyreactive and bind with low affinity to a wide variety of antigens (52, 54, 100). Yet, this does not hold for CpG stimulation *in vitro*, as these are internalized in a BCR-independent fashion. See below in the section *in vitro TLR signaling* for additional observations that TLR and BCR signaling may cooperate to promote CLL proliferation in um-CLL.

The absence of TIR8, a negative regulator of TLR signaling, was shown to accelerate disease progression in TCL1 mice (101). Moreover, epidemiological studies found an increased risk for the development of CLL following episodes of sinusitis, pharyngitis, influenza, cellulitis, and herpes zoster, where risk increased with increasing severity or frequency of infection (102–104). These studies suggest that infectious agents can promote disease onset and progression of CLL, which may be related to TLR activation (102).

It is not yet clear to what extent these non-BCR signals contribute to proliferation in situ, but it is apparent that both BCR, CD40, and TLR activation all show marked similarities in the downstream signaling pathways involved, including the MAPK/ERK, PI3K/AKT/mTOR, and NF-κB pathways (43) **(Figure 2)**. Perhaps a combination of stimuli is what may really drive CLL proliferation *in vivo* or helps develop it into more aggressive disease.

In Vitro CLL Proliferation

Despite CLL being a proliferative disease with significant cell turnover, primary CLL cells rapidly undergo apoptosis in the absence of microenvironmental survival signals (105). What further illustrates that essential *in vivo* factors are missing in *in vitro* systems, is that co-culture with stromal cells or the addition of soluble factors can significantly extend CLL survival, yet only for a limited amount of time and thus far, no system permits the long-term expansion of CLL cells *in vitro* (7, 19, 94, 106, 107). In vitro studies typically analyze CLL cells isolated from PB and consequently, the contribution of the host microenvironment to the proliferation of CLL cells *in vivo* remains ill-defined (19). The difficulties of mimicking a physiologic microenvironment supporting CLL proliferation hinder *in vitro* studies and as a result, a large variety of culture systems have been developed in order to investigate CLL proliferation (105). This raises difficulties in comparing data achieved with these highly variable approaches and hinders systematic characterization of culture systems or back-to-back comparisons in terms of CLL proliferation (105, 106). In the next section, we will elaborate on CLL proliferation in the light of *in vitro* data and we will specifically discuss models utilizing BCR, CD40 or TLR stimulation in combination with costimulatory cytokine signals.

In Vitro BCR Signaling

Despite the consensus regarding the role of BCR signaling in the biology of CLL, the response of CLL cells to BCR stimulation *in vitro* is notoriously heterogeneous among patient samples (43). In vitro BCR stimulation of CLL cells is performed using anti-IgM, either soluble or immobilized. In vitro responses to BCR

stimulation differ between um-CLL and m-CLL as demonstrated by several groups using multiple assays, including global protein tyrosine phosphorylation, gene expression profiling, cellular metabolic activity, apoptotic response and proliferative activity (29–31). Several studies have reported that response to BCR stimulation was correlated with sIgM levels (31). Some studies found higher sIgM levels in um-CLL (108), whereas others found little to no differences between um-CLL and m-CLL (39, 46), claiming a role for additional factors contributing to BCR responsiveness (39). Many studies showed a correlation of CD38 and ZAP-70 expression with BCR responsiveness, however, CD38 does not influence BCR signaling *in vitro* and ZAP-70 is not required for response. Therefore, these correlations most likely do not result from functional interactions, but are a result of um-CLL expressing higher levels of CD38 and ZAP-70 (39). Some discrepancies found in literature concerning *in vitro* responses to BCR stimulation can, at least partially, be attributed to the lack of a standardized protocol to trigger the CLL BCR *in vitro* (109). For example, it has been shown that immobilized anti-IgM provides a more potent *in vitro* CLL stimulus than soluble anti-IgM (109, 110).

Another possibility is that the variability in BCR responses stems from variable degrees of sIg clustering, which may be associated with natural genomic heterogeneity in BCRs and/or response to antigen (71). Indeed, induction of stable BCR clustering on healthy B cells modulated BCR responsiveness. In fact, by titrating the amount of anti-IgM crosslinking, healthy B cells could be induced to recapitulate the diversity in signaling observed in CLL cells, confirming that BCR clustering can modulate BCR responsiveness and thereby phenocopy the signaling dysfunction in CLL (71). As for the heterogeneity of BCR responsiveness, CLL cells could be divided into 3 subgroups of SHP-1^{low}/pPLCG2^{high} to SHP-1^{high}/pPLCG2^{low} expression, where each subset displayed unique deviations in their BCR signaling responses (71). As increasing levels of SHP-1 and decreasing levels of pPLCG2 correlated with weakened BCR responsiveness, this suggests that phenotypic variability within isogenic populations of cells may result from heterogeneous levels of signaling regulators (71).

Whereas BCR stimulation of healthy B cells significantly induces their proliferation, *in vitro* BCR stimulation of CLL cells does not lead to proliferation (107), which is another reminder that the *in vitro* CLL systems are missing a crucial aspect that is active in patients. In vitro engagement of the BCR in CLL promotes G1 cell cycle progression as shown by increased levels of cyclin D2 and CDK4, but does not induce cell division, associated with constitutively high levels of the cell cycle inhibitor p27 (30). Accordingly, CLL cells within proliferation centers of the LN showed high expression of cyclin D2 and downregulation of p27 (111). Therefore, CLL proliferation may depend on costimulatory signals such as those delivered through CD40 or TLRs, possibly in combination with cytokines (6, 7, 16). The interleukins are a family of cytokines that serve as key regulatory elements within the immune system and a number of specific interleukins have been identified as being associated with the proliferation of CLL cells *in vitro* (43). Importantly, IL-4 promotes the expression and function of surface IgM in CLL cells, thereby enhancing *in vitro* BCR responsiveness (112). However, proliferation of CLL cells has been mostly described in an antigen-independent context using combined stimulations with CD40L/IL-21, CD40L/CpG, or CpG/IL-15 (107), which we will describe in the next sections.

In Vitro CD40 Signaling

In vitro CD40 stimulation of CLL cells can be performed using either soluble agonists or *via* co-culture with CD40L-presenting cells. Importantly, soluble agonists like anti-CD40 antibodies or soluble CD40L are inferior to co-culture with CD40L-presenting cell lines which are able to support proliferation more efficiently (85, 113, 114). Again, as various soluble CD40 agonists and CD40L co-culture systems are used, this hinders direct comparison of *in vitro* studies on CD40-mediated CLL proliferation (85).

Similar to BCR stimulation, crosslinking of CD40 provides only weak proliferative responses in CLL whereas healthy B cells proliferate well (87). In contrast to single BCR or CD40 stimulation, co-culture of CLL cells with activated CD4 T cells promoted CLL proliferation to the same extent of that in healthy B cells, which respond equally well to single CD40 stimulation or co-culture with T cells (87, 93). Although a monolayer of CD40L-presenting fibroblasts induced a highly similar gene profile as induced by co-culture with T cells (7), it did not induce CLL proliferation like activated T cells (106), highlighting the role of additional T cell-derived signals in the proliferation of CLL.

CD40 triggering upregulates the IL-21 receptor, making CLL cells more receptive for the T cell cytokine IL-21, which has been implicated in CLL proliferation (115). IL-21 significantly increased CD40-mediated proliferation, and CLL proliferation by activated T cells was shown to be IL-21-dependent as well (7, 116). Importantly, *in vitro* T cell activation induced IL-21 mRNA production, specifically in follicular T helper cells, which have been shown to be present and produce IL-21 in LNs *in vivo* (7, 117). Another T cell cytokine implicated in CD40-mediated CLL proliferation is IL-4. In vitro stimulation with soluble CD40L caused a slight increase of CD40 expression on CLL cells but stimulation with IL-4 resulted in a significant increase of CD40 expression (118). Combined stimulation of CD40 and IL-4 however results in only modest proliferation of CLL cells (116), but it primes cells for proliferation *via* IL-21, as combined stimulation of CD40L, IL-4 and IL-21 results in increased CLL proliferation (117).

Interestingly, *in vitro* BCR stimulation of CLL cells also resulted in increased expression of CD40, suggesting potential crosstalk between BCR and CD40 signaling (31, 93). In fact, whereas BCR stimulation rapidly activates pSYK in both healthy B cells and CLL cells, stimulation with CD40L also activates pSYK in CLL cells but has no effect on pSYK in healthy B cells (119). Likewise, inhibition of SYK hampers CD40-mediated proliferation of CLL cells but not in healthy B cells. In addition to SYK, BTK is also activated upon CD40 stimulation, suggesting that CLL cells exploit CD40 stimulation by increasing BCR

pathway activity (119). A possible explanation may be that CD40 acts as a gatekeeper for BCR signaling by inhibiting negative feedback components like LYN and SHP-1, so that CD40-dependent activation of the BCR pathway is required to overcome negative feedback signals in anergic CLL cells (119) **(Figure 3)**.

CD40 stimulation in combination with IL-21 or IL-4 is sufficient to induce CLL proliferation *in vitro* whereas for BCR stimulation this is not the case (6, 7). However, CLL cells treated with ibrutinib *in vivo* did not proliferate upon CD40/IL-21 stimulation *in vitro*, an effect which can be recapitulated by *in vitro* inhibition of either BTK, PI3K or SYK, indicating the requirement of BCR kinases in CD40-mediated proliferation (107). CLL proliferation is significantly increased further when BCR stimulation is added to the combined stimulation of CD40 and cytokines whereas for healthy B cells this is not the case, showing that these separate signaling nodes may potentially cooperate to drive CLL proliferation *in vivo* (6).

In Vitro TLR Signaling

In vitro activation of TLR signaling is carried out using CpG, resulting in endocytosis and subsequent binding of TLR9 in the endosomal compartment, thereby mimicking the interaction of CLL cells with bacteria (120). In vitro stimulation with CpG activates p-p65 and decreases IRAK1 levels, recapitulating the effects of TLR activation as observed in LN CLL cells *in vivo* (24). In vitro stimulation of CLL cells with CpG resulted in quite variable reports of proliferation (99, 107, 121, 122). Similar to *in vitro* BCR activation, um-CLL cells can more often be induced to proliferate upon CpG stimulation (97, 123). Importantly, response is not correlated with TLR9 mRNA or protein expression (97). The *in vitro* proliferative response to CpG was found to be highly predictive of progression-free survival, time to treatment and overall survival in m-CLL, whereas prognosis of um-CLL was equally worse, with or without proliferative response to CpG *in vitro* (124). Although we still do not fully understand the mechanistic basis of TLR signaling in CLL proliferation, these findings do support the relevance of TLR signaling in driving CLL.

In vitro CpG stimulation was shown to upregulate CD122, which is a shared subunit of the receptor for IL-2 and IL-15 (125). Consistently, addition of the T cell cytokine IL-2 significantly enhances CpG-mediated proliferation of CLL cells (7). IL-15, produced by monocytes, synergistically promotes CpG-mediated CLL proliferation independent of CLL mutation status, thus reversing the difference as usually seen between um-CLL and m-CLL (8, 107). Moreover, *in vitro* proliferation mediated by CpG/IL-15 could not be linked to prior treatment or *in vivo* growth rates. As for genetic abnormalities, only TRI-12 was associated with a significantly greater propensity for proliferation in response to CpG/IL-15 (8). Examples of robust

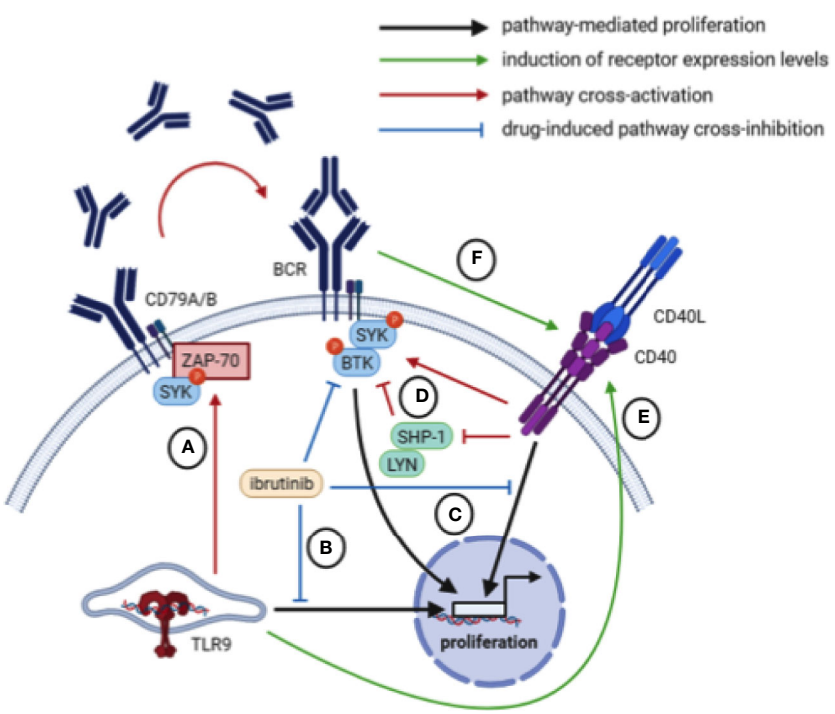

FIGURE 3 | Overview of potential crosstalk mechanisms between BCR, CD40, and TLR signaling. **(A)** TLR signaling in um-CLL may mount an autocrine feedback loop mediated by ZAP-70 and SYK involving the production and secretion of IgM which may subsequently trigger BCR signaling in an autocrine/paracrine manner. **(B)** Inhibition of BCR signaling *via* BTK inhibits CpG-mediated proliferation. **(C)** Inhibition of the BCR-associated kinases BTK or SYK inhibits CD40-mediated proliferation. **(D)** Upon CD40 stimulation, the BCR-associated kinases BTK and SYK are activated, possibly *via* inhibition of negative feedback components LYN and SHP-1. **(E)** TLR9 stimulation *via* CpG increases expression of the CD40 receptor. **(F)** Activation of BCR signaling increases expression of the CD40 receptor.

in vitro proliferation of clones with slow *in vivo* growth rates support the notion that the availability of stimulatory signals within the *in vivo* microenvironment may be as relevant as a cell's intrinsic potential for proliferation (8). Notably, the abundant presence of IL-15-expressing cells in LNs of CLL patients makes this mechanism clinically relevant (125). Similar to CD40 stimulation, CpG induces upregulation of the IL-21 receptor (126). Whereas healthy B cells proliferated much more than CLL cells following single CpG stimulation, addition of IL-21 rescued enhanced the proliferative activity of CLL cells to the same level of healthy B cells (123).

Links between TLR and BCR signaling have also been described, as for example *in vitro* stimulation of CLL cells with CpG induced phosphorylation of CD79A, LYN and SYK which appears to rely on the expression of ZAP-70 (127). The strong association between CpG-mediated CLL proliferation and IGHV mutation status may suggest that TLR stimulation is modulated by BCR signaling (97, 123). For example, anergic cells that are usually m-CLL and lack expression of ZAP-70, show reduced proliferative responses to CpG *in vitro* (124). Studies have shown that engagement of TLR signaling in CLL is able to mount an autocrine feedback loop involving the production and secretion of IgM leading to activation of the cell's own BCR, which reinforces the concept of tonic BCR signaling in the absence of antigen (107, 127) **(Figure 3)**.

Similar to *in vitro* BCR activation, stimulation with CpG also caused upregulation of CD40 on CLL cells, showing links of TLR stimulation with CD40 signaling as well (99, 122, 128, 129). Activation of TLR9 *via* CpG significantly increased CLL proliferation when combined with CD40 stimulation, similar to CD40/IL-21 stimulation (6, 7, 128, 130). Importantly, combined TLR/CD40 stimulation overcomes the hyporesponsiveness to CpG as often seen in m-CLL (130). On the contrary, CLL cells that do not proliferate *in vitro* in a T-cell dependent manner, can be triggered to proliferate upon addition of CpG/IL-2 (93).

Finally, inhibition of BCR signaling *via* treatment with ibrutinib or *via* inhibition of SYK significantly inhibits both CD40- and CpG-mediated CLL proliferation *in vitro*, showing the role of BCR-associated kinases (36, 107, 131). CD40 and CpG-induced proliferation do however differ in their involvement of the BCR complex. CD40 involves the recruitment of BTK independent of upstream BCR components whereas CpG indirectly triggers the BCR *via* IgM secretion (107). Therefore, BCR-targeted agents effectively target the aforementioned TLR-BCR feedback loop (107).

Concerning the potential cooperation of signaling pathways, it is important to note that a divergence exists between crosstalk of separate pathways and active rewiring of distinct signaling pathways. For example, in diffuse large B cell lymphoma it was found that the GC and ABC subsets depended on the BCR subunits CD79A/B, but engaged divergent downstream signaling pathways (132). In patients with MYD88 mutations, a new complex consisting of TLR9 and MYD88 was found, revealing interactions with the BCR subunits CD79A/B. It was shown that TLR and BCR signaling cooperate to assemble MYD88 in a signalosome which activates mTOR and NF-κB signaling, providing a mechanistic insight as to why these patients were particularly sensitive to the BCR-targeted agent ibrutinib. This same rewiring was not found in CLL samples, but the expression of ZAP-70 represents another example where single activation of TLR9 is sufficient to fully engage BCR signaling (127, 132). ZAP-70 therefore represents an important candidate for signaling pathway rewiring in CLL. It was shown that TLR-mediated BCR activation was not dependent on the kinase activity of ZAP-70, which is compatible with ZAP-70 functioning as a scaffold in a signaling complex that relays TLR9 signals to SYK, thereby integrating innate into adaptive immune responses (42, 127). However, studying rewiring of signaling pathways is difficult and usually requires techniques such as mass spectrometry or proximity ligation assays to study the interactome of proteins, as ultimately protein-protein interactions assemble and regulate signaling pathways (133). Finally, multiomic analyses allow to study changes in signaling networks under specific conditions, and a novel kinomics approach applied in CLL revealed that rewiring of signaling pathways is not strictly oncogenic but can also be influenced by therapy (134). Comparison of kinase fingerprints between treatment-naïve patients and patients who had undergone prior chemoimmunotherapy, revealed SYK as a critical kinase to be differentially active upon BCR stimulation which correlated with proliferative capacity *in vitro* (134).

In summary, various crosstalk mechanisms between BCR, CD40, and TLR signaling have been described in CLL based on receptor expression levels, the activation of downstream mediators as well as the use of targeted inhibitors **(Figure 3)**.

SUMMARY AND OUTLOOK

In summary, most evidence for driving CLL proliferation *in vivo* is currently attributed to BCR engagement. A major discrepancy is that BCR stimulation *in vitro* does not induce proliferation, indicating that BCR-induced CLL proliferation *in vivo* likely requires additional (costimulatory) signals that are missing *in vitro*. We have highlighted the role of T cells in the proliferation of CLL cells *in vivo*, as T cell-derived signals including CD40L, IL-21 and IL-4 significantly promote *in vitro* proliferation of CLL cells (6). Moreover, it is important to consider that crosstalk between BCR, CD40, and TLR signaling occurs *in vitro*, and may thus play an important role *in vivo*. Consequently, we propose that combined triggering of multiple nodes of BCR, CD40, and TLR signaling in combination with costimulatory signals by cytokines orchestrate CLL proliferation, both *in vitro* and *in vivo* (**Figure 3**).

Modeling the CLL microenvironment is an area of intense investigation, and most studies have been performed using CLL cells isolated from PB as tissue-residing CLL cells are not easily obtained. Current experimental methods relying on the investigation of PB CLL cells lack the ability to appropriately mimic the lymphoid microenvironment due to a lack of *in vitro* cultures that allow the long-term expansion of CLL cells. Crucially, this indicates that essential factors or aspects are missing in current *in vitro* models. Despite these limitations,

many *in vitro* observations have helped to elucidate the role of (the combination of) individual signals in the proliferation of CLL *in vivo*, including the development of new therapies to target CLL proliferation.

New innovative *in vitro* CLL models continue to be developed and most promising could be 3D cultures that may overcome some of the current limitations of *in vitro* studies. Current 2D culture systems do not reflect the true 3D microenvironment present in human tissues, where various types of cell-cell interactions and interactions with the extracellular matrix occur, which may be fundamental to study CLL proliferation (135). Although the use of 3D models is new in the field of CLL, a recent study reported a significant increase in proliferative response, proliferation rates and number of cell generations compared to 2D cultures, irrespective of the biological characteristics of CLL cells (6). Therefore, 3D models may contribute pathophysiological relevance to *in vitro* culture systems of CLL and will be valuable for future studies. Similar to 2D CLL culture systems, many types of 3D models have been developed for solid tumors, including the use of scaffolds, gels, spheroid cultures and fluidic systems (136). However, unlike solid tumors, secondary lymphoid tissues do not derive from a single stem cell progenitor and thus the advantages and limitations of each of these systems have to be evaluated in terms of accurate mimicking of the CLL microenvironment. We may safely predict that in the near future a variety of 3D CLL systems will be reported. The ultimate goal is to implement a standardized system for *in vitro* proliferation, that will allow novel drug testing, as well as meaningful study of various CLL clonotypes.

AUTHOR CONTRIBUTIONS

MH wrote the review and prepared figures. EE conceptualized and wrote the review. AK edited the review and contributed text. All authors contributed to the article and approved the submitted version.

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ZAP-70 Shapes the Immune Microenvironment in B Cell Malignancies

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Zeta-chain-associated protein kinase-70 (ZAP-70) is a tyrosine kinase mainly expressed in T cells, NK cells and a subset of B cells. Primarily it functions in T cell receptor (TCR) activation through its tyrosine kinase activity. Aberrant expression of ZAP-70 has been evidenced in different B cell malignancies, with high expression of ZAP-70 in a subset of patients with Chronic Lymphocytic Leukemia (CLL), associating with unfavorable disease outcomes. Previous studies to understand the mechanisms underlying this correlation have been focused on tumor intrinsic mechanisms, including the activation of B cell receptor (BCR) signaling. Recent evidence also suggests that ZAP-70, intrinsically expressed in tumor cells, can modulate the cross-talk between malignant B cells and the immune environment, implying a more complex role of ZAP-70 in the pathogenesis of B cell malignancies. Meanwhile, the indispensible roles of ZAP-70 in T cell and NK cell activation also demonstrate that the autologous expression of ZAP-70 in the immune environment can be a central target in modulation of tumor immunity. Here we review the evidences of the link between ZAP-70 and tumor immunology in the microenvironment in B cell malignancies. Considering an emerging role of immunotherapies in treating these conditions, understanding the distinct molecular functions of ZAP-70 in a broader cellular context could ultimately benefit patient care.

Keywords: ZAP-70, tumor microenvironment, immunotherapy, B cell lymphoma, CLL

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INTRODUCTION

Zeta-chain-associated protein kinase-70 (ZAP-70) is a tyrosine kinase mainly expressed in T cells and NK cells (1, 2). The function of ZAP-70 in T cell receptor (TCR) activation through its tyrosine kinase activity has been well-studied through pioneering works by the Weiss laboratory and others [for review see (3)]. In the early 2000s, the aberrant high expression of ZAP-70 was identified in a subset of Chronic Lymphocytic Leukemia (CLL) patients (4), which turned out to also reflect an unfavorable clinical outcome (5). Much work has been done to establish ZAP-70 as a prognostic marker in CLL, assuming that assessment of its expression was somehow less time and labor-consuming than *IGHV* mutation analyses (6). However, the variation of expression levels and the lack of harmonized tests have hampered this development (7), consequently ZAP-70 expression is not routinely assessed to guide clinical decisions. Subsequent studies further revealed the expression of ZAP-70 in other B cell malignancies, such as Acute Lymphoblastic Leukemia (ALL), Burkitt-lymphoma and Mantle Cell Lymphoma (MCL) (8, 9). Although studies have shown the involvement of ZAP-70 in IgM-mediated B cell

receptor (BCR) signaling in CLL, the role of ZAP-70 in the pathogenesis of CLL and other B cell malignancies is still arguable. Recently studies have implied that tumor intrinsic ZAP-70 expression modulates the cross-talk between malignant B cells and their environment, suggesting a new angle to understand the role of ZAP-70 in these diseases. We will review here how ZAP-70 expression in malignant B cells has an impact on cell migration, innate immune response, and T cell infiltration. In contrast, its expression in T cells and NK cells can affect tumor immune responses. Therefore, targeting ZAP-70 may exert anti-tumor effects not only through the modulation of signaling cascades in malignant B cells, but also through inhibition of cells resident or recruited to the tumor microenvironment.

ZAP-70 EXPRESSION IN B CELL MALIGNANCIES

The expression of ZAP-70 in B cell malignancies was first detected in CLL with 20–80% of leukemic B cells having ZAP-70 expression levels equivalent to autologous CD3+ T cells in patients, correlating with unmutated *IGHV* gene and poor clinical outcomes (5, 6, 10, 11). Notably, the expression of ZAP-70 in CLL cells frequently varies across the entire clone and a somewhat arbitrary threshold of >20% is required to classify a patient by flow-cytometry as "ZAP-70-positive." Importantly, the expression levels of ZAP-70 in CLL cells are relatively stable over time (6, 10, 12). The aberrant ZAP-70 expression has further been found to associate with sIgM expression in CLL (13), which further suggested an essential role of ZAP-70 in CLL pathogenesis and progression. Importantly, discordant cases of ZAP-70 expression in *IGHV*- mutated CLL indicated that it possesses a higher predictive value for a poor clinical outcome and therefore strongly suggest that it may actively contribute to the pathogenesis (5, 6). In addition to CLL, ZAP-70 is also expressed in a fraction of B-ALL cases, including most of the childhood pre-B cells ALL (14, 15) and adult ALL cases with different maturation phenotypes (9, 16). Notably, ZAP-70 level in ALL is associated with CD38 expression, but no correlation was observed to specific cytogenetic abnormalities (9, 17). Moreover, ZAP-70 expression was identified in a subset of other B cell malignancies, including, Follicular Lymphoma (FL), Mantle Cell Lymphoma (MCL), Hairy Cell Leukemia (HCL), and Diffuse Large B-cell Lymphoma (DLBCL) by western blotting, flow cytometry (14) and immunohistochemistry assessment (8), and in very rare cases of classic Hodgkin lymphoma (18).

The presence of ZAP-70 in subsets of B cell malignancies also with immature phenotypes may reflect their cellular origin, since ZAP-70 expression is also evidenced in normal B cells, especially developing and differentiating B cells. Using a ZAP-70 deficient mouse model, the protein was found to be expressed in pro-B and pre-B cells and to play a role in the process of pro-B to pre-B cells transition in the bone marrow through engaging in the pre-BCR complex formation (19). Notably, ZAP-70 and SYK were functionally redundant in B cell development, since only mice with both ZAP-70 and SYK deficiency displayed a complete B cell developmental block (19, 20). This finding was further supported by a study analyzing B cell populations from human bone marrow, peripheral blood, and tonsils, which found ZAP-70 expression in pro-B and pre-B cells but not in the majority of normal mature B cells (9). Notably, similar to malignant B cells, ZAP-70 expression in normal B cell populations is also modulated by phosphorylation upon BCR activation (14, 21).

Since ZAP-70 is normally not expressed in mature B cells, its expression in CLL and other mature B cell-derived neoplasms likely points to their different cellular origin (9, 15). Interestingly, point mutations in *ZAP-70*, which can result in the lack of ZAP-70 protein expression in human T cells, were not identified in normal human B cells and ZAP-70 negative malignant B cells (9). Therefore, the down-regulation of ZAP-70 through B cell development may represent a physiological process of B cell maturation.

The aberrant high ZAP-70 expression found in some mature B cell malignancies may be caused by epigenetic modulation and clonal evolution during tumor transformation. In CLL, hypomethylation on CpG sites in the *ZAP-70* gene 5′ regulatory regions have been identified to be associated with high ZAP-70 expression and predictive of a poor disease outcome (22–24). Alternative mechanisms leading to the aberrant expression of ZAP-70 relate to tumor-microenvironment mediated induction of ZAP-70: In B cells derived from peripheral blood, which have consistently low ZAP-70 levels, BCR-activating stimuli (e.g., anti-IgM, sCD40L, IL-4, IL-6, and IL-10) upregulate the expression of ZAP-70 (14). Unmethylated CpG oligodeoxynucleotides, which can trigger an innate immune response through TLR9 activation, promote proliferation in a subset of CLL cells, accompanied by ZAP-70 induction (25, 26).

TUMOR ZAP-70 EXPRESSION MODULATES THE TUMOR- AND IMMUNE MICROENVIRONMENT

Efforts have been made to understand the molecular role of tumor-intrinsic ZAP-70 expression in B cell malignancies. In CLL, ZAP-70 expression is associated with enhanced BCR signaling upon IgM activation, evidenced by a positive correlation between ZAP-70 expression, phosphorylation of SYK, BLNK, and PLCγ2 and calcium response (4, 27). Notably, the kinase activity of ZAP-70 is dispensable for BCR signaling in CLL, since the phosphorylation of ZAP-70 catalytic sites appears negligible compared to that of SYK (28). In addition an introduced mutation abrogating kinase activity of the ZAP-70 catalytic site had no significant effect on IgM-mediated BCR signaling activation (29). This suggests that the role of ZAP-70 in B cell malignancies is different from that in T cells. Interestingly, despite the dispensable nature of its kinase activity, ectopic expression of ZAP-70 in the Burkitt lymphoma line BJAB enhanced the phosphorylation and activation of BCR-related signaling cascades under conditions of IgM activation (28). These findings have led to the suggestion that ZAP-70 acts mainly as an adaptor protein to recruit downstream protein

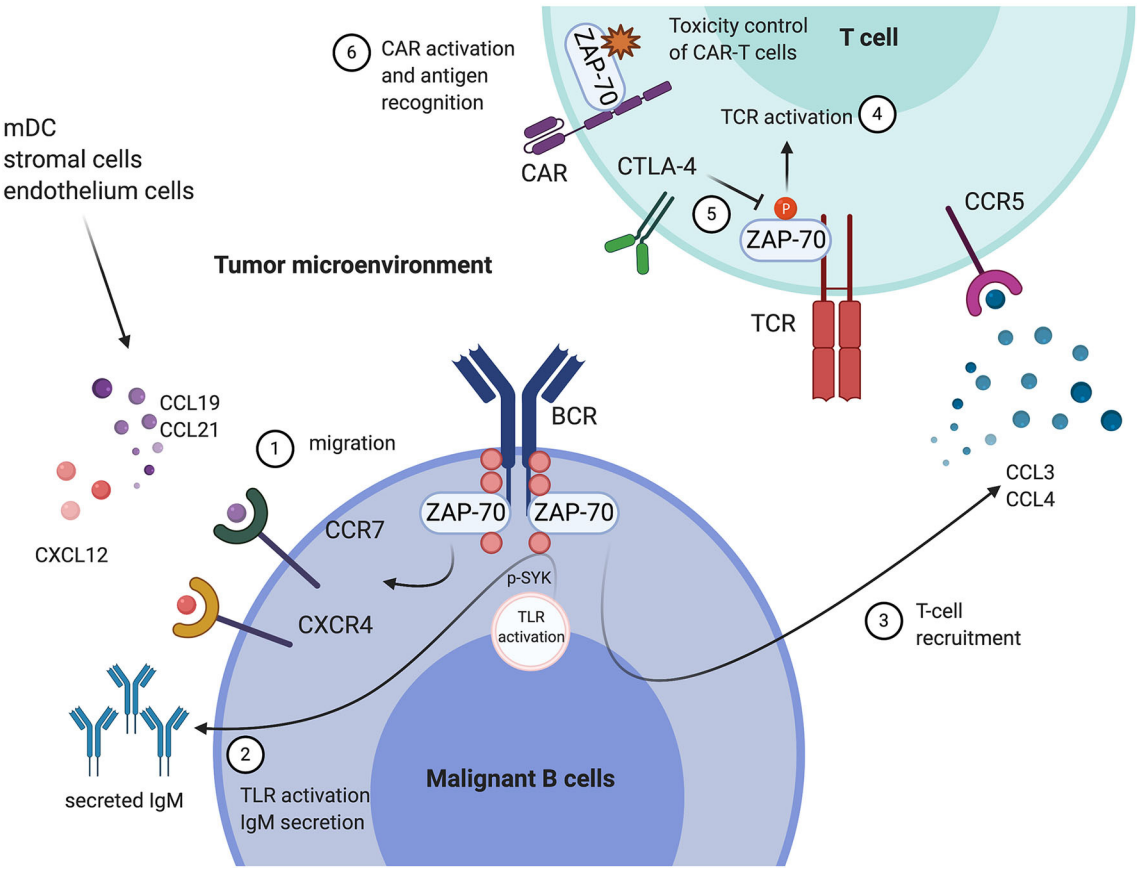

FIGURE 1 | Tumor expression of ZAP-70 modulates the immune microenvironment, and the functional roles of ZAP-70 in environmental T cells. In Chronic Lymphocytic Leukemia (CLL) cells, ZAP-70 mediates BCR signal transduction through its kinase activity or as a scaffold protein recruiting other tyrosine kinases. In addition, ZAP-70 modulates the communication between malignant cells and their tumor microenvironment. (1) ZAP-70 is involved in the regulation of CCR7, CXCR4 expression on CLL cells, which promote the migration of tumor cells toward an environment niche, secreting CCL19, CCL21, and CXCL12; (2) ZAP-70 in CLL is engaged in TLR9 activation-mediated anti-apoptotic effects and cell proliferation, likely through mediating SYK activation and IgM secretion; (3) ZAP-70 expression in CLL cells is associated with high levels of CCL3 and CCL4 secretion, which engage in the recruitment of T cells. In T cells in the tumor microenvironment, (4) ZAP-70 expression and phosphorylation is essential for TCR-mediated T cell activation; (5) CTLA-4 is a negative regulator of ZAP-70 phosphorylation, thus suppressing T cell activation; (6) ZAP-70 expression in CAR-T cells is important for cell proliferation and antigen recognition. This Figure has been created with Biorender.com.

kinases, such as PI3K, c-Cbl, Cbl-b, and Shc (28). In contrast, in B-ALL, ZAP-70 is constitutively phosphorylated, suggesting the tyrosine kinase activity is continuously involved in ALL biology (16). However, the detailed role of ZAP-70 in B-ALL is still unknown.

In addition to engaging in tumor cell intrinsic signaling, likely improving the cellular fitness of tumor cells, evidence suggest that ZAP-70 expression is also involved in the cross-talk between malignant B cells and their microenvironment (**Figure 1**).

Cell Migration

C-C chemokine receptor type 7 (CCR7) and C-X-C chemokine receptor type 4 (CXCR4) expression on B cells is essential for cell migration and homing during B cell development through the binding to their putative chemokine ligands CCL19/CCL21 and CXCL12, respectively (30). High receptor expression on malignant B cells correlates with advanced disease stage in CLL (31), and in Diffuse Large B cell Lymphoma (DLBCL), associated with increased bone marrow infiltration and poor outcomes (32). Other studies have shown ZAP-70 expression correlates with enhanced T- and B cells migration and chemotaxis in the microenvironment. In a recent study deciphering the molecular cues which modulate inflammation-dependent oligomerization of the chemokine receptor CCR7 in dendritic- and T cells, ZAP-70 has been identified as an interactor of CCR7 under chemokine stimulation, suggesting a role of ZAP-70 in CCR7 related cell migration and chemotaxis (33). This finding is consistent with previous studies showing ZAP-70 expression in CLL cells correlates with CCR7 expression, induced by IgM-mediated ERK activation, thus enhancing the migratory ability to CCL19 and CCL21 (34, 35). A recent study has further evidenced this in CLL patients, and observed that ZAP-70 positive CLL cells migrated more to CCL19, CCL21, and CXCL12 by controlling the chemokine-driven clustering of the integrins VLA-4 and

LFA-1 (36). Moreover, ZAP-70 expression also correlates with CCR7/CXCR4 expression in B cell precursor ALL disease and here promotes migration toward CCL19/CXCL12 in the central nervous system (37).

Innate Immune Responses

Besides BCR mediated signals, Toll-like receptor (TLR) signaling, which can bridge innate and adaptive immune responses, has been found to play a role in CLL activation and proliferation (38, 39). Interestingly, ZAP-70 appears to play a role to determine the environmental triggered TLR response in CLL: A recent study from our lab has elucidated that expression of ZAP-70 in CLL is strongly predictive of TLR9 agonists-mediated anti-apoptotic effects and cell proliferation, likely through mediating SYK activation, IgM secretion and Bim degradation (26).

T Cell Infiltration

Mounting evidence indicates that the ZAP-70 expressed in tumor cells has ramifications for the composition of immune cells in the microenvironment, especially for the number of infiltrating T cells. In studies comparing the immune-phenotype of ZAP-70 positive and ZAP-70 negative CLL patients, tumor ZAP-70 expression was associated with increased CD4 central memory T cells and CD3/CD69+ T cells with decreased CD4/CD8 ratio in the peripheral blood (40–42). However, since high ZAP-70 expression is normally observed in only a subpopulation of CLL cells and varies substantially between patients, it is possible that subtle changes in different T cell populations between ZAP-70 positive and negative patients are partly impacted by inconsistencies in the definition of ZAP-70 positive in these studies. Interestingly, studies have evidenced that CLL cells secrete the C-C motif chemokine ligands, CCL3 and CCL4, which enable the recruitment of T cells and monocytes, under the stimulation of IgM and in co-culture with nurse-like cells (NLC) (43). In addition, in CLL, ZAP-70 positive patients have significantly higher CCL3 and CCL4 plasma levels (43), and CCL3 plasma levels correlate with other risk factors (44). These findings suggest a potential role of tumor autologous ZAP-70, mediating immune-responses and fostering a tumor-supportive microenvironment through modulation of the expression of T cell chemokines.

ZAP-70 EXPRESSION IN T CELLS AND NK CELLS, AND THEIR ROLES IN B CELL MALIGNANCIES

Immunotherapy, including checkpoint blockade inhibition and cell-based immunotherapies, is a fast developing area in cancer treatment. Such treatment modalities have been applied to treat B cell malignancies and demonstrated significant improved outcomes in smaller subsets of patients, who previously relapsed from chemotherapies and targeted therapies (45). Notably, despite showing some promising effects, the molecular mechanisms that inhibit T cell and NK cell activation in B cell malignancies and block anti-tumor immunity are far from being comprehensively described.

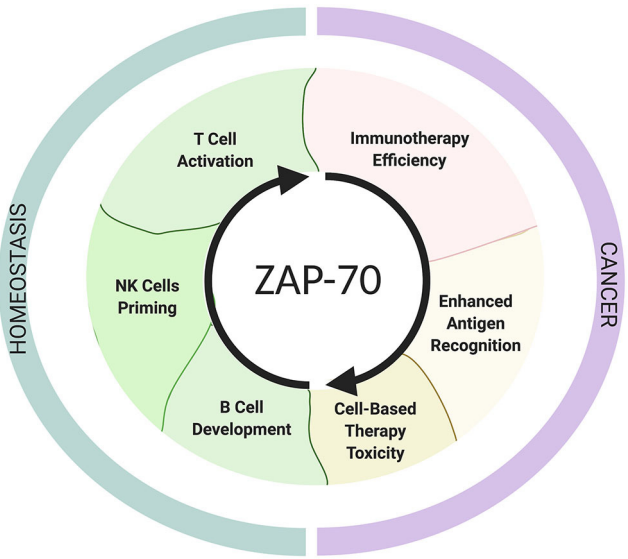

FIGURE 2 | The roles of ZAP-70 in homeostasis and cancer. The schematic shows ZAP-70 involvement in physiological contexts and in cancer. This Figure has been created with Biorender.com.

Because of its indispensible role for TCR activation, deficiency, or aberrantly high expression of ZAP-70 in T cells expectedly result in immune deficiency [ZAP-70-related severe combined immunodeficiency syndromes (SCID)] (46) and autoimmunity, respectively (47). To date, it remains an important but unanswered question whether ZAP-70 expression levels in T and NK cells are associated with patient responses to immunotherapies, but an increasing amount of evidence suggests that ZAP-70 deficiency or inhibition can contribute to impaired tumor-surveillance (**Figure 2**).

T Cells

Structurally, ZAP-70 has been found to play a central role in immunological synapse formation in cytotoxic T lymphocytes (CTL) (48). CTLA-4 is a well-established inhibitory checkpoint for T cell activation (49). It has been suggested that the inhibition of ZAP-70 tyrosine-phosphorylation is a mechanism of CTLA-4 mediated suppression of CD4+ T activation, indicating a central role of ZAP-70 kinase activity in T cell activation and anti-tumor immune responses (50). In a recent study the GTPase-activating protein (GAP) Rasal1, which inhibits ZAP-70, has been identified to suppress anti-tumor immune-responses. Antagonizing ZAP-70 inhibition by siRNA against Rasal1 increased the number of CD8+ tumor-infiltrating T-cells expressing granzyme B and interferon gamma (no '1') and enhanced tumor killing (51). Notably, in a DLBCL case, ZAP-70 deficiency caused complete ablation of the CD8 population in the tumor environment (52), suggesting a profound effect of ZAP-70 in tumor immune-responses.

NK Cells

Recently, NK cells have been applied for cancer-immunotherapies, benefitting from its antigen-independent host immune responses and cytotoxicity against malignant cells (53). ZAP-70 is a kinase that is also involved in the activation of NK cells upon engaging with ligands on targeted cells (54) and downregulation of ZAP-70 is associated with inhibition of NK cell responses under prolonged activation and continuous DNA damage stress (55). A very recent study revealed that ZAP-70 is engaged in immunomodulatory drug pomalidomide induced granzyme-B secretion and cytolytic activity of NK cells (56). However, ZAP-70-independent pathways exist which modulate NK cell mediated cytotoxicity, primarily through signaling modulated by non-ITAM-based receptors, like NKG2D (57). It has also been described that NK cells from SYK$^{-/-}$ ZAP-70$^{-/-}$ mice still maintain natural cytotoxicity, which suggests a redundant role of ZAP-70 in this process, despite driving the activation of NK cell receptor signaling (58).

CAR-T Cells

Chimeric antigen receptor T cells (CAR-T) are T cells expressing artificial T cell receptors which contain both tumor specific- as well as T cell activating motifs (59). Promising results from clinical trials had led to several CAR-T cell therapies approved by the United States Food and Drug Administration and European Medicines Agency for treating relapsed or refractory B cell malignancies (60). In spite of the similarity between chimeric antigen receptors (CARs) and natural TCRs, reduced efficiency of antigen-recognition and affinity remain major issues in CAR-T cell therapies. Third generation CAR-T cells are potentially more efficient than second generation through engaging additional co-stimulatory molecules. Evidence from a comparative study indicates that activation and phosphorylation of ZAP-70 in CAR-T cells is associated with enhanced cell proliferation and expansion of third generation CARs, containing both CD28 and 4-1BB motifs, compared to second generation CARs (61). The importance of ZAP-70 in CAR-T activation has been further addressed by a very recent study: Using quantitative single-molecule live-cell imaging, CAR-T cells have been shown to have ∼1,000 times reduced antigen sensitivity compared to normal T cells, and data suggest that the underlying mechanism relates to reduced recruitment of ZAP-70 to CARs. This study enlightens the importance of ZAP-70 in CAR-T activation and suggests it as a promising target for improving CAR-T antigen recognition (62).

ZAP-70 AS THERAPEUTIC TARGET

Considering the importance of ZAP-70 in T cell and NK cell activation, great effort has been put to target ZAP-70 in order to control diseases derived from abnormal T or NK cell activation, such as immune disorders and autoimmune diseases (3, 63). ZAP-70 has been found not only to function through its kinase activity, but also as an important scaffold protein to associate with TCR or BCR related molecules, independent of its catalytic activity (28, 64), suggesting that kinase-inhibition may not completely abolish protein function. Several *in vitro* studies have previously investigated inhibitors which can suppress ZAP-70 kinase activity or disrupt its protein-binding ability to access downstream TCR related activators (65, 66). These inhibitors have been well-described in a recent review (63).

Although the expression of ZAP-70 in tumor cells has been linked to a dismal outcome, there have only been few attempts to inhibit ZAP-70 as a treatment, partly because the biological functions of ZAP-70 in B cell malignancies remain elusive. Tyrosine kinase inhibitors have been assessed to treat ZAP-70 positive CLL, for example, gefitinib has been tested for inducing apoptosis of ZAP-70 positive CLL cells and cell lines *in vitro*. These studies demonstrated that gefitinib inhibits the basal and BCR activation-mediated phosphorylation of ZAP-70 at the micromolar level and that ZAP-70 expression sensitizes cells to gefitinib induced cell apoptosis (67). However, it is arguable whether these pro-apoptotic effects of gefitinib were achieved through the inhibition of ZAP-70 or other related tyrosine kinases, such as SYK.

While ZAP-70 constitutes an interesting and attractive target for therapeutic interventions in cancer patients, especially in those with aberrant expression in B cell malignancies, the simultaneous inhibition of T and NK cells appears to be inevitable and may be less desirable and potentially even harmful. While T cell subsets may promote tumorigenesis (e.g., through CD40 stimulation) and their inhibition may therefore be therapeutically beneficial, blockage of cytotoxic T cells and NK cells may be less so. Whether different immune cells display different susceptibilities to ZAP-70 inhibition, thus allowing for a wide-enough therapeutic window of antagonists to be beneficial, is unknown, but at least seems possible. We believe this is a substantial problem to be considered in the design of ZAP-70 directed therapies.

Immunotherapies, including CAR-T cells, immune checkpoint blockade, and adaptive T cell therapies, have been applied in clinical treatment for B cell malignancies. The safe and precise control of over-reactions of anti-tumor immune responses has been a major issue for the toxicity of cell-based immunotherapies (68). Based on the essential role of ZAP-70 in TCR activation, some studies suggest targeting ZAP-70 in order to control effector T cells, which could potentially be applied for developing safer adaptive T cell therapies (**Figure 1**). A recent study defined ZAP-70 as a target to control the toxicity caused by over-reacting CAR-T cells such as cytokine release syndrome (CRS). Dasatinib, a tyrosine kinase inhibitor has been found to attenuate CAR-T toxicity by suppressing ZAP-70 activation (69). However, inhibition of other kinases by dasatinib, such as Abl and Src tyrosine kinases, may likely contribute. An engineered ZAP-70 construct has been established by the Weiss lab to specifically study the role and requirement of ZAP-70 kinase activity in different biological processes. The so-called analog-sensitive ZAP-70 mutant (ZAP-70 AS), which contains an engineered binding pocket around the kinase domain, sensitive to an analog of the small molecule kinase inhibitor PP1, conserves the normal ZAP-70 catalytic activity and can be specifically inhibited (70). This specificity has a great

TABLE 1 | Cell-type specific functions of ZAP-70 in B cell malignancies.

	Cell types	Proposed functions
Malignant B cells	CLL	✧ ZAP-70 enhances BCR signaling upon IgM activation; ✧ Interactions with BCR-related proteins; ✧ ZAP-70 correlates with CCR7, CXCR4 expression and enhanced cell migration; ✧ Modulation of TLR-induced response through mediating SYK activation, IgM secretion, and Bim degradation; ✧ Associates with CCL3 and CCL4 secretion and T cell infiltration in the tumor microenvironment.
	ALL	✧ Constitutively phosphorylated, detailed role of ZAP-70 in ALL is unknown.
	Others	✧ Undefined
Tumor-environment immune cells	T cells	✧ Essential for TCR activation through its tyrosine kinase activity; ✧ Plays central role in immunological synapse formation in CTL. ✧ CTLA-4 in CD4+ T cells inhibits ZAP-70 activity.
	NK cells	✧ NK cell activation upon receptor engagement; ✧ May be redundant in NK cell mediated cytotoxicity.
Immunotherapy	CAR-T cells	✧ Associates with enhanced cell proliferation and expansion in the 3rd generation CAR-T cells contacting both CD28 and 4-1BB; ✧ Reduced recruitment of ZAP-70 to CARs is associated with less antigen sensitivity compared to normal T cells.

potential to be applied for the safe control of adaptive cell-based immunotherapies (71).

CONCLUSION

ZAP-70 is not only critical for T cell and NK cell activation, but also associated with poor outcomes of B cell malignancies, especially in CLL. Tumor intrinsic expression of ZAP-70 in B cell malignancies has been shown to enhance cellular signals under ligand stimulated BCR activation. However, the underlying mechanisms known so far cannot fully explain the correlation between ZAP-70 expression and dismal outcome. More evidence has pointed to ZAP-70 driven environmental changes, which may play a central role for triggering innate immune responses and immune cell infiltration (**Figure 1** and **Table 1**).

Growing evidence also indicates that the modulation of ZAP-70 activity can be applied to control T cell activation, which has translational potential to mitigate the toxicity associated with cell-based immunotherapies (**Figure 1**). However, since most of the evidence has only been compiled from *in vitro* experiments, more *in vivo* studies are needed to fully characterize whether such therapies can be applied in a clinical setting.

A thorough review of the published evidences focusing on defining the role of ZAP-70 in health and disease clearly indicates that it remains an attractive target for therapeutic interventions, more than ever. More experimental evidence is needed to fully understand the biology behind ZAP-70 in B cell malignances in a holistic cellular approach. The simultaneous targeting of ZAP-70 in tumor cells, T and NK cells, may be beneficial in some instances, but also bears the risk to promote tumor growth through impairing immune surveillance.

AUTHOR CONTRIBUTIONS

JC and IR designed and wrote this review. AM provided critical editing on the manuscript and the graphs. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Linking Immunoevasion and Metabolic Reprogramming in B-Cell–Derived Lymphomas

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Lymphomas represent a diverse group of malignancies that emerge from lymphocytes. Despite improvements in diagnosis and treatment of lymphomas of B-cell origin, relapsed and refractory disease represents an unmet clinical need. Therefore, it is of utmost importance to better understand the lymphomas' intrinsic features as well as the interactions with their cellular microenvironment for developing novel therapeutic strategies. In fact, the role of immune-based approaches is steadily increasing and involves amongst others the use of monoclonal antibodies against tumor antigens, inhibitors of immunological checkpoints, and even genetically modified T-cells. Metabolic reprogramming and immune escape both represent well established cancer hallmarks. Tumor metabolism as introduced by Otto Warburg in the early 20th century promotes survival, proliferation, and therapeutic resistance. Simultaneously, malignant cells employ a plethora of mechanisms to evade immune surveillance. Increasing evidence suggests that metabolic reprogramming does not only confer cell intrinsic growth and survival advantages to tumor cells but also impacts local as well as systemic anti-tumor immunity. Tumor and immune cells compete over nutrients such as carbohydrates or amino acids that are critical for the immune cell function. Moreover, skewed metabolic pathways in malignant cells can result in abundant production and release of bioactive metabolites such as lactic acid, kynurenine or reactive oxygen species (ROS) that affect immune cell fitness and function. This "metabolic re-modeling" of the tumor microenvironment shifts anti-tumor immune reactivity toward tolerance. Here, we will review molecular events leading to metabolic alterations in B-cell lymphomas and their impact on anti-tumor immunity.

Keywords: B-cell-derived Non-Hodgkin lymphoma, chronic lymphocytic leukemia, metabolism, immune escape, immune therapeutics

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INTRODUCTION

Metabolic reprogramming is a well-established hallmark of cancer (1). In fact, emerging evidence suggests that metabolic reprogramming does not only confer bioenergetic advantages but also impacts immune surveillance, thus being closely interconnected with immune escape, another hallmark of cancer (1). In this mini-review on B-cell-derived lymphomas, we will focus on

metabolic alterations, the underlying molecular mechanisms, and their impact on anti-tumor immunity.

In general, cells meet their energetic demands in form of adenosine triphosphate (ATP) by different degrees of either glycolysis or mitochondrial oxidative phosphorylation (OxPhos). Although both metabolic axes happen simultaneously, there is a distinct fine-tuning of the balance between glycolysis and OxPhos. Non-proliferating, quiescent or differentiated cells primarily rely on OxPhos in the presence of oxygen and only switch to glycolysis under hypoxia. Highly proliferative cells obtain most of their ATP by aerobic glycolysis despite the availability of oxygen, a mechanism introduced by Otto Warburg in the 1920s and referred to as the "Warburg effect" (2).

Although aerobic glycolysis is less efficient, energy generation is faster and provides intermediates such as nucleotides, amino acids, and carbons for fatty acid (FA)/lipid synthesis; all crucial components for dividing cells. Consequently, aerobic glycolysis is predominantly found in activated immune cells such as effector T-cells (3) but also in most dividing malignant cells (4).

OxPhos provides an enhanced metabolic flexibility as it can be fueled by different sources, primarily glucose, glutamine, and FAs. Cell types that preferentially utilize OxPhos are slow-dividing, long-lived immune cells such as regulatory and memory T-cells (5) or leukemia stem cells (6).

Apart from the energy provision, metabolic processes impact a plethora of cellular functions by amongst others interfering with translation, epigenetics, and post-translational modifications as reviewed by Patel et al. (7). As such, metabolism represents a central regulator of cell fate and function.

Owed to its vital importance metabolism is tightly regulated by both intrinsic and extrinsic mechanisms. Malignant cells display aberrations in those regulatory circuits leading to metabolic profiles that favor survival, growth, and immune escape. In the follow, we will discuss the most prominent metabolic alterations described in B-cell-derived Non-Hodgkin lymphomas (B-NHLs) including chronic lymphocytic leukemia (CLL).

METABOLIC ALTERATIONS IN B-NHLs AND CLL

Diffuse Large B-cell Lymphoma (DLBCL)

As the most common form of B-cell lymphomas, aggressive DLBCL accounts for about 35% of all newly diagnosed B-NHLs (8). The DLBCL displays a pronounced heterogeneity in terms of genetic background and outcome. The cell of origin (COO) algorithm categorizes DLBCLs based on the gene expression profiles (GEPs) into "germinal center B-cell like" (GCB) types resembling normal germinal center B-cells and "activated B-cell like" (ABC) types with GEPs reminiscent of *in vitro* activated B-cells (9–11). An additional classification framework known as consensus cluster classification (CCC) revealed three separate clusters with distinct metabolic fingerprints: OxPhos-DLBCL, B-cell receptor (BCR)-DLBCL, and host response (HR)-DLBCL (12). OxPhos-DLBCLs display a prominent mitochondrial component, with elevated OxPhos, an overall increased mitochondrial contribution to the total energy turnover, and a greater incorporation of carbons derived from FAs and glucose into the tricarboxylic acid (TCA) cycle. In contrast, non-OxPhos-DLBCLs are metabolically rewired toward aerobic glycolysis (13). Immunohistochemical studies in DLBCL revealed expression of transporters of lactate (i.e., MCT1 and TOMM20) that can fuel the TCA cycle of malignant cells in a process better known as the "reverse Warburg effect" (14). Interestingly, OxPhos-DLBCL exhibited marked susceptibility toward inhibition of mitochondrial FA oxidation (FAO) and of PPARγ that regulates FA uptake and storage (13). BCR-DLBCLs were susceptible to pharmacological SYK inhibition (15), which in turn leads to a downregulation of glycolytic components (such as GLUT1 and hexokinase 2) (16).

As a central hub for the integration of metabolic processes, mammalian target of rapamycin (mTOR) controls nutrient/amino acid sensing, glycolysis, OxPhos, and consequently proliferation and survival. It serves as the core component of two multi-protein complexes (mTORC1 and mTORC2) that regulate different cell processes [reviewed in (17)]. Non-GCB DLBCLs depict increased mTOR-activity, which is linked to inferior survival (18). However, an *in vitro* study conducted on different DLBCL cell lines demonstrated therapeutic efficacy of mTOR inhibitors independent of COO. Overall, no clear link between COO- or CCC-based classifications and mTOR activity could be established yet.

Furthermore, DLBCLs (over-)express indoleamine-2,3-dioxygenase (IDO), which catalyzes breakdown of the essential amino acid L-tryptophan into the catabolite L-kynurenine (19). The latter one could promote expression of the pro-oncogenic Bcl-6 in DLCBL (20). In fact, both enhanced IDO expression (21) as well as elevated serum L-kynurenine levels (22) were linked to reduced response rates and inferior 3-yr overall survival (OS).

Follicular Lymphoma (FL)

The second most common type of B-NHL is the indolent follicular lymphoma (FL) (23). In FL SYK is, similar to DLBCL, highly activated and regulates mTOR (24). In addition, recurrent somatic mutations of *RRAGC* that encodes for the Ras-related GTP-binding protein C are the leading cause for mTORC1 activation in FL (25) and render FL cells more susceptible toward mTOR-induced cytotoxicity (26). Beyond that, metabolism of FLs remains largely unexplored. Notably, transformation into DLBCL is associated with an enhanced expression of the glycolytic machinery, which is in line with the increased glucose uptake as revealed by ^{18}F-FDG PET/CT in transformed lymphomas (27, 28).

Mantle Cell Lymphoma (MCL)

MCL represents about 5-10% of B-NHLs. Despite being classified as indolent, it has a rather aggressive disease course. MCL cell lines display constitutive mTOR activation (29). A dysregulation of the upstream PI3K/AKT pathway has been implicated as a driver of mTOR in MCL. This notion is further corroborated by the observation that the phosphatase and tensin homologue (PTEN), which acts as an intrinsic PI3K/AKT inhibitor, can be

reduced or undetectable in MCL (30). Inhibiting mTOR was effective in targeting MCL metabolism (31) and is approved for the relapsed/refractory (r/r) situation based on positive data from clinical studies (32). The Bruton tyrosine kinase (BTK) inhibitor ibrutinib abolishes BCR signaling and has emerged as a potent therapeutic option for r/r MCL. BTK-blockade markedly affected the (ibrutinib-responsive) MCLs' metabolic activity including glycolysis and the TCA cycle (33). Interestingly, Zhang et al. reported that ibrutinib-resistant MCLs depict a metatobolic rewiring toward glutaminolysis-fueled OxPhos (34). These drug-resistant cells were readily targeted by a OxPhos inhibitor, showing promising efficacy in patient-derived preclinical models.

Chronic Lymphocytic Leukemia (CLL)

CLL as the most common adult leukemia of the Western world is a heterogeneous disease characterized by accumulating monoclonal B-lymphocytes (35). Circulating CLL cells are mainly quiescent and proliferation predominantly occurs in lymph nodes (LNs) and the bone marrow (BM). Nevertheless, circulating CLL cells possess a marked metabolic activity that differs from healthy B-lymphocytes. As they traffic between hypoxic (i.e., LN and BM) and normoxic compartments (i.e., peripheral blood), CLL cells were found to constitutively express hypoxia-inducible factor (HIF-1α), which gets further upregulated within LNs thus promoting aerobic glycolysis (36, 37). Hypoxia-induced upregulation of glycolytic genes is further supported by adenosine signaling, which is triggered by the CLL cells' ectonucleotidases CD39 and CD73 (37).

CLL cells *per se* contain more mitochondria than conventional B-lymphocytes. Endogenous nitric oxide (NO) levels correlate positively with mitochondrial mass (38). In fact, NO can drive mitochondrial biogenesis, as NO supplementation increases mitochondrial mass in B-NHL-derived cell lines, whereas NO inhibition antagonizes this process (39). Correspondingly, Jitschin et al. demonstrated enhanced mitochondrial OxPhos, respiration, and respiratory capacity (40). The thereby amplified electron turnover *via* the mitochondrial electron transport chain yielded increased levels of ROS within the CLL cells but also systemically. Oxidative stress led then to a compensatory upregulation of heme-oxygenase-1 in CLL cells, a key cellular antioxidant, which also functions as a positive switch for the key activator of mitochondrial transcription factor A. Mitochondrial biogenesis, increased respiration, and oxidative stress appear to form a positive self-reinforcing feedback loop. As previously shown for solid tumors, pyruvate can act as a scavenger of mitochondrial superoxide in CLL cells. Increased oxidative stress under hypoxia led to enhanced pyruvate uptake while normoxic conditions led to a pyruvate release (36).

In fact, CLL cells from patients with higher disease stages and those with molecular features associated with a poor prognosis, like unmutated IGHV (U-CLL) and ZAP-70 positivity, showed higher mitochondrial respiration (41, 42). Both aforementioned genetic risk factors foster BCR signaling. Consistently, BCR-targeting reduces the metabolic activity (41). In general, CLL cells and conventional B-cells did not differ in terms of their basal glycolytic rate. However, CLL cells showed an elevated glycolytic capacity and glycolytic (together with respiratory) reserve. Furthermore, patients with U-CLL had higher lactic acid serum concentrations and displayed an elevated glycolytic capacity as compared to their mutated counterparts (M-CLL) (43). This suggests that CLL cells are better equipped to adapt to fluctuations of bioenergetic resources. In fact, microenvironmental stimuli further support the CLL cells' metabolic flexibility as contact to LN- or BM-resident stromal cells elicits a glycolytic switch in a Notch/Myc-dependent manner (44). Transferring these findings into the clinics, patients with higher glycolytic capacity and reserve have a worse OS and a shorter time-to-treatment (43). Furthermore, CLL samples with higher glycolytic flexibility showed an increased resistance to novel drugs affecting the mitochondria, such as venetoclax and navitoclax (43).

Another metabolically important aspect is the role of free fatty acids (FFAs). Lipoprotein lipase (LPL) is the major enzyme catalyzing hydrolysis of triglycerides into FFAs and is mainly present in adipose tissue, playing a key role in lipid metabolism. CLL cells carry LPL on their cell membrane, while LPL gene expression is elevated in U-CLL cells (45, 46). LPL facilitates lipoprotein uptake, which enables CLL cells (unlike normal B-cells) to store and metabolize FFAs (46). Intracellular FFAs can then be used to promote the already more active OxPhos in CLL cells (47). Moreover, FFAs may themselves drive mitochondrial biogenesis through activation of PPARγ (48). Upregulation of LPL in CLL cells is at least partly mediated by STAT3, since STAT3 binds to the LPL promotor and STAT3 knockout downregulated LPL protein levels (46). In addition to LPL, CD36 (a cell surface FA translocase) density is higher on CLL cells as compared to non-malignant B-cells. CD36 expression (and the consecutive FA uptake) were driven by STAT3 and inhibition of FA uptake reduced CLL cell viability (49). Again, BTK-inhibition reduced LPL levels and FFA metabolism both *in vitro* and in patients at least partly through interfering with STAT3 (50). Interestingly, CLL ibrutinib-resistant cases could be targeted by an FAO-inhibitor highlighting the importance of FAO metabolism and OxPhos (51). Emphasizing the relevance of FFA metabolism in CLL, the OS of patients with high LPL levels was worse than for those with low ones (45).

Taken together, metabolic adaptations and flexibility occur in multiple facets in DLBCL, FL, MCL, and CLL (**Figure 1**). They confer enhanced survival, proliferation, and therapeutic resistance but at the same time, we can therapeutically exploit them.

LYMPHOMA METABOLISM AND ITS POTENTIAL IMPACT ON ANTI-LYMPHOMA IMMUNITY

Tumors including B-cell malignancies have developed a variety of mechanisms to evade the anti-tumor immunity. Amongst others, four major "metabolic strategies" have been identified (**Figure 2**): 1) competition over nutrients, 2) production of bioactive metabolites, 3) induction/promotion of regulatory, tolerogenic immune cells, and 4) metabolic control of immune checkpoints (ICPs).

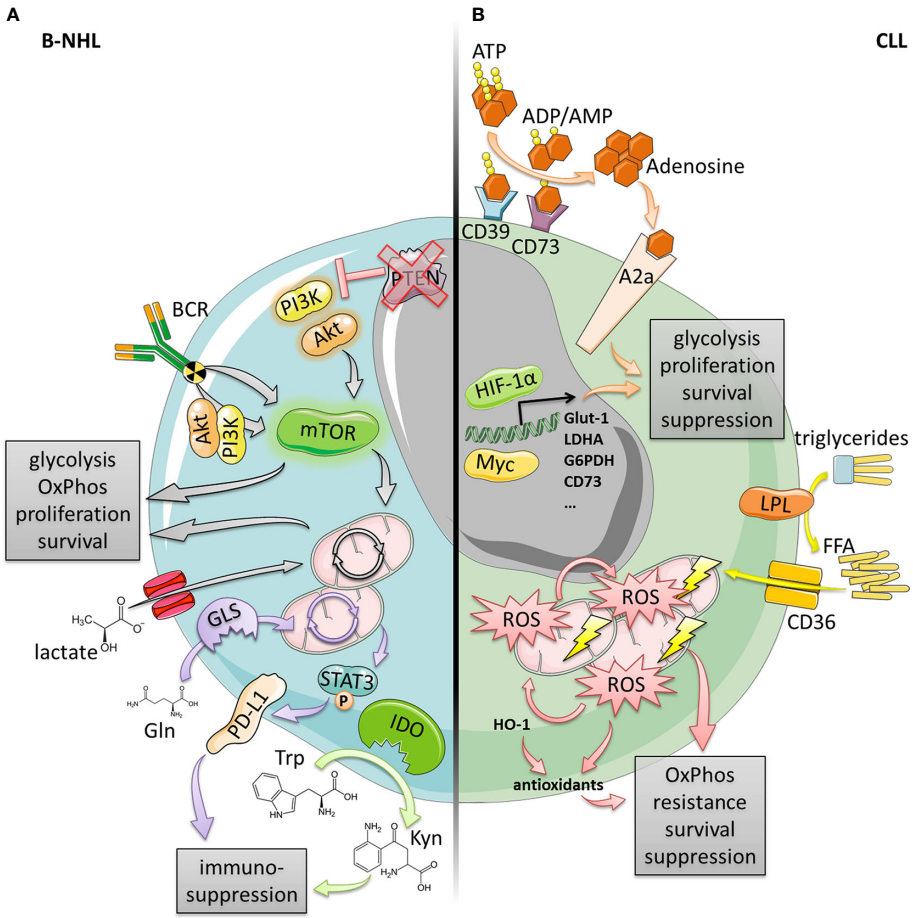

FIGURE 1 | Metabolic alterations in B-cell lymphomas and CLL. **(A)** B-NHL (DLBCL, MCL, FL) often exhibit an elevated mTOR signaling activity enabling increased glycolysis, OxPhos, proliferation and survival. This can be driven by the BCR in a PI3K/AKT-dependent or independent manner, or by genetic events such as the loss of PTEN expression resulting in constitutively active PI3K/AKT. Increased OxPhos was also found to be fueled by elevated lactate shuttling into the TCA cycle due to increased lactate importer expression. Additionally, both increased expression of IDO as well as glutaminolysis-driven PD-L1 induction provide enhanced immune-suppression. **(B)** CLL cells display a high mitochondrial biomass and high levels of OxPhos generating large amounts of energy and ROS that in turn drive mitochondrial biogenesis and generation of antioxidants (at least partly by HO-1). This vicious cycle confers enhanced oxidative stress resistance, survival and suppression. OxPhos and mitochondrial biogenesis could also be driven by increased activity of LPL and CD36 consuming triglycerides and importing free fatty acids (FFA). In contrast, microenvironmental trigger (e.g., hypoxia or LN-/BM-stroma) can induce transcription factors, such as Myc or HIF-1α leading to a glycolytic switch (enabled by high metabolic flexibility), and an increase of the adenosinergic axis culminating in enhanced survival, proliferation, and suppression.

The most studied example of nutrient competition is the increased glucose consumption by malignant cells caused by elevated expression levels of glucose transporters and enzymes of the glycolytic machinery {as seen in BCR-DLBCL [e.g., GAPDH expression (52) and lactate secretion (13)], transformed FL [e.g., GAPDH and aldolase A (27, 28)], MCL [e.g., glycolytic flux (33)], and CLL in the LN-/BM-niche [e.g., glycolytic flux and key glycolytic enzymes (44)]}. This is detrimental for T- and NK-cells as their proliferation, activation, and differentiation is highly dependent on glucose as a fuel for both aerobic glycolysis and OxPhos [reviewed in (53)]. Similarly, tumoricidal (M1) macrophages depend on glucose to fully mount their effector cytokine response [reviewed in (54)]. Additionally, increased activity of lipid/FFA-consumptive enzymes (i.e., LPL and CD36 as seen in CLL) as well as of glutaminase (as seen in DLBCL) contribute to a nutrient-poor environment. Apart from the depletion of basic bioenergetic substrates, increased expression of the ectonucleotidases CD39/CD73 (as seen in CLL) reduces extracellular ATP (exATP) by enzymatic conversion to adenosine. exATP was shown to be particularly important for long-lived T-cell immunity through purinergic signaling promoting mitochondrial fitness (55). Expression of L-tryptophan-depleting IDO (as seen in DLBCL) has been linked to inferior progression free survival and OS in B-cell malignancies (21). In T-cells L-tryptophan shortage leads to cell cycle arrest (56) at least partly due to a stress response caused by uncharged transfer-RNAs (57), and to reduced proliferation caused by mTOR inhibition (58). Furthermore, tryptophan is essential for clonal

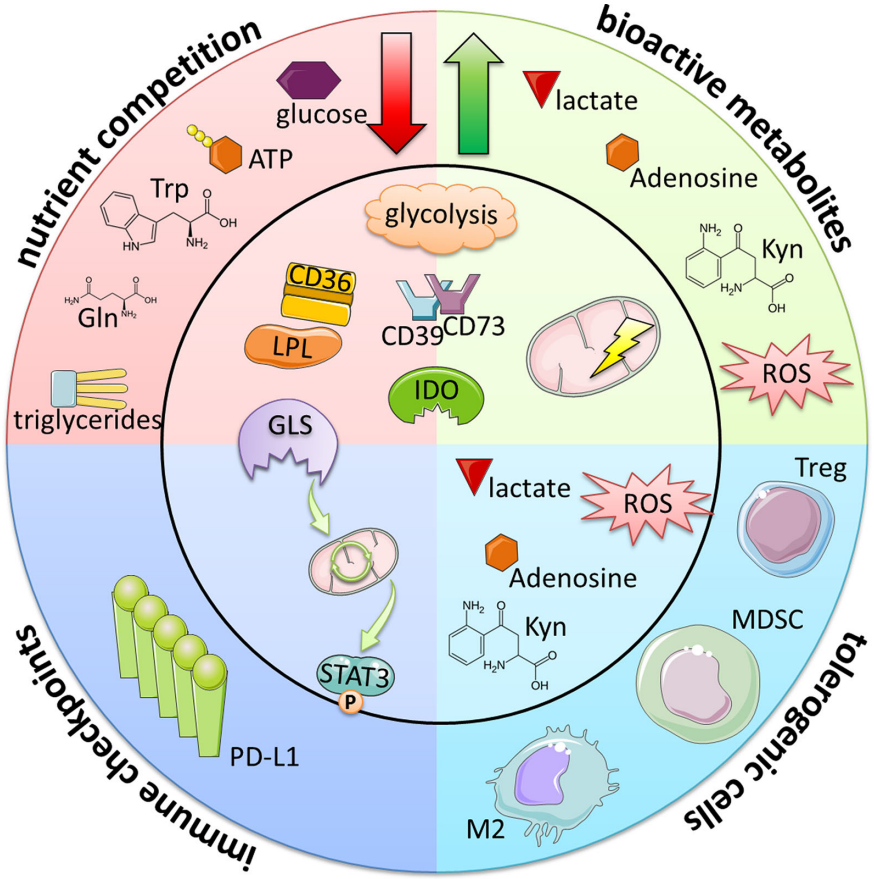

FIGURE 2 | Immunometabolic evasion mechanisms of B-cell lymphomas and CLL. Metabolic alterations lead to the reduction of essential immune cell nutrients and at the same time to the generation of bioactive metabolites. In addition to their direct immuno-suppressive nature, these byproducts also increase the frequencies of tolerogenic, suppressive immune cells (such as Tregs, myeloid-derived suppressor cells [MDSCs] and M2-like macrophages) either indirectly *via* the creation of favorable conditions or directly inducing their differentiation. Furthermore, certain metabolic programs can promote the expression of immune checkpoints such as PD-L1. Taken together, these events ultimately lead to the overall suppression of immune attack and increase the tumor cells' survival and persistence.

expansion and effector T-cell differentiation *via* metabolic reprogramming through mTOR (59).

Simultaneously, the different rewired metabolic activities lead to accumulation of various bioactive (and potentially immuno-modulatory) metabolites within the tumor microenvironment (TME). This includes lactate (as a result of enhanced glycolytic activity) which leads to the inhibition of T- and NK-cells *via* blunted lactate export acidifying the cytoplasm (60) as well as reduced NFAT levels (61) resulting in diminished cytokine production and effector function. Similarly, the IDO catabolite L-kynurenine inhibits immune-cell function (62), e.g., by induction of T-cell exhaustion (63, 64) and deregulation of NK-cell activating receptors (65). In fact, increased L-kynurenine serum concentration is associated with inferior OS in DLBCL (22). Accumulating extracellular adenosine converted from exATP by CD39/CD73 (as seen in CLL) can blunt immune responses by activating adenosine receptors that signal *via* cyclic AMP and protein kinase A. T-cells respond with reduced proliferation (66, 67), NFκB activity (68), and cytokine production (67, 69, 70) as well as increased exhaustion (71). NK cells are similarly affected by adenosine (67, 72). Abundant ROS (as seen in CLL, DLBCL, and FL) regularly leads to oxidative stress in malignancies. Again, T- and NK-cells are particularly sensitive toward ROS-induced cytotoxicity (73, 74), e.g., through impairment of T-cell receptor signaling (75, 76) leading to reduced cytokine production (77). This is reflected by the negative prognostic impact of oxidative stress in DLBCL (78) and FL (79).

However, those bioactive metabolites are not only capable of direct immune cell suppression, but can also favor preferential survival and/or induction of tolerogenic cell types. Actually, regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs), and pro-tumorigenic M2-like macrophages accumulate in B-cell-derived malignancies. As a prime example, kynurenine directly promotes reprogramming toward Tregs by inducing their master transcription factor FOXP3 (80). At the same time, Tregs are more resistant than conventional (potentially tumor-directed) T-cells toward detrimental effects caused by abundant lactate (81) or ROS (82) thereby enjoying a survival benefit.

As stated above, a novel, fourth mechanistic axis has been established interconnecting metabolic activity and expression of ICPs such as PD-L1 that prevent mounting of an effective anti-tumor immunity. PD-L1 is found in B-NHL and can have a negative prognostic impact (e.g., for DLBCL) (83, 84). Consequently, ICP blockade is currently undergoing clinical evaluation. Recent studies have demonstrated that glucose uptake and glutaminolysis are required for a stable PD-L1 expression. In DLBCL, glutaminolysis contributes to STAT3 induction, which positively regulates PD-L1 (85). Glucose serves as a substrate for posttranslational protein glycosylation, while N-glycosylation of PD-L1 maintains its stability and interaction with its cognate receptor (86).

Overall, metabolic reprogramming is closely linked to immunoevasion. However, many of the here described phenomena are extrapolated from basic studies or translational research within different disease contexts. Thus, immunometabolic research in B-cell malignancies needs to be further extended to build a sound basis for novel treatment strategies.

CONCLUSION AND FUTURE PERSPECTIVES

The concept of immunometabolic regulation has emerged as an important research field. Interplay between cells does not only occur *via* signaling molecules and receptor-ligand interactions but also through metabolic communication. Tumor cells have adapted their metabolic regulatory circuits (**Figure 1**), which improves their survivability and resistance toward anti-tumor immunity and/or (immune-based) therapies (**Figure 2**). However, metabolic reprogramming can cause novel metabolic dependencies and/or vulnerabilities rendering malignant cells more susceptible toward interferences within their metabolic framework as already described for DLBCL (13, 15), FL (24, 26), MCL (31, 32) and CLL (41, 43, 50, 51).

Targeting key (dysregulated) metabolic molecules would represent one very obvious strategy for re-establishing a (for immune cells) more favorable environment without lack of nutrients or presence of detrimental catabolites. However, it needs to be taken into consideration that bioenergetic processes of malignant cells and of activated immune cells are very similar. As such, mTOR as a central hub for nutrient sensing and bioenergetic regulation in various types of B-NHLs would represent a *bona fide* target. Activated T-cells are also strongly dependent on mTOR-regulated uptake of glucose and amino acids [reviewed in (87)]. Thus, targeting mTOR would inevitably affect the T-cell's metabolic competence (and consequently anti-tumor function) as seen in preclinical models (88). Here, focusing on pathways that are not directly associated with the cell's bioenergetics and self-evidently do not overlap between malignant and immune cells is more promising. Blockade of IDO (that is not expressed in T- and/or NK-cells) for example is currently investigated in a number of malignancies (89). Notably, reducing ROS production (by histamine application) coupled with IL-2-triggered T- and NK-cell activation has led to solid clinical effects in patients with acute myeloid leukemia (90) and comparable observations were reported when combining bicarbonate that neutralizes an acidic milieu with ICP blockade in preclinical melanoma models (91).

Nowadays, adoptive transfer of genetically engineered chimeric antigen receptor (CAR) T-cells has heralded a new era in the immunotherapy of cancer in particular of B-NHLs (92). The efficacy of CAR T-cell treatment and their adequate anti-tumor effect rely on sustained metabolic activity and energy supply as well as *in vivo* persistence. In analogy to the intrinsic anti-tumor immune responses, the TME can represent a metabolic barrier for CAR T-cells as convincingly shown for IDO and anti-CD19 CAR T-cells in a preclinical lymphoma model (93). Manipulating the metabolic equipment of CAR T-cells itself to empower their function in the TME [reviewed in (94)] poses a promising approach for optimizing CAR T-cell therapy in the foreseeable future. Strategies to do so, include additional genetic manipulation that, e.g., has led to the design of ROS-resistant CAR T-cells, the expansion of CAR T-cells in presence of cytokines that promote metabolic fitness such as IL-21 (95), and their combined use with agents such as adenosine receptor antagonist that target tumor metabolism-triggered detrimental effects (96).

In summary, the multifaceted metabolic alterations in B-NHL and CLL have been the subject of intense research. However, more research is needed to better understand the complex immunometabolic interactions in order to help us to further improve the efficacy of emerging immunotherapies such as CAR T-cells or immune cell engaging antibodies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Role of Specific B-Cell Receptor Antigens in Lymphomagenesis

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The B-cell receptor (BCR) signaling pathway is a crucial pathway of B cells, both for their survival and for antigen-mediated activation, proliferation and differentiation. Its activation is also critical for the genesis of many lymphoma types. BCR-mediated lymphoma proliferation may be caused by activating BCR-pathway mutations and/or by active or tonic stimulation of the BCR. BCRs of lymphomas have frequently been described as polyreactive. In this review, the role of specific target antigens of the BCRs of lymphomas is highlighted. These antigens have been found to be restricted to specific lymphoma entities. The antigens can be of infectious origin, such as *H. pylori* in gastric MALT lymphoma or RpoC of *M. catarrhalis* in nodular lymphocyte predominant Hodgkin lymphoma, or they are autoantigens. Examples of such autoantigens are the BCR itself in chronic lymphocytic leukemia, LRPAP1 in mantle cell lymphoma, hyper-N-glycosylated SAMD14/neurabin-I in primary central nervous system lymphoma, hypo-phosphorylated ARS2 in diffuse large B-cell lymphoma, and hyper-phosphorylated SLP2, sumoylated HSP90 or saposin C in plasma cell dyscrasia. Notably, atypical posttranslational modifications are often responsible for the immunogenicity of many autoantigens. Possible therapeutic approaches evolving from these specific antigens are discussed.

Keywords: B-cell receptor, antigen, lymphoma, autoreactivity, posttransnational modification, antigens of infectious origin

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B-CELL DEVELOPMENT AND DIFFERENTIATION IN THE CONTEXT OF LYMPHOMA GENESIS AND AUTOREACTIVITY

B Cell Development and Generation of B-Cell-Receptor Diversity

B-lymphocytes are part of the adaptive immune system. Their main function is the production of antigen-specific antibodies during humoral immune responses. They also function as antigen presenting cells (APC) for T helper cells and can have regulatory tasks. In the course of immune responses, activated B cells can differentiate into memory B cells or antibody-secreting plasma cells. B cell development is initiated when hematopoietic stem cell-derived common lymphoid progenitors in the bone marrow differentiate into pro-B cells. Here, mediated by the lymphocyte-specific recombinases RAG1 and RAG2, and other DNA-modifying enzymes such as KU70/KU80

(1) and artemis (2), a V(D)J gene recombination of individual immunoglobulin (Ig) variable (V), diversity (D), and joining (J) genes is initiated (3). First, the Ig heavy chain is assembled through a D_H to J_H recombination, followed by a V_H to D_HJ_H joining. Multiple genes of each of the three types of genes are available for recombination, causing combinatorial diversity. As further diversification mechanisms, individual nucleotides can be deleted from the joining ends of the rearranging genes, or non-germline-encoded nucleotides (N nucleotides) are randomly inserted between the V_H, D_H, and J_H genes by terminal deoxynucleotidyltransferase (TdT) (4, 5). These processes of combinatorial and junctional diversity represent key mechanisms enabling a large variety of possible B cell receptor (BCR) reactivities given a relatively limited number of genes for immunoglobulins. After V_H to D_HJ_H joining, the heavy chain rearrangement is expressed as a pre-BCR with a non-rearranged surrogate light chain, and tested for functionality (this is needed, as about two thirds of rearrangements are out-of-frame and hence unproductive). If the first rearrangement is non-productive, a further attempt is made on the second heavy chain allele. Pre-B cells with a completed heavy chain rearrangement then perform Ig light chain gene rearrangements, beginning at the Igκ locus. The same diversification mechanisms as described for the heavy chain take place, with the exception that the light chains lack a D gene, so that V_L genes are directly joined to J_L genes. If the first rearranged VκJκ light-chain gene is not functional, further rearrangements can occur on the same allele, or on the second Igκ locus. If all these attempts fail, rearrangements of the Igλ locus occur. The combination of a rearranged heavy chain with a rearranged light chain (Igκ or Igλ) represents a further mechanism of combinatorial BCR diversity. After successful light chain rearrangement the differentiation stage of immature B cell is reached and the BCR is expressed as an IgM surface receptor. These cells are then selected against autoreactivity of the BCR (further discussed below), and the cells surviving this selection process exit the bone marrow and become mature, naive B cells, co-expressing the BCR as IgM and IgD molecules, mediated by differential splicing of the IgH transcripts.

Oncogenic Translocations During the V(D)J Rearrangement

The rearrangement processes of Ig heavy and light chain genes, which are accompanied by DNA double strand breaks, bear the inherent risk of causing oncogenic chromosomal translocations of protooncogenes, which bring the translocated oncogenes under control of the Ig enhancers. As these are highly active in B cells, this causes a constitutive, deregulated expression of the oncogenes. These translocations are often characteristic for certain B-cell Non-Hodgkin's lymphoma entities: In mantle cell lymphoma (MCL) the gene encoding cyclin D1 (*CCND1*) is characteristically translocated into the IgH locus (t(11;14) (q13; q32)) and in follicular lymphoma (FL) *BCL2*-IgH translocations (t (14;18)(q31;q21)) are seen in more than 90% of cases (6). Despite these translocations, a functional BCR is regularly still expressed by the respective lymphomas, strongly indicating that the cells, despite carrying these oncogenic translocations, still depend on expression of a BCR (7).

Activation of Mature B Cells and Germinal Center Reaction

If mature B cells are activated through binding of an antigen to the BCR, and if T cell help is available, a T cell-dependent humoral immune response is initiated. After an initial encounter of antigen-specific B cells and cognate T cells in the T cell area of secondary lymphoid organs (e.g. lymph nodes), antigen-activated B and T lymphocytes migrate into B cell follicles and establish germinal centers (GCs). In the dark zone of these structures, the activated B cells proliferate (8). These dark zone GC B cells also activate the process of somatic hypermutation (SHM), which introduces mutations at a very high rate (10^{-3} to 10^{-4} mutations/bp per cell division (9)) into the Ig heavy and light chain V region genes. The key enzyme for this process is activation-induced cytidine deaminase (AID) (10, 11). As the mutations are largely random, most will be disadvantageous and will result in death of the respective B cells. Only B cells expressing a BCR with improved affinity will be positively selected through interactions with follicular dendritic cells and follicular T helper cells. This interaction takes place in the light zone of the GC, where the GC B cells are mostly non-proliferating. GC B cells typically undergo multiple rounds of proliferation/mutation and selection, resulting in a stepwise improvement of BCR affinity. In the course of the GC reaction, many B cells undergo class switch recombination (CSR) to change the isotype of the Ig heavy chain from IgM and IgD to IgG, IgA, or IgE (10). Also for this process, AID is an essential enzyme. Migration of the B cells within the GC is controlled by dynamic expression of the chemokine receptors CXCR4 (highly expressed on B cells in the dark zone) and CXCR5 (highly expressed on B cells in the light zone), and gradients of their ligands CXCL12 and CXCL13, respectively (12).

The transcription factor BCL6 is the master regulator of the GC B cell gene expression program (13). BCL6 activates PAX5, BACH2, and MITF, and it inhibits the plasma cell master regulators IRF4, BLIMP1 and (indirectly) XBP1 (14). Strong BCR activation leads to a shift from BCL6 dominance to upregulation of BLIMP1 (PRDM1) (14). BLIMP1 represses transcription of BCL6 and PAX5, and induces expression of IRF4 (MUM1) and XBP1, leading to differentiation of GC B cells into plasma cells. Other positively selected GC B cells differentiate into long-lived memory B cells, but the responsible transcription factor networks are less well understood (15).

Mechanisms of Loss of Immunological Self-Tolerance

The mechanisms of BCR diversity inevitably have the side effect of generating also autoreactive BCRs (16, 17). Immature B cells with strongly autoreactive BCR are usually deleted (18), which is referred to as central tolerance. Furthermore, B cells with autoreactive BCRs can change into an anergic state (19, 20) and immature B cells with autoreactive BCRs can modify their light chain genes by new rearrangements, which is called receptor editing, and thus escape clonal deletion (21–23). Failure of the tolerance process leads to the generation of naive mature autoreactive B cells (24–26). Furthermore, peripheral self-reactive B cells receiving proliferative signals *via* MHCII/T

cell receptor (TCR) and CD40/CD40L interactions can be depleted in a Fas/FasL-dependent manner (27–29). Altered pro-inflammatory, B-cell-stimulating signals such as BAFF, IL-6 or CpG or anti-inflammatory, immunosuppressive signals such as IL-10 can influence these peripheral self-tolerance checkpoints (29–31).

The presence of certain types of HLA (32) is a crucial prerequisite for most autoimmune phenomena. In addition, there are a large number of single nucleotide polymorphisms (SNPs) or mutations in susceptibility genes associated with autoreactivity, including PTPN22, CTLA4, A20, TLR7, TLR9, MYD88, CD40/CD40L, ICOS/ICOSL or genes in the BCR signaling pathway (33–39). In addition, external factors can create an inflammatory environment, reverse the segregation of certain antigens, or activate autoreactive bystander cells. In the presence of certain HLA types, immune responses against certain infectious pathogens can lead to autoreactivity *via* molecular mimicry (40, 41).

Another mechanism of loss of self-tolerance is the occurrence of alterations in self-proteins, either by somatic mutations or by atypical secondary modifications (42). The secondary occurrence of RPC1 autoantibodies and scleroderma in patients with a precancerous disease or cancer with somatically mutated *POLR3A* gene are examples (43). Besides somatically mutated neoantigens, posttranslational modifications (PTM) can characteristically lead to antigen-specific breaks of tolerance, (44) such as modified wheat gliadin in celiac disease (45), N-terminal acetylated myelin basic protein in multiple sclerosis (46), citrullinated fibrin/vimentin in rheumatoid arthritis (47, 48), phosphorylated SR proteins in systemic lupus erythematosus (49, 50), or phosphorylated enolase in pancreatic carcinoma (51–53) (**Table 1**). Not all of these autoantibodies differentiate between modified antigens and wildtype isoforms. It is assumed that PTM-specific T cells, in contrast to non-PTM-specific T cells, escape central negative selection in the thymus (59).

Germinal Center Reaction and Lymphoma Genesis

The two processes modifying IgG genes in GC B cells – SHM and CSR – have not only very important roles for an efficient humoral immune response, but they also bear an inherent risk for mutations. SHM is not completely restricted to the IgV genes and can also target some non-Ig genes, including the proto-oncogene *BCL6*. This off-target SHM is particularly extensive in some types of lymphomas, including diffuse large B cell lymphoma (DLBCL), and is therefore termed aberrant SHM (60–62). Both SHM and CSR are mechanistically linked to DNA strand breaks, which is why both of them can also cause chromosomal translocations (63). Translocation of *BCL6* or *MYC* into the Ig loci are prototypical examples of such translocations mediated by misguided SHM or CSR (64). Notably, also the translocation events in GC B cells are mostly targeted to the non-expressed Ig alleles (as described earlier for V(D)J recombination-associated translocations), indicating that also at this stage of B cell differentiation, the occurrence of an oncogenic translocation does not inevitably prevent the selection for expression of a functional BCR by the lymphoma cells. Two further vulnerabilities of GC B cells for lymphoma genesis are the intrinsically high and fast proliferation activity of GC B cells, and the transient down-regulation of DNA damage responses. This allows SHM to occur without automatic induction of apoptosis (65). All these features together likely explain why about 90% of lymphomas are of B cell origin, mostly induced during a GC reaction.

Key signaling pathways frequently affected in lymphoma genesis are the following ones: the BCR- pathway with *CD79B* and/or *MYD88* mutations in the activated B cell (ABC) type of DLBCL (66), the latter also being typically involved in lymphoplasmocytic lymphoma (67), the canonical and the alternative NF-κB signaling pathway in a variety of different lymphomas including classical Hodgkin lymphoma (68–70), the NOTCH1 signaling pathway in chronic lymphocytic leukemia (CLL) (71) and a DLBCL subgroup with poor prognosis (72), the NOTCH2 signaling pathway in splenic MZL (73), as well as the JAK-STAT pathway, especially in classical Hodgkin lymphoma (74) and in primary mediastinal B-cell lymphoma (75). Furthermore, frequent mutations are described in genes encoding factors of the apoptosis signaling pathway (76) and in genes encoding for important molecules of immune surveillance (77–79).

Typically, the malignant lymphoma cells retain many characteristics of their origin counterparts, including their morphology, surface markers and gene expression profiles (7). For example, the differentiation between GC B cell-like (GCB) and ABC type of DLBCL is based on gene expression profiles (80) and immunophenotypical profiles (81). These original cell characteristics, which transformed cells can retain as established cell lines even after decades of cell culture, mostly also include the expression of the BCR. Subgroups of some lymphomas (e.g.

TABLE 1 | Examples of post-translationally modified B-cell receptor (BCR) antigens in diseases other than lymphoma.

Disease	Antigen	Posttranslational Modification
Rheumatoid arthritis	fibrin/vimentin	citrullination (47, 48)
Juvenile idiopathic arthritis	DEK protein	acetylation (54)
Multiple sclerosis	myelin basic protein	N-terminally acetylated (46)
	MOG	malondialdehyde (55)
SLE	SR proteins	phosphorylation (49, 50)
Celiac disease	Gliadin	deamidated (45) by transglutaminase
Goodpasture syndrome	collagen IV	sulfilimine bonds (56, 57)
IgA nephropathy	IgA	galactose-deficient IgA (58)
Pancreatic adenocarcinoma	Enolase	phosphorylation (51–53)

Burkitt lymphoma (BL), primary central nervous system lymphoma (PCNSL), DLBCL, marginal zone lymphoma (MZL), MCL, and CLL) express a functional BCR, partly despite persistent AID expression with variable persistent SHM. This indicates a certain dependence or a selection advantage by BCR expression, possibly even a permanent BCR stimulation by an antigen in subgroups of the above mentioned lymphoma. In addition to the typical translocations as well as activating mutations of proto-oncogenes and inactivating mutations of tumor suppressor genes, the involvement of the BCR in lymphoma genesis was suspected early on (7, 82). The strong upregulation of the NF-κB signaling pathway in many B-cell lymphomas could also be partly explained by BCR activation.

Two principal types of BCR signaling are being distinguished. Tonic signaling is a constitutive and presumably antigen-independent signaling that is crucial for B-cell survival. In contrast, crosslinking of the BCR by direct binding to the cognate antigen induces activation of the B cell and plays an important role in humoral responses inducing B cell proliferation, AID expression, affinity maturation and differentiation. Whereas tonic signaling mainly relies on the PI3K/AKT pathway, the NF-κB pathway plays a major role in antigen-mediated active BCR signaling.

If the concept of two types of BCR signaling is applied to lymphoma, a tonic BCR stimulation pattern plays a major role in GCB-DLBCL, mainly mediated by a Y188 mutation within *CD79A*, and in a relevant subgroup of BL accompanied by mutations in *TCF3* and *ID3*, and activation of the PI3K pathway (83, 84). Active BCR signaling in lymphomas shares similarities with BCR stimulation by exogenous cognate antigens and plays an important role in ABC-DLBCL, where it is called chronic active BCR signaling (80). In ABC-DLBCL, mutations of components of the BCR pathway, including members of the CARD11/BCL10/MALT1 (CBM) complex (85, 86), and of *MYD88* (87) were frequently found. In ABC-DLBCL (88) and in CLL (89) constitutive BCR clustering is observed as it is seen in normal B cells after BCR binding of an antigen. For a particular genotypic subgroup of ABC-DLBCL with *MYD88* L265P and *CD79B* mutations, an interaction of the BCR with MYD88 was reported, mediated by TLR9 (90), which is located in the endosome and normally senses CpG DNA. This was named My-T-BCR supercomplex (91).

SPECIFIC BCR ANTIGENS IN LYMPHOPROLIFERATIVE DISEASES

When considering BCR stimulation by antigens in lymphomas, several questions arise: is the BCR polyreactive or specific for one antigen? Are there random antigens for each individual patient with lymphoma or is there an over-representation of certain antigens? If so, are these over-representations entity-specific? And what are the possible underlying causes of the misdirected immune responses? We will highlight antigens of infectious origin as possible triggers of (mainly indolent) lymphomas. Subsequently, the involvement of autoantigens and underlying mechanisms of autoreactivity will be discussed.

The Role of Infectious Agents in B-Cell Neoplasia

The suspected relevant BCR target antigens could be antigens of persistent or recurrent infections. The most prominent example for this is the involvement of *Helicobacter pylori* in the pathogenesis of MALT lymphoma (a subtype of MZL) of the stomach (92–95). However the BCR were found to resemble rheumatoid factor (96, 97) or in other reports were polyreactive to autoantigens including IgG and *Helicobacter* sonicate (95). This indicates a mainly indirect role of bacterial infection for triggering lymphoma growth, presumably mediated to *H. pylori*-stimulated T helper cells. Regarding the gastrointestinal tract, *Campylobacter jejuni* was also associated with the genesis of lymphoproliferative diseases in the small intestine (98). It was furthermore speculated that *Campylobacter jejuni* may also contribute to duodenal FL, as it is typically restricted to this anatomic site. Considering the usually favorable outcome, this is often managed with a watch & wait strategy. Beside gastric MALT lymphoma other entities of MZL are triggered by chronic infections. For MALT lymphoma of the ocular adnexae, a strong association with *Chlamydia psittaci* was reported in specific regions (99), and for primary cutaneous MZL, *Borrelia spec.* infections were reported to be potentially causative. Splenic MZL shows an over-representation of the IGHV1-2*04 gene, has recurrent mutations in *NOTCH2*, and in a fraction of cases its development may be triggered by hepatitis C virus (HCV) (73, 100, 101).

Successful therapeutic concepts for infection caused MZL have been demonstrated by eradication of *H. pylori* with proton pump inhibitors with or without bismuth, in combination with clarithromycin and metronidazole or amoxicillin for gastric MALT lymphomas. These drugs have been incorporated into the current therapeutic standard of ESMO/EHA for gastric MALT lymphomas regardless of stage (94, 102, 103). Furthermore, the efficacy of antibiotic eradication of *Chlamydia psittaci* for ocular adnexal MALT lymphomas by doxycycline or clarithromycine has been demonstrated (104, 105). Moreover, preliminary reports about successful treatment of *Borrelia*-spec. associated primary cutaneous MZL were published (106). Similarly, the eradication of HCV and thus elimination of viral antigens as triggers of lymphoma BCRs can lead to regression of HCV-associated splenic MZL, and this is currently recommended as first line therapy in the current ESMO/EHA guidelines (101, 103). In summary, the examples presented here highlight the potential of lymphoma regression upon anti-viral or anti-bacterial treatment. This is a strong argument for a causative role of the respective infections for sustained triggering of lymphoma growth.

Using BCR expression cloning and subsequent antigen screenings, we could extend this list of infection-triggered lymphomas. We identified a specific antigen of a common bacteria as BCR antigen of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), a rare type of B-cell lymphoma, which frequently manifests at cervical lymph nodes and with regular expression of functional BCRs. This target antigen was DNA-directed RNA polymerase beta' (RpoC) of the Gram-

negative cocci *Moraxella catarrhalis (*107). *Moraxella catarrhalis* is known to co-express MID/hag, a superantigen activating IgD^+ B cells by binding to the Fc domain of IgD. RpoC and MID/hag additively activate the BCR and the NF-κB pathways and induce proliferation of lymphocyte predominant (LP) tumor cells of NLPHL with RpoC-specific BCRs. In particular, RpoC was a frequent antigen of BCRs of IgD^+ LP cells, whose IgHV genes had extraordinarily long complementary determining region 3 (CDR3s). Moreover, patients showed a predominance of HLA-DRB1*04/07, suggesting existence of a permissive MHC-II haplotype (107, 108). Interestingly, this haplotype is also known from autoimmunity for its association with rheumatoid arthritis as shared epitope (109). Patients had high-titer, and light-chain-restricted anti-RpoC serum-antibodies, further supporting infection of the patients by *M. catarrhalis* and mislead immune responses against this bacteria (**Figure 1**). These results suggest to conduct clinical trials examining a potential effect of antibiotic therapy for relapsed or refractory IgD^+ NLPHL. Moreover, if no B-cell depletion was therapeutically induced in the patients, active vaccination might make sense after local therapy. For this potential strategy the target antigens, i.e. RpoC and MID/Hag of the lymphoma BCRs must not be present in the vaccine, to avoid stimulation of remaining LP cells.

Regarding aggressive lymphomas like BL, potential infectious triggers of the BCR are discussed for many years. Expression of sIg is a hallmark of all types of BL, despite *MYC*-involving translocations with one Ig gene allele as t(8;14), t(2;8) or t(8;22). However, for BL no direct BCR stimulation by an antigen has been suspected, but actually tonic BCR pathway activation amplified by mutations in *ID3* and *TCF3* genes has been reported in BL (84). CRISPR-screening identified CD79B-dependency in the BL Ramos cell line (110). For endemic BL a frequently preceding coincidence of malaria and latent EBV infection was observed, which both likely contribute to BL pathogenesis (111, 112). However, in this case EBV is not a BCR stimulating antigen, but it infects B cells and can contribute to their transformation through expression of EBV-encoded genes in latently infected B cells. For BL, in general EBV latency phase I is observed with expression of just EBNA1, so that the pathogenetic role of EBV in BL is still not fully understood (113). In sporadic BL the frequent extranodal manifestation in the appendix vermiformic and ileocoeliac junction area raised speculations about a possible infectious trigger, but a causative infectious agent has not yet been identified.

The role of infections can go far beyond direct BCR stimulation and influence lymphoma genesis in other ways. The BCR often does not seem to play a significant role in EBV-associated B-cell lymphomas, e.g. in classical Hodgkin lymphoma, functional BCR are often lost. In classical Hodgkin lymphoma, typically EBV latency II is present with expression of EBNA1, and LMP1 and LMP2a. In post-transplant lymphoproliferative disease (PTLD), EBV latency III is observed with expression of EBNA1, -2A, -3A, -3B, -3C, and LMP1 and LMP2a (113). LMP2a contains an ITAM mimicking motive (potentially) relevant for proximal BCR pathway activation, and LMP1 is a viral oncogenic mimic of CD40, recruiting among others the signaling factors TRAF2 and TRAF3, but in contrast to CD40 not TRAF3 (114–120). Besides EBV, other viruses can play important roles in lymphoma genesis by transformation of lymphocytes by latent viral infections, such as HTLV1 in adult T-cell leukemia (121) and HHV8 (122) in primary effusion lymphoma. Another important mechanism is immunosuppression by HIV attenuating control of EBV- or HHV8-infected B cells (123).

The Role of Autoantigens in Lymphoproliferative Diseases

Autoantigens in Indolent Lymphoma

Endogenous immunogenic proteins could contribute to permanent growth advantages of lymphoma cells with the appropriate BCR autoreactivity by their inexhaustible supply. MZL is a $CD5^-$ and $CD10^-$ indolent lymphoma often accompanied by a paraprotein. Extranodal MZL is frequently associated with infectious triggers as described above. Beside recurrent mutations in *MLL2*, *NOTCH2*, *PTPRD*, and *KLF2*, nodal MZL correlates (shows) over-represented usage of *IGHV4-34* in around 30% of cases (124), which is known to be linked with autoreactivity. This autoreactivity is also demonstrated by MZL emerging from Sjögren's disease (125).

FL is a $CD5^-$ and $CD10^+$ indolent lymphoma characterized by the presence of t(14;18)(q32;q21) leading to overexpression of BCL2. Regarding the BCR pathway Freda Stevenson et al. described a gain of N-glycosylation sites within the IgV genes by SHM leading to chronic activation of the BCR pathway by binding of N-hyperglycosylated BCRs to lectins in the lymphoma microenvironment (126). Subtypes of FL with a distinct manifestation and clinical course may have a different underlying biology. Here, pediatric FL, with regular cervical nodal manifestation and without *BCL2* translocation, and duodenal FL have to be mentioned. Both characteristically do not spread beyond initial local manifestations.

CLL is the most common hematological cancer in adults in the Western world and clinically shows considerable heterogeneity (127). It is characterized by a population of ≥ 5,000 clonal B cells/μl in the peripheral blood. Nodal, extranodal or splenic manifestation with < 5,000 clonal B cells/μl is called small lymphocytic lymphoma (SLL). The monoclonal tumor cells express CD5, CD23, CD200, and low levels of sIg, and lack CD10 expression. By analysis of IGHV genes of a very large number of CLL cases, it became clear that unrelated CLL patients can have highly similar if not identical BCRs (128). This phenomenon of groups of CLL with highly similar IGHV and IGHL gene rearrangements is termed BCR stereotypy. It is considered the strongest evidence that antigen selection plays an important role in the pathobiology of CLL. CLL patients whose disease belong to a specific stereotypic subset often show similar clinical and biological characteristics, including disease progression. Interestingly, for several of the stereotypic groups, autoantigen specificity of the BCR has been demonstrated (129–131). There is evidence to suggest that these BCR enable specific recognition of an (auto)antigen, which leads to increased proliferation of the malignant B-cell clone and thus plays a crucial role in the pathophysiology of CLL (132). Indeed, inhibitors of BCR signaling pathway are of great importance in

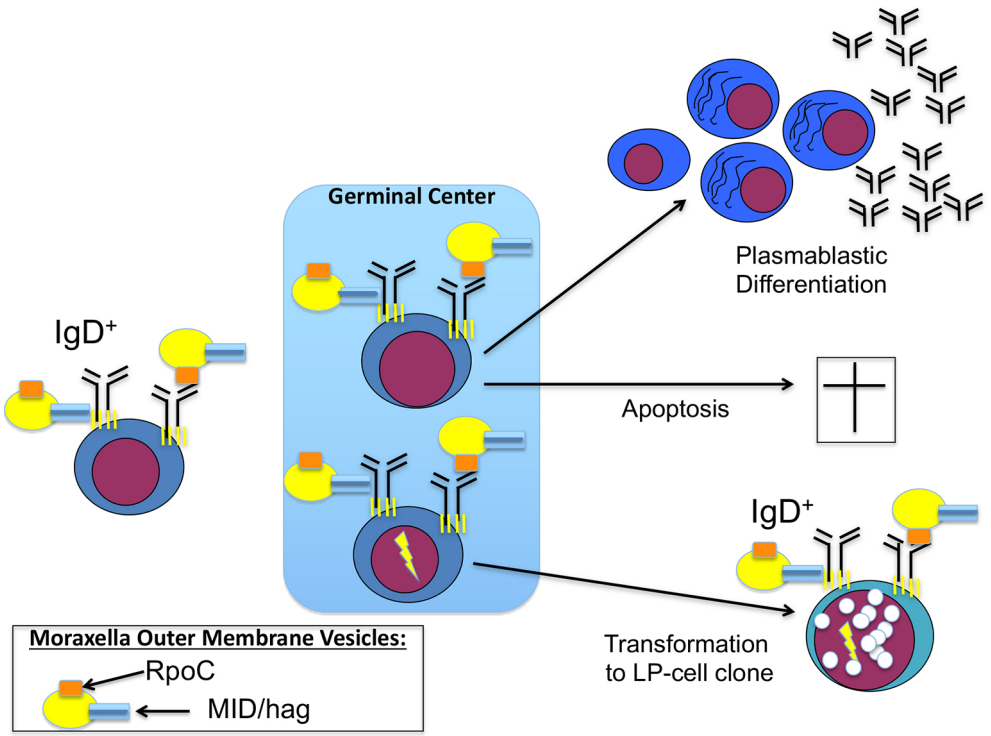

FIGURE 1 | Contribution of *Moraxella catarrhalis* to IgD$^+$ nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) pathogenesis: costimulation of IgD-positive B cells by *Moraxella catarrhalis* RNA polymerase beta' (RpoC) *via* the Fab fragment and MID/hag *via* the Fc fragment of the B-cell receptor (BCR). Naive IgD$^+$ B cells with a BCR specific for RpoC encounter *M. catarrhalis* outer membrane vesicles. Binding of RpoC to the Fab and of MID/hag to the Fc of membrane IgD induces activation of RpoC-specific IgD$^+$ B cells, which is supported by CD4$^+$ T cells particularly in patients with an HLA-DRB1*04 haplotype. The persistent/recurrent presence of *M. catarrhalis* presumably induces a germinal center (GC) reaction resulting in differentiation of memory B cells and plasmablasts and production of class-switched anti-RpoC serum antibodies and apoptosis of some GC B cells due to disadvantageous mutations. Subsequently, transformation into lymphocyte predominant (LP) cells may occur, accompanied by mutations in proto-oncogenes and tumor suppressor genes, and by chromosomal translocations (e.g. *BCL6* translocations).

clinical practice for CLL patients (133). OxLDL, Fam32a, SMCHD1, MAZ, vimentin, myosin chains, and pUL32 have been identified as (auto)antigens that can specifically bind to CLL BCRs (134–137). The mutation status of the BCR of CLL clones represents a strong prognostic marker. CLL with no or few somatic BCR mutations within their rearranged IGHV genes ("unmutated" CLL, U-CLL) experience a significantly more aggressive disease than patients with >2% mutation load ("mutated" CLL, M-CLL) (138). However, this subdivision might be more complex regarding the clinical heterogeneity of CLL. If the findings of the subsets are combined with the findings of U-CLL vs. M-CLL, there are three categories: CLLs with stereotypic BCRs (about 1/3 of the cases and mostly U-CLL), CLLs with specific IGHV genes (U- and M-CLL) and those with heterogeneous and no particular IG features (mainly M-CLL). U-CLL have polyreactive BCRs specific for autologous neoantigens (e.g. myosin chains, vimentin, oxLDL, PC9, Fam32A, SMCHD1, and MAZ) (134), while the BCRs of some M-CLL react with foreign antigens, such as yeast derived glucans (139), or autoantigens as Fc parts of rheumatoid factors (140).

There are also indications that CLL cells show antigen-independent, cell-autonomous signaling (141, 142); a behavior that has not been shown in normal B cells and other B-cell malignancies. This cell-autonomous signaling is based on the recognition and self-association of the BCR of CLL cells to itself, the ultimate autoantigen. Various CLL-derived BCR bind to specific, different epitopes of themselves and thus initiate intracellular signal transduction. The avidity of BCR self-recognition seems to have an influence on the course and severity of the disease. In summary, for CLL different models and ideas exist for the significance of the BCR in CLL. It is likely that all models of BCR reactivity have their justification, whereby, depending on the situation, one or the other mechanism may be more important. All models emphasize the importance of BCR antigen recognition in conjunction with BCR auto-stimulation in addition to genetic lesions in the pathogenesis of CLL. However, it should not be forgotten that additional effects with an influence on the pathogenesis have to be considered, such as the existence of specific effector functions for IgM and IgD. Nevertheless, the use of inhibitors of the BCR signaling pathway (e.g. BTK inhibitors) and thus the proliferation of CLL cells has significantly improved the therapeutic options and led to permanent remissions, even in high-risk and refractory CLL patients.

Hairy cell leukemia (HCL) is a rare indolent lymphoma typically affecting middle-aged to old males. Beside its name-giving feature of protruding villi on the surface of the leukemic cells, it has an immunophenotype characterized by expression of CD103, CD11c, CD22, and CD123, and lack of CD5 and CD10 expression. Expression of CD25 distinguishes a classical and a variant form of HCL (143). Classic HCL always carry *BRAF* mutations (144). HCL cells express a BCR and the IGHV genes IGHV3-21, IGHV3-30, and IGHV3-33 are overrepresented. HCL is frequently associated with Igλ light chains (145, 146). Variant HCL lack *BRAF* mutations, and often use the IGHV4-34 gene.

MCL is a rare B-cell neoplasia, which accounts for about 6-8% of all Non-Hodgkin lymphoma (127). Male, elderly patients are over-represented, and extra-lymphatic manifestations are common (147, 148). MCL cells typically show a $CD5^+$, $CD23^-$, $CD200^-$ immunophenotype with strong expression of CyclinD1, due to translocation of the *CCND1* gene into the IgH locus (t(11;14)(q13;q32)) (149). Regarding its IGHV mutational and DNA methylation status, MCL can be distinguished into pre- and post-GC-derived cases (150). Over-representation of specific IGHV gene groups and stereotypic rearrangements has been described similar to CLL, but with a lower frequency (151–153). In accordance with this, a strong BCR and NF-κB pathway activation in MCL was reported (154), and antigen-induced activation was stronger compared to other B-cell neoplasia (155). Pharmacological targeting of the BCR pathway by inhibition of PI3K or BTK is established for relapsed/refractory MCL (156–159). Recently, we identified human LDL receptor-related protein associated-protein 1 (LRPAP1) as frequent autoantigen of recombinant BCRs in MCL cases (8/21) and two of seven MCL cell lines (MAVER1 and Z138) (160). LRPAP1 consists of 357 amino acids and has a molecular weight of 39 kDa. LRPAP1 functions as antagonist and chaperon of the family of LDL-receptors and it takes part in Megalin/Cubilin endocytosis (161, 162). Immunization of rats with LRPAP1 results in Heymann-Nephritis (163, 164).

Autoantigens in Aggressive Lymphomas

Regarding autoantigenic targets of BCRs of aggressive lymphomas, several examples exist. DLBCL is the most common aggressive B-cell Non-Hodgkin lymphoma. According to the WHO classification, DLBCL can be classified based on gene expression profiling into ABC–like type, GCB–like type and primary mediastinal B-cell lymphoma (80, 165). In contrast to relatively well studied genetic or epigenetic pathway alterations, little is known about specific external stimuli of distinct subgroups of DLBCL (166, 167). In particular, DLBCL of the ABC-type or the recently specified MCD-type or cluster 5 harbor recurrent mutations in *MYD88* and *CD79B* with dependency on constitutive BCR signaling (72, 88, 168, 169). For systemic DLBCL a cis and trans stimulation of the BCR by a so far non-characterized autoantigen was reported for the HBL1 cell line. Moreover, an anti-idiotype reactivity of the BCR of the TMD8 cell line against an epitope within its own FR2 ($V^{37}R^{38}$) was described, and for the U2932 and OCI-LY10 cell lines BCR reactivity against apoptotic cell debris was reported (169–172). Recently, Arsenite resistance protein 2 (ARS2) was identified as the BCR target of ABC-DLBCL. Compared to controls, ARS2 was hypo-phosphorylated exclusively in cases and cell lines with ARS2-specific BCRs (**Figure 2** and **Table 2**). In a validation cohort, hypo-phosphorylated ARS2 was found in 8/31 ABC-DLBCL, but only 1/20 GBC-DLBCL. Incubation with ARS2 induced BCR-pathway activation and increased proliferation, while an ARS2/ETA' toxin conjugate induced killing of cell lines with ARS2-reactive BCRs.

PCNSL represents a specific extranodal subtype of DLBCL with molecular similarities to systemic DLBCL of MCD or C5 type with frequent mutations in *MYD88* and *CD79 (*195, 202). PCNSL show strong over-representation of IGVH4-34, and poly-reactivity against a plethora of antigens was reported (196, 203). In addition, sterile a-motif domain containing protein 14 (SAMD14) and neural tissue-specific F-actin binding protein I (neurabin-I) with a homologous SAM domain were identified as specific auto-antigenic targets of recombinant BCRs of PCNSL and SAMD14/neurabin-I specific autoantibodies were detected in sera and cerebrospinal fluid of patients. In the respective cases, SAMD14 and neurabin-I were atypically hyper-N-glycosylated (SAMD14 at ASN339 and neurabin-I at ASN1277), explaining their auto-immunogenicity (**Figure 2**) (173). Primary intraocular lymphoma (PIOL) is biologically closely related to PCNSL and can progress after a various period of time into PCNSL (198), but it is not clear whether SAMD14/neurabin-I are altered as well in this subgroup, or if a different antigenic trigger exists.

Although tonic BCR activation is characteristic for BL (84), preliminary results suggest the involvement of post-translationally modified specific autoantigens that contribute to pathogenesis in at least a subgroup of sporadic EBV-negative BL (191).

Autoantigens in Plasma Cell Dyscrasia

Multiple myeloma (MM) accounts for 1% of all malignancies, and for over 10% of hematological malignancies. The disease is characterized by neoplastic proliferation of a single plasma cell clone producing a large amount of a monoclonal antibody termed paraprotein, M-protein or M-component (204). Malignant gammopathies are often preceded by monoclonal gammopathy of undetermined significance (MGUS), a benign disorder with a strikingly elevated monoclonal Ig level in individuals lacking evidence of MM or other lymphoproliferative malignancies. Long-term follow-up of patients with MGUS reveals a 1% to 3% annual risk of developing MM or, to a lesser extent, other lymphoproliferative malignancies (204). In MGUS and plasma cell dyscrasia hyperphosphorylated SLP2 and sumoylated HSP90 were found to be the targets of paraproteins (171, 175) both in MM and lymphoplasmocytic lymphoma (LPL) (**Tables 2** and **3**). These "paratargets" with their atypical PTMs were found with different frequencies in different ethnics (205). Of interest, PTMs for both antigens had an autosomal dominant pattern of inheritance (**Figure 2**), and pedigrees with family members as carriers and affected with MGUS or plasma cell dyscrasia were

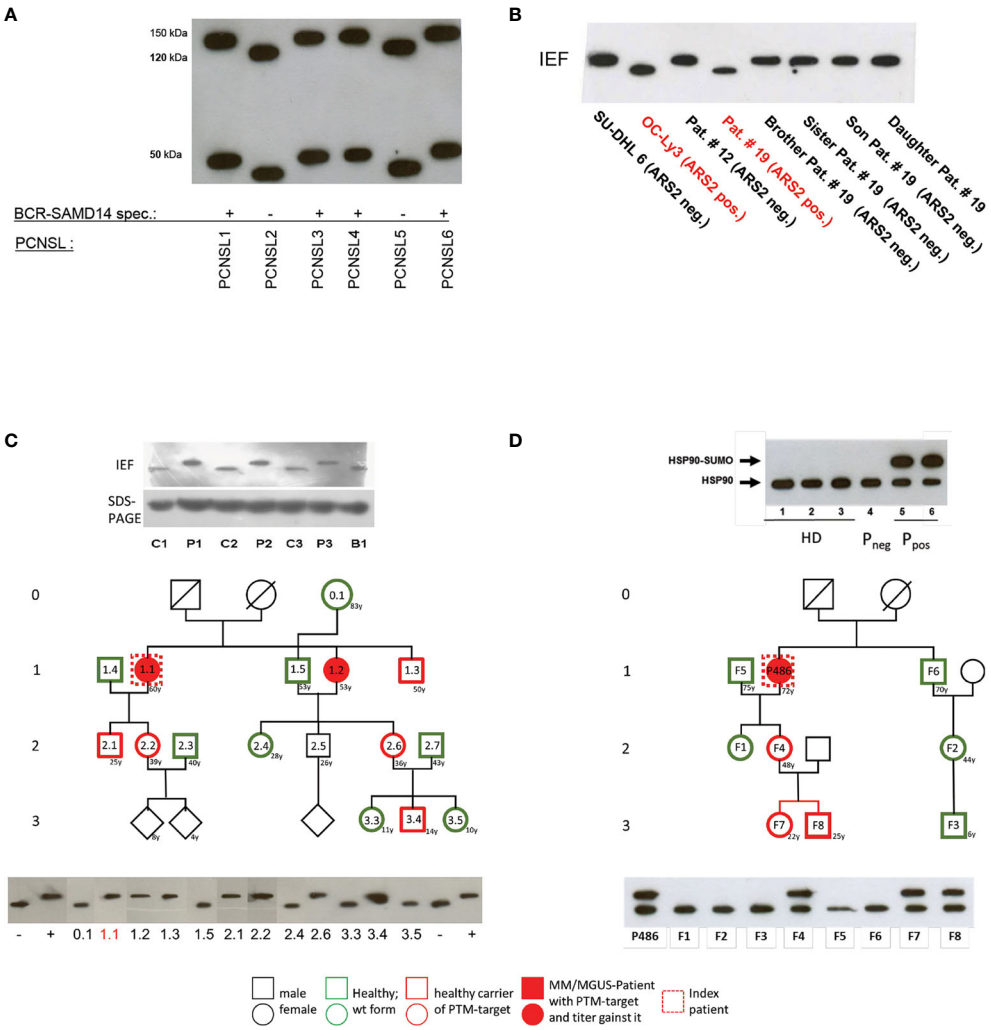

FIGURE 2 | Examples of posttranslational modification of lymphoma B-cell receptor (BCR) target antigens: **(A)** Representative Western blot of hyper-N-glycosylated Neurabin-I and SAMD14 in patients with PCNSL first reported by Thurner et al (173). Patients with primary central nervous system lymphoma (PCNSL) and SAMD14/Neurabin-I reactive lymphoma BCRs had exclusively hyper-N-glycosylated isoforms of both antigens. **(B)** Representative isoelectric focusing (IEF) of hypophosphorylated Arsenite resistance protein 2 (ARS2) in diffuse large B cell lymphoma (DLBCL) first reported by Thurner et al (174). DLBCL cell lines and peripheral blood lysates of DLBCL of patients and family members. ARS2 was found to be hypo-phosphorylated in a cell line and a patient, but this phenotype of an atypical posttranslational modifications (PTM) was not inherited in a Mendelian manner. **(C)** Representative analysis of hyperphosphorylated SLP2 (paratarg-7), which was first reported by Preuss et al (171). Hyperphosphorylated SLP2 was detected by IEF and not by SDS-PAGE. The hyperphosphorylation introduces an additional phosphate group in the molecule leading to a different isoelectric point of the protein; the increase in molecular weight is too small to be detected by SDS PAGE. Shown are immunoblots incubated with anti-human-STOML2 (paratarg-7). P1-3: MM/monoclonal gammopathy of undetermined significance (MGUS) patients with immunoreactivity against paratarg-7; C1-3: MM/MGUS patients without SLP2 (paratarg-7) immunoreactivity; B1: healthy blood donor. Inheritance: SLP2-phosphorylation state in patient families (example). The family of a relevant patient was analyzed for its SLP2 phosphorylation state by IEF. Carriers of hyperphosphorylated SLP2 (patient and persons of risk) were indicated in red. **(D)** Representative SDS PAGE of sumoylated HSP90 first reported by Preuss et al (175). Sumoylation of HSP90 does not change the isoelectric point of the molecule but leads to an increase in molecular weight which is detected by SDS PAGE. HD: healthy donor; P_{neg}: MM/MGUS patient without immunoreactivity against HSP90-SUMO; P_{pos}: MM/MGUS patients with immunoreactivity against HSP90-SUMO.Inheritance: HSP90 sumoylation state in patient family (example). The family of a relevant patient was analyzed for HSP90-SUMO by SDS PAGE. Carriers of HSP90-SUMO (patient and persons of risk) are indicated in red.

described for both hyperphosphorylated SLP2 and sumoylated HSP90 (175, 179). Interestingly, SLP2-reactive paraproteins do not differentiate between the normally phosphorylated SLP2 and the hyperphosphorylated SLP2 isoform in contrast to HSP90-reactive paraprotein, which is specific for the sumoylated isoform.

The question as to why the respective paraprotein antigen is present in post-translationally modified form in this group of people and to what extent this influences the development and progress of the disease remains unsolved. It is remarkable that these post-translationally modified antigens were detected

TABLE 2 | Post-translationally modified B-Cell Receptor (BCR) antigens in lymphoma.

Disease	Antigen	Posttranslational Modification
CLL	LDL	oxidization (176)
PCNSL	SAMD14/neurabin-I	N-glycosylation (173)
DLBCL	ARS2	Hypophosphorylation
BL	HSP40, Bystin	sumoylation and acetylation
LPL/MM	SLP2, ATG	phosphorylation (177–179)
	HSP90	sumoylation (175)
	LGL1, sapC	deficiency glucocerebrosidase (180)

almost exclusively in MM/MGUS patients and their blood relatives as well as in approx. 1-2% of the healthy population; in all other examined subjects (patients and healthy persons) the antigen is present in wild-type form, i.e. unchanged and does not induce an immune response. Nair et al. had different findings and described glycosphingosine as a frequent antigenic target structure of paraproteins in sporadic MGUS and MM as well as in monoclonal gammopathies associated with Gaucher disease (180, 206). Data from our laboratory rather suggest post-translationally modified saposin C as a paraprotein target structure in Gaucher-associated MM/MGUS (207).

Role of T-Helper Cell Co-Stimulation in Lymphoproliferative B-Cell-Diseases

Sequence and structure of the BCR antigens of malignant plasma cells found to date, whether post-translationally modified or not, indicated the need for the involvement of T-helper cells for stimulation at the beginning and during the course of pathogenesis. Furthermore, several studies provided evidence for a causal relationship between MGUS/MM and chronic antigenic stimulation (208). In addition, when SLP2 is used as a model antigen in MM, the patient's paraprotein binds to both the wild type SLP2 and the actually immunogenic post-translationally hyper-phosphorylated variant of SLP2. Thus, SLP2-specific B cells cannot be the initiators of the postulated chronic stimulation or pathogenesis.

In vitro stimulation of $CD4^+$ T-helper cells of MGUS/MM patients with a paratarg-7-specific paraprotein induced distinct paratarg-7-specific responses: 65% of these patients had a paratarg-7-specific TH1 response. 89% of these TH1 cells specifically recognized the modified hyperphosphorylated SLP2. 42% of the stimulated patients also had modification-specific TH2 cells (**Figures 3A, B**). Hence, with the T-helper cells the contribution of the adaptive immune system was found, which specifically recognize the post-translational modification and thus are at the beginning of chronic stimulation. Further characterization of the hyperphosphorylated SLP2-specific T-helper cells showed that there are (at least) six HLA-DR subtypes, named "permissive", that can present phosphorylated peptide epitopes to the T helper cells for stimulation. Compared to a healthy reference population, patients with SLP2-specific MM express these six permissive DR subtypes significantly more frequently. Thus, expression of a hyperphosphorylated-SLP2-permissive DR subtype is, besides posttranslational modifications, the second important prerequisite for the development of this disease (209).

The interaction of these modification-specific T-helper cells from patients with non-modification-specific paraprotein and their corresponding B cells is a new type of epitope spreading. In contrast to classical epitope spreading, which extends horizontally across the amino acid sequence of an antigen, this is a vertically spread modification of the same amino acid.

Physiologically, however, both phenomena are based on the same fact that the epitope of an antigen that is specifically bound by the BCR of a B cell does not have to be the same epitope that the B cell presents to the T-helper cells in its MHC-II molecules after antigen processing. When a T-helper cell finds its antigenic epitope in the appropriate MHC-II context on the B cell, it provides the help necessary for the maturation of this B cell. Applied to the situation with SLP2, this means that even those SLP2-specific B cells whose BCR does not differentiate between the modified version of this antigen phosphorylated on serine17 and the non-phosphorylated wild-type, can be stimulated by modification-specific T-helper cells (**Figures 3C, D**).

Since all posttranslational paratarg modifications found to date (including SLP2) are always consistently expressed in all cells of the organism, the B cells of the respective patients with a paratarg-specific BCR as well as all other antigen-presenting cells (e.g. macrophages or DC) can present only the modified variant to their T-helper cells. This can be seen by the fact that, except in the TH1 response of patient 8, the induced SLP2-specific T-cell responses were significantly modification-specific after *in vitro* stimulation of the T-helper cells (**Figures 3A, B**).

B-Cell Lymphoma Without a Role of BCR Antigens

For some other lymphoma entities, stimulation of the BCR by antigens is likely not important. These are for example classical Hodgkin lymphoma, whose malignant Reed-Sternberg und Hodgkin cells have lost their B-cell-phenotype including functional BCR genes (187), PTLD with crippled BCR genes (210), or primary mediastinal B cell lymphoma (211, 212), which usually does not express sIg either (**Figure 4**).

Suspected Role of Specific Antigens in T Cell Neoplasia

The search for TCR antigens is much more complicated as it requires screening of peptides presented on correct HLAs. This is probably the main reason why no TCR target antigens of PTCL have been found. For certain peripheral T cell lymphomas (PTCL) there is evidence for a potential role of antigen stimulation in their pathogenesis. One example is angioimmunoblastic T cell lymphoma, in which clonal B cell populations and paraproteins are often also found. It is unclear if the paraproteins in AITL might be a simple epiphenomenon of accompanying EBV^+ B-cell-clones, or if they target the same antigen as the TCRs of the T follicular helper cells of origin of AITL. Another example is enteropathy-associated T cell lymphoma, which is rare and typically occurs after a long

TABLE 3 | B cell lymphoma and B-cell receptor (BCR) antigens.

B-cell neoplasia	Expression of sIg	Indications for chronic BCR stimulation by an antigen or alternative BCR pathway activation
CLL	Yes, dim	• subsets with stereotypic CDR3 (181) • specific autoantigens for individual subsets (129, 134, 176, 182) • specific microbial antigens (139) • concept of autonomous, antigen-independent BCR signaling mediated by anti-framework region reactivity (142) • clinical effectivity of BCR pathway inhibition (133)
MCL	Yes	• subgroup with stereotypic CDR3s (152) • subgroup with BCR-reactivity and autoantibodies against LRPAP1 (183) • reactivity against protein A of *Staphylococcus aureus* (184) • clinical effectivity of BCR pathway inhibition (156, 159)
FL	Yes	• gains of N-glycosylation sites in BCR yield in binding to lectins (126) • pediatric FL: cervical manifestation, speculation about infectious trigger • duodenal FL: speculations about infectious trigger
HCL	Yes	• classic HCL hints for affinity maturation (145, 185) • variant HCL regularly *IGHV4-34*
MZL	Yes	• splenic MZL: strong association with HCV (100, 101) • MALT-lymphoma of the stomach: strong association with *H. pylori* (94, 95) • MALT-lymphoma of ocular adnexes: reported association with *Chlamydia psittaci* (99) • MALT-lymphoma of salivary glands after Sjögren's syndrome: autoreactive BCR (125) • effectivity of BCR-pathway inhibition (186)
cHL	No	• destructive IgV gene mutations in 25% of cases (187, 188) • *ITAM-signal of EBV-encoded LMP2a mimicking activated BCR (117)
NLPHL	Yes	• reported predominance of Igκ-light chains (189) • IgD^+ subgroup with cervical manifestation (190) • *Moraxella catarrhalis* RpoC as antigen of IgD^+ LP-cells with extraordinary long CDR3s (107) • However, clinical trials with BTK-inhibition in r/r NLPHL failed
BL	Yes	• concept of tonic BCR activation by mutation in *ID3* and *TCF3 (*83) • suspected stimulation by *Plasmodium falciparum* of EBV-infected centroblasts in endemic BL (111, 112) • reports of modified autoantigens in sporadic BL (191)
DLBCL	in subgroups Yes	ABC-type • activating mutations in *CD79B* und *MYD88* (66, 87) of MCD type, cluster 5 or ABC-type • reported autoreactivity of OCI-Ly10, U2932 lines (169), reactivity against FR2 of TDM8 line, cis and trans stimulation by an autoantigen for HBL1, reactivity of CDR3 of TDM8 against FR2 (V (37)R (38)) of TDM8 (169) • ARS2 identified as frequent target antigen of ABC-type DLBCL. ARS2 hypophosphorylated in these cases. • effectivity of BCR-pathway inhibitors (192, 193) PCNSL • overrepresentation of auto-reactivity associated *IGHV4-34 (*194) • activating mutations in *CD79B* und *MYD88 (*195) • reported poly-reactivity of BCR (196) • SAMD14/neurabin-I identified as target of BCRs SAMD14/neurabin-I hyper-N-glycosylated in these patients (173) • effectivity of BCR-pathway inhibitors (197, 197) • PIOL shares biologic characteristics with and frequently progresses to PCNSL and shares overrepresentation of IGHV4-34 (198) PTL • frequently shares activating mutations in *CD79B* und *MYD88*, with other aggressive lymphomas of immunologically privileged sites
PMBCL	No	• probably independent of BCR (199)
LPL	Yes	• clinical effectivity of BCR pathway inhibition (200, 201) • post-translationally modified SLP2 and HSP90 as specific antigens (paratargets) of IgM paraproteins (171, 175, 180)
MM	only secreting, no sIg	• posttranslationally modified SLP2, HSP90, sapC as specific antigens (paratargets) of paraproteins (171, 175, 180)

period of celiac disease. Breast implant-associated anaplastic T cell lymphoma usually shows a significantly more favorable course than ALK-negative ALCL and the role of antigen stimulation is partly shown by remission after explanation of the breast implants alone. However, a TCR-reactivity against components of breast implants has not been shown.

LYMPHOMA BCR AND THERAPEUTIC IMPLICATIONS

Inhibition of the BCR Pathway

In the treatment of B-cell lymphomas and leukemias and increasingly in the treatment of autoimmune diseases, the

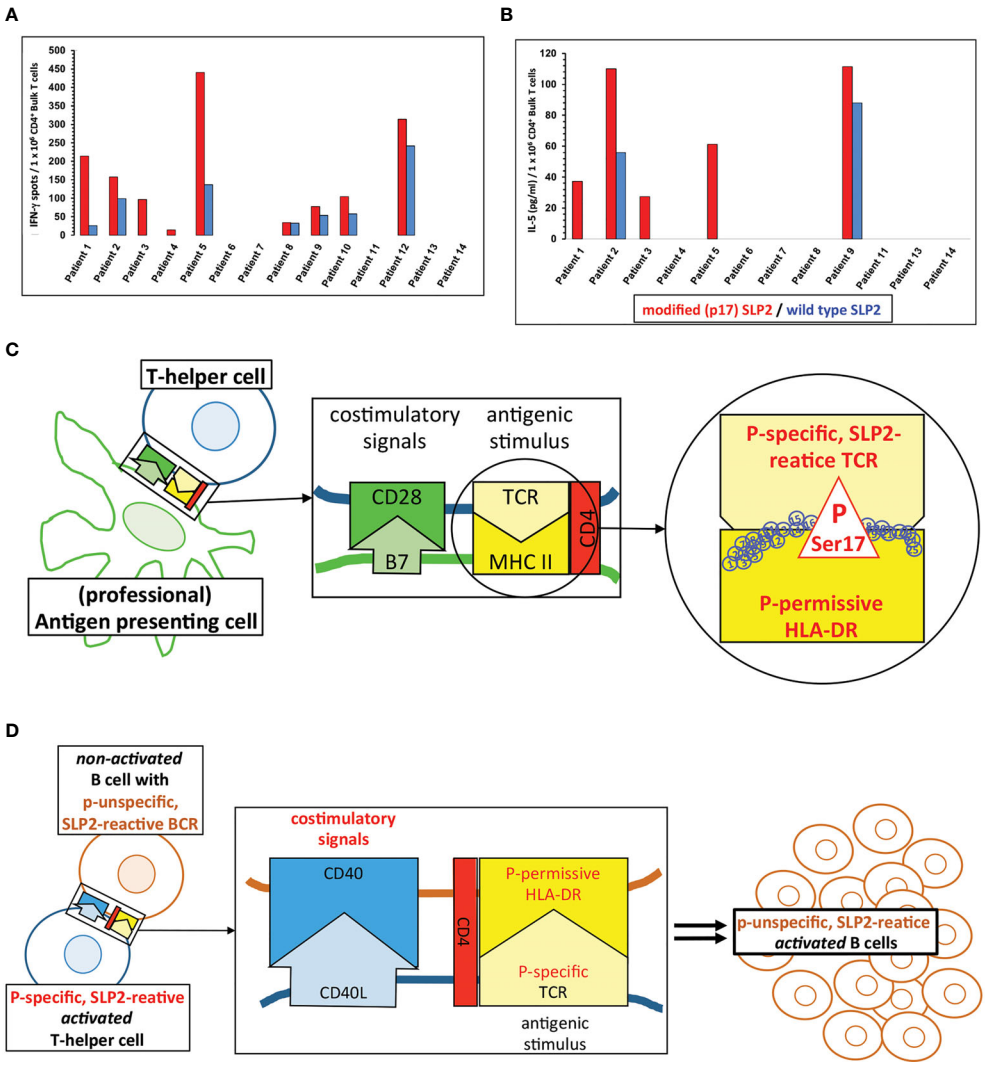

FIGURE 3 | Paratarg-7-specific T-helper cells in myeloma/monoclonal gammopathy of undetermined significance (MGUS) patients with a SLP2-specific paraprotein as a new type of epitope spreading. **(A)** Representative IFN-g ELISPOTs first reported by Neumann et al. showed by *in vitro* stimulation for SLP2(paratarg-7)-specific TH1 cells in 9/14 patients specific responses to the antigen. 8/9 patients had a significant ($p << 0.05$.) stronger response against the phosphorylated variant of the SLP2-peptides used for stimulation (red columns) compared to the peptides derived from the non-phosphorylated wild type (blue columns) (209). **(B)** T-helper cells from 5/12 patients showed a significant stronger TH2 response against the modified peptides compared to the wild type peptides. Again, these are the results of *in vitro* stimulation of myeloma/MGUS patients' T-helper cells with a paratarg-7/SLP2-specific paraprotein using overlapping 15 amino acids long peptides covering the first 30 amino acids of the SLP2 sequence. Wild-type peptides and peptides with a phosphorylated Ser17 of the posttranslational modifications (PTM) variant were used. Subsequently, the culture supernatant was analyzed by ELISA for the TH2 cytokine IL-5 (209). **(C)** T-helper cells with a T-cell receptor (TCR) specific for Ser17-phosphorylated version of SLP2 are primed by antigen-presenting cells, equipped with the corresponding permissive MHC-II molecules offering all necessary costimulatory signals for full maturation. **(D)** Subsequently, T-helper cells with these properties (phosphospecific SLP2-reactive) can stimulate all B cells presenting a phosphorylated Ser17 epitope. The specificity of the receptor of these B cells for the hyperphosphorylated isoform of the antigen is not important. Thus, B cells are also stimulated whose BCRs bind the unmodified wild type of SLP2. This form of epitope spreading comprises the same amino acid of the antigen, but with the difference of posttranslational modification. Therefore, this type of spreading is vertically oriented. For some other posttranslationally modified antigens, the lymphoma BCRs are specific for modified isoform/variant depending on the PTM, i.e. HSP90-SUMO.

inhibition of the main signaling pathway of B cells, the BCR pathway, plays a crucial role. SYK inhibitors make pathogenetically sense because the SYK kinase lies relatively far upstream in this pathway. SYK inhibitors showed *in vitro* and *in vivo* activity against B-cell lymphomas and various other hematological neoplasias. However, SYK inhibitors have not yet been able to gain clinical importance in the treatment of B-cell lymphomas (213). Interestingly, SYK inhibitors were investigated for autoimmune diseases with major B-cell involvement, including rheumatoid arthritis (214), and the first FDA approval of a SYK

inhibitor, fostamatinib was granted for treatment of immune thrombocytopenia (ITP) (215). The BTK inhibitor ibrutinib initially represented a new standard in the therapy of r/r CLL showing even enduring responses in CLL with *TP53* mutations or del17p (133), and later it was shown that even in the therapy of CLL with mutated IGHV status the results were superior to the previous gold-standard immunochemotherapy concepts (216). In these cases, the accumulation of cancer cells is usually slowed down, but no complete remission in terms of negative minimally residual disease (MRD) is achieved. The C481S mutation in BTK and various PLCg2 mutations as well as mutations in the PI3K signaling pathway have been identified as resistance mechanisms (217, 218). Further indications for which ibrutinib is approved are LPL in combination with rituximab (201) and r/r MCL (156), for which the combination with the BCL2 inhibitor venetoclax was particularly impressive (159). In aggressive lymphomas, BTK inhibitors appear to be of particular benefit in lymphomas with activating mutations in *MYD88* and *CD79B (*91). In PCNSL, BTK inhibitors have been used as monotherapy (197). Unfortunately, in combination with immunochemotherapy, increased mold infections were observed (219). In a DLBCL first line trial, the combination of ibrutinib with immunochemotherapy led to increased toxicity, so that immunochemotherapy could often not be completed in a relevant proportion of patients, but a subgroup analysis showed a significant improvement of overall survival for younger patients (193). Acalabrutinib is a second generation BTK inhibitor, which is also approved in CLL and shows a different spectrum of side effects as compared to ibrutinib (220–222). In contrast to ibrutinib and acalabrutinib, the non-covalent BTK inhibitors Loxo-305, Vecabrutinib and ARQ 531 do not require the presence of the C481 wild type configuration, but may also be active in C481S BTK mutated disease (223–225). Similar to SYK inhibitors, BTK inhibitors are also being investigated in autoimmune diseases and some are approved for this purpose as for ITP, multiple sclerosis or graft-versus-host disease (GVHD) (226–228). A further prominent target for inhibition is PI3K with idelalisib, copanlisib and duvelisib approved for r/r CLL/SLL and FL, the first inhibits selectively PI3kdelta, the latter inhibits PI3Kalpha and PI3Kdelta and the last PI3Kdelta and PI3Kgamma (229–231). However, the use of idelalisib was decreased due to toxicity problems, mainly related to autoimmune phenomena such as pneumonitis and colitis (232). Further potential targets for inhibition are Lyn and the components of the CBM complex, i.e. CARD11, BCL10, and MALT1 (233).

It is presently unclear why some types of lymphomas respond well to BCR inhibiting treatment and others not. Perhaps, in non-responding lymphomas, BCR pathway activity is less essential for lymphoma cell survival and proliferation, or this pathway is activated by genetic and/or epigenetic mechanisms further downstream, so that upstream inhibition of BTK does not cause a downregulation of this pathway, an example is r/r FL lymphoma with mutation in CARD11 showing poor response to ibrutinib (234).

Attenuation of BCR Activation by Eradication of Antigenic Trigger

A possible therapeutic strategy for antigen-driven lymphomas is to remove the antigenic trigger. This can be possible for infectious antigenic triggers as typically in MZL by antibiotics or antiviral therapy (101, 103). A similar approach of antibiotic eradication could be investigated for *Moraxella spec.*-reactive NLPHL in clinical trials for patients with relapsed/refractory IgD^+ NLPHL, or a consolidating vaccination against *Moraxella catarrhalis* in early stage NLPHL patients who have only been treated locally and have not received therapeutic B-cell depletion. Of course, this vaccination must not contain the antigenic triggers themselves.

Attenuation of Stimulation by Modulation of Immunogenic PTMs

For lymphoplasmocytic lymphoma, for ABC-DLBCL, and for PCNSL, the search for specific substances to modulate the immunogenic PTMs of hyper-phosphorylated SLP2 and sumoylated HSP90 in LPL, of N-hyperglycosylated SAMD14/neurabin-I in PCNSL and of hypophosphorylated ARS2 in DLBCL would be useful. The aim would be to reverse the PTMs as permanently as possible and thus weaken the immunogenic stimulus. These substances could, for example, be investigated in secondary prophylaxis.

Targeting Lymphoma-Cells by Their BCR-Reactivity

The specific BCR antigens identified so far could possibly be used as basis for therapeutic approaches using retrograde BCR targeting - which has been proposed as the BAR (BCR antigen for reverse targeting) concept. This approach has parallels of targeting lymphoma BCRs by anti-idiotypes (235) and exploits the entity-specific BCR reactivity of lymphoma clones. Possibilities would be, for example, immunotoxins consisting of fusion proteins with the epitope region of the target antigen coupled to a toxin or an enhancer of the immune response (236–238), or T or NK cells with chimeric antigen receptors with the epitope region of the target antigens as extracellular capture domain (**Figure 5**) (239). This approach was demonstrated *in vitro* using immunotoxins consisting of the epitope of the respective BCR target antigen and a shortened form of *Pseudomonas aeruginosa* exotoxin A (ETA'). The role of pre-existing serum autoantibodies against the corresponding target antigens is critical here. After infusion of immunotoxins, consisting of epitope region and toxin, deposits of toxic immune complexes could develop. When using chimeric antigen receptor (CAR) T cells with an epitope region of the lymphoma BCR target antigen as part of the CAR ectodomain, the antibodies could possibly cause strong stimulation with cytokine release syndrome. *In vivo*, however, such CAR T-cell constructs appear to function despite the presence of autoantibodies (239). A standard anti-CD19-scFv/CD28/CD3ζ second-generation CAR construct might be used as a basis. The target antigen epitopes are combined or replace the anti CD19 scFv.

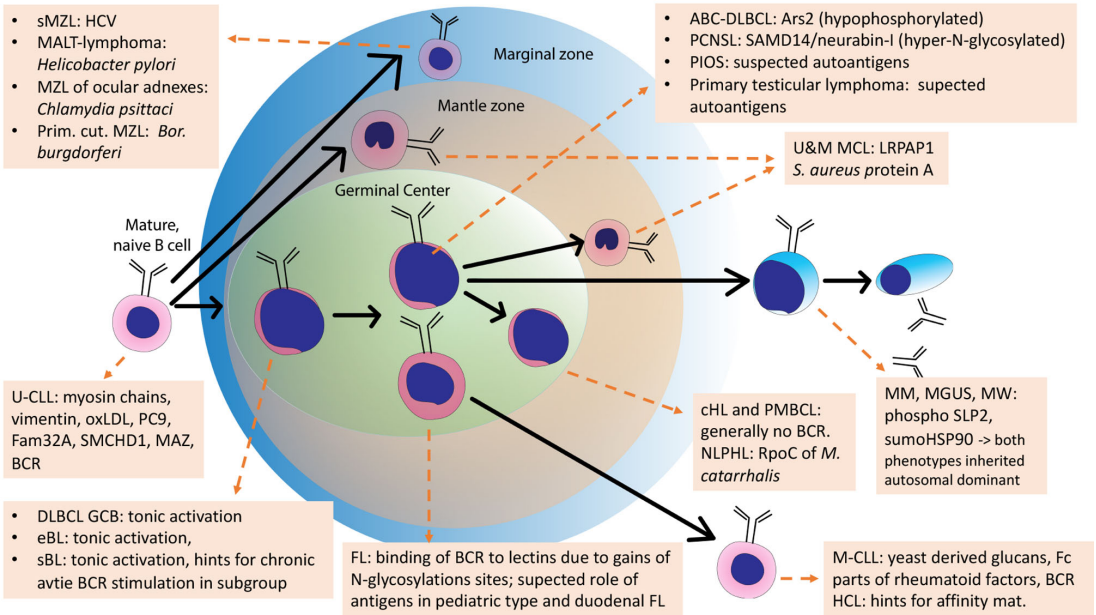

FIGURE 4 | Schematic overview of the development of normal B cells and their malignant counterparts. Arrows: Normal B-cell differentiation; dotted arrows: normal B-cells as cell of origins of specific lymphomas. This scheme is an adaption of the scheme from Küppers et al., 2005, with added B-cell receptor (BCR) antigens identified in the meantime.

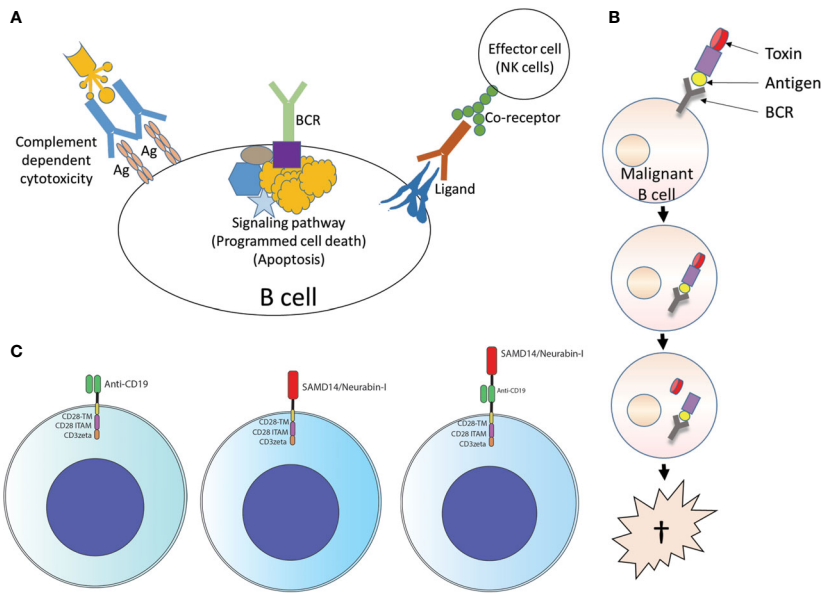

FIGURE 5 | Usage of lymphoma B-cell receptor (BCR) antigens for targeting lymphoma. **(A)** Forward targeting: Classical antibody therapy cannot differentiate between malignant and benign B cells. mAbs against B cell surface antigens such as CD20 bind to their target present on all B cells and activate complement, antibody-dependent cell-mediated cytotoxicity or direct cell death. The same is also true for antibodies against receptors with immunomodulatory functions. Independently of this, tumor cell death could also be induced antibody-independently by drugs that interfere with critical signaling pathways (such as ibrutinib, which interferes with BTK, a step in the BCR signaling pathway). Ag, antigen. **(B)** Reverse targeting: The BAR (BCR antigen for reverse targeting) concept is based on the highly specific interaction of a BCR found exclusively on malignant B cells with its highly specific target antigen; benign B cells do not possess this BCR. Synthetic conjugates of BCR antigen with a toxin (BAR toxin) bind exclusively to the malignant cells, are internalized and release the toxin that kills the cell. **(C)** CARs: Conventional CAR with CD19 scFv/CD28 4-1BB CD3ζ CAR backbone (left); the anti CD19 scFv was exchanged by the frequent BCR antigen of MCL resulting in the construct SAMD14/neurabin-I/CD28 4-1BB CD3ζ CAR backbone (middle) or combined with anti CD19 scFv (right).

AUTHOR CONTRIBUTIONS

LT wrote the manuscript. K-DP wrote the section on plasma cell diseases, FN on the role of Th cells, SH on NLPHL, and MB and K-DP on reverse targeting. RK and SS revised the manuscript and contributed significant and very important additional information. MH revised the manuscript and contributed information concerning effector cells and therapeutic mechanisms. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Modeling the Leukemia Microenviroment *In Vitro*

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Over the last decade, the active role of the microenvironment in the pathogenesis, development and drug resistance of B cell malignancies has been clearly established. It is known that the tissue microenvironment promotes proliferation and drug resistance of leukemic cells suggesting that successful treatments of B cell malignancies must target the leukemic cells within these compartments. However, the cross-talk occurring between cancer cells and the tissue microenvironment still needs to be fully elucidated. In solid tumors, this lack of knowledge has led to the development of new and more complex *in vitro* models able to successfully mimic the *in vivo* settings, while only a few simplified models are available for haematological cancers, commonly relying only on the co-culture with stabilized stromal cells and/or the addition of limited cocktails of cytokines. Here, we will review the known cellular and molecular interactions occurring between monoclonal B lymphocytes and their tissue microenvironment and the current literature describing innovative *in vitro* models developed in particular to study chronic lymphocytic leukemia (CLL). We will also elaborate on the possibility to further improve such systems based on the current knowledge of the key molecules/signals present in the microenvironment. In particular, we think that future models should be developed as 3D culture systems with a higher level of cellular and molecular complexity, to replicate microenvironmental-induced signaling. We believe that innovative 3D-models may therefore improve the knowledge on pathogenic mechanisms leading to the dissemination and homing of leukemia cells and consequently the identification of therapeutic targets.

Keywords: chronic lymphocytic leukemia (CLL), B cell malignancies, microenvironment, *in vitro*, 3D models (three dimensional)

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INTRODUCTION

To date, a cure for cancer remains a major unmet clinical need and the possibility to achieve it relies on an increasing knowledge of the fundamental biological and molecular mechanisms leading to neoplastic transformation. In recent decades, novel experimental strategies have allowed for great advancements in cancer research providing major insights into the complexity of tumor development (1, 2). Particular attention has been dedicated to the design and implementation of experimental models that may allow for the study of human tumors in the setting of a research

laboratory in a reproducible and consistent manner, through both the generation of tumors in living organisms (*in vivo* models) and the creation of culture systems of increasing complexity (*in vitro* models).

For many years, cancer pathophysiology studies and drug testing have relied on conventional 2-dimensional (2D) cell cultures *in vitro* systems and animal models (3). Although widely used, these models have a number of limitations thus poorly reflecting the *in vivo* situation and the actual responses to therapies. In particular, by definition, 2D cultures are lacking the physiological 3-dimensional (3D) structure of human tissues, whereby cell-cell and cell-extracellular matrix (ECM) interactions occur creating the so-called microenvironment. Not only the microenvironmental interactions but also the 3D structure itself are considered key for cell proliferation, differentiation and mobility, as they occur in the context of cancer development (4). In addition, animal models are expensive, time consuming and may not adequately reproduce the features of human tumors, present the correct immune activation or predict drug responses. *In vitro* 3D tissue models could provide a third approach that bridges the gap between traditional 2D culture and animal models (5). 3D cultures have obtained popularity in the study of solid tumor biology, being able to address several questions that are difficult to unravel by using conventional 2D culture models, such as in the event of metastasis and invasion, aggressiveness, dormancy and cell-cell interactions (6). In particular spheroids and organoids are the most established systems for different cancers. Spheroids represent the simplest model of 3D organization; as the name suggests, tumor cells, including primary cells, aggregate in spherical shapes. Organoids are more complex and are developed from embryonic induced-pluripotent and somatic stem cells and cancer cells or from primary tumor biopsy. The latter have the advantage of preserving the intact structure of the original tumor tissue along with its heterogeneity, morphology and gene pathways (7). These 3D models are widely used for solid tumor but it is now becoming clear that they may also be relevant for hematological cancers, in particular when assessing *in vitro* responses to drugs where 2D models poorly predict the actual clinical outcome (8).

Recently, *in vitro* 3D models mimicking specialized microenvironments of lymphoid tissues and incorporating advanced biomaterials and microfluidics, helped identify novel cellular, biochemical, and biophysical interactions and elucidate new regulatory mechanisms and potential therapeutic targets that could not otherwise have been studied in conventional 2D cultures (9). Several 3D systems have also been applied to the study of different B cell malignancies; however, this has only recently been used for CLL and with rather limited attempts.

In this review, we will discuss the design and propose of the use of new 3D *in vitro* models for B cell malignancies, in particular in the context of CLL research, discussing the key molecules/signals present in the tissue microenvironment that are likely needed to reliably replicate microenvironmental-induced signaling in such systems with increasing complexity *in vitro* (**Figure 1**).

The Tissue Microenvironment in B Cell Malignancies

The specialized microenvironments of lymphoid tissues affect immune cell function and play an important role in the pathogenesis of hematological cancer by providing protective and supportive niches for malignant cells as paradigmatically shown in the case of chronic lymphocytic leukemia (CLL). Signals occurring in tissue compartments (Bone Marrow - BM) and secondary lymphoid organs are pivotal for survival, proliferation, homing and trafficking of the malignant cells (10). Calissano et al., by using *in vivo* deuterium labelling as an indicator of cells that had divided, documented the dynamic cellular kinetics and intraclonal complexity of CLL cells (11). These studies demonstrate that CLL is a dynamic disease where cells traffic and home between the peripheral blood (PB), bone marrow (BM), and the lymphoid tissues and here proliferate and die at variable rates. In particular, with the same deuterium labelling method, Herndon et al. identified the lymph node (LN) as the anatomical site where the majority of the proliferation

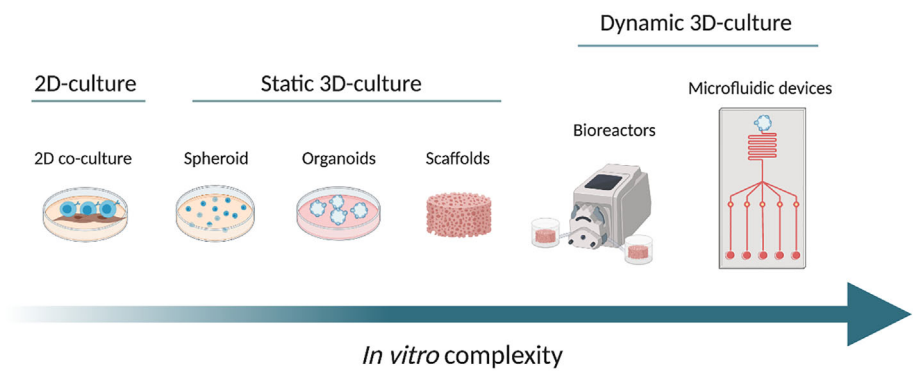

FIGURE 1 | From left to right, the scheme shows the increasing complexity of the *in vitro* models, from a conventional 2D co-culture model (on the left), through different static 3D culture systems namely spheroid, organoids and scaffolds. On the right side, we propose examples of dynamic cultures: bioreactor and microfluidic device (at the far right). The arrow from left to right shows the increase in complexity. Created with BioRender.com.

occurs in contrast to the BM (12). In tissues, CLL cells make contacts with various types of cells, such as non-malignant stromal cells, nurse-like cells (or lymphoma associated macrophages), T lymphocytes, and mesenchymal-derived stromal cells. There, cells are also exposed to integrins, chemokines, cytokines and other survival factors (13). In particular, the LN microenvironment promotes B-cell receptor signaling, NF-kB activation leading to CLL cells proliferation (14). One has to note that only a small proportion of CLL cells proliferate in the so-called proliferation centers within the LN while the vast majority are resting or anergic (15) thus suggesting a very specialized and segregated structural organization within the tissues. For all these reasons, it is very challenging to reproduce *in vitro* what occurs *in vivo* possibly leading to CLL cells proliferation. Recently, Haselager et al. gave an overview of *in vivo* versus *in vitro* signals involved in CLL cells proliferation that we should take into consideration for the development of more complex *in vitro* models (16).

Mimicking the Microenvironment: A Story of Increasing Complexity

It is then clear that the complexity of the interactions occurring *in vivo* and the cell heterogeneity present in the leukemic microenvironment pose a number of challenges when trying to dissect the specific role of each component in the development of CLL. Based on this, it becomes obvious that traditional preclinical *in vitro* modeling using only primary CLL cells or even cell lines have become obsolete. This is clearly evident by the fact that CLL cells undergo spontaneous apoptosis when cultured *in vitro* unless substitutes of survival signals found in the tumor microenvironment are provided (17). It is also interesting to note that many novel therapeutic agents currently under development for CLL are targeting not only intrinsic CLL signaling pathways, but also key CLL-microenvironment interactions. Because of this, traditional read-outs, such as *in vitro* cell death, are therefore limited and unable to fully evaluate these agents and unravel novel mechanisms of action. For example, the low rate of cell apoptosis induced *in vitro* by the BTK inhibitor ibrutinib would not have allowed for the identification of this compound as a revolutionary therapeutic agent through a traditional *in vitro* drug screening approach (18).

With this in mind, a series of improvements in 2D cultures have been implemented throughout the years by adding progressively more microenvironmental components aiming at improved reproducibility of the *in vivo* complexity and function.

The Cellular and Molecular Components of the Tissue Microenvironment

Describing the components present in the tissue microenvironment is not the focus of this review [recently reviewed by Haselager et al. (16)] and we will provide a brief overview on few components that may have more relevance when considering building a functional system *in vitro*.

Nurse-like cells (NLCs) are found in secondary lymphoid organs where they activate the BCR signaling in CLL cells and secrete CXCL12 and CXCL13 that attract CLL cells into the tissue microenvironment. They also express BAFF and APRIL that promote survival and proliferation of CLL cells, thus providing full support for the leukemic cells as seen *in vitro*. Similarly, BM stromal cells (BMSCs), which are crucial for the well-being of normal haematopoietic cells, also regulate the survival of malignant cells in the bone marrow. The interaction between VCAM-1 on the BMSCs and VLA-4 integrin on CLL cells leads to the upregulation of the anti-apoptotic molecule MCL1. In addition, BMSCs, similarly to NLCs, secrete CXCL12 that interacts with CXCR4 on CLL cells, promoting tissue homing and regulating cell trafficking (19, 20). Keep in mind that FDCs and endothelial cells play a non-redundant role for tissue homing and CLL retention in tissues as well (21). In particular, adhesion to microvascular endothelial cells promotes CLL cells survival, activation and drug resistance along with two neuroendocrine secretory polypeptides that enhance the endothelial barrier function, for instance chromogranin A (CgA) and its N-terminal fragment (called vasostatin-1, CgA1-76), which circulate in variable amounts in the blood of patients with CLL (22).

Activation of malignant B cells through CD40 and by IL4 secreted by $CD4^+$ T cells also promote CLL survival in lymphoid tissues (23). Activated CLL cells secrete CCL3, CCL4, CCL17, and CCL22 that recruit T cells and monocyte/macrophages to the tissue sites allowing the interaction with the leukemic cells (24). Although playing a pro-tumor effect, it is interesting to note that, in patients with CLL, despite an increased number of circulating $CD4^+$ and $CD8^+$ T cells, their functionality is compromised as T cells fail to form immune synapses (25) thus providing an explanation for the diminished immune surveillance. Similarly, NK cells have a defective cytotoxic activity in CLL, due to the overexpression of HLA-G in the plasma of CLL patients that impairs NK cytotoxicity and induces NK apoptosis (**Figure 2**) (26).

Cell Cocktails: Combination of Chronic Lymphocytic Leukemia Cells and Microenvironmental Elements

Over the last two decades, accumulated experimental evidence demonstrated that bone marrow mesenchymal stromal cells (BM-MSCs) in general can protect different types of leukemia from spontaneous and chemotherapy-induced apoptosis (27).

In the case of CLL, the most relevant, and indeed the most utilized cells in co-culture systems, are stromal and nurse-like cell (NLC). Stromal co-culture systems were first described by Panayiotidis et al. in 1996. They showed that culturing CLL cells in contact with BMSCs could increase the percentage of viable cells after 10 days of culture by more than 30% compared to controls. Similarly, CLL cells incubated *in vitro* with FDCs were protected from spontaneous apoptosis as a result of the ligation of CD44 on CLL cells and up-regulation of MCL1, a member of the BCL2 family of anti-apoptotic proteins (28). Later, the same positive effect in terms of leukemic cell survival *in vitro* has been demonstrated with different BM stromal cells (29). In other instances, in an attempt to simplify the culture systems and avoid

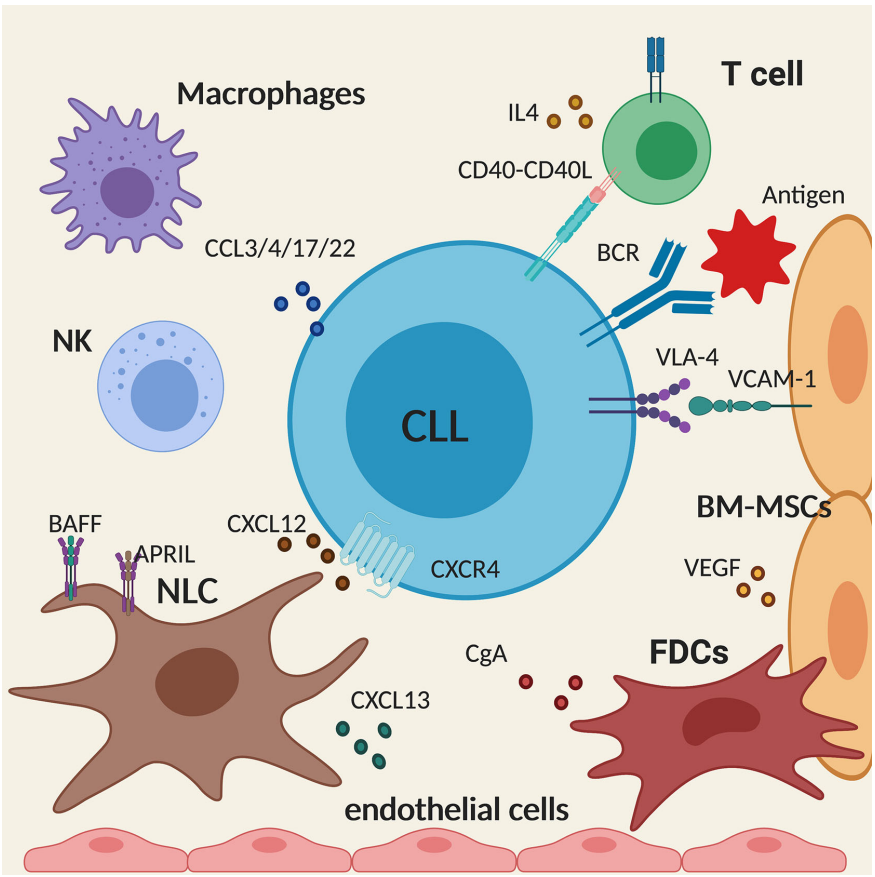

FIGURE 2 | Schematic summary of the main cellular and molecular components of the tissue microenvironment in chronic lymphocytic leukemia (CLL). The figure shows a CLL cell (in the centre) interacting with the different components of the microenvironment described in the text. Created with BioRender.com.

the use of different types of cells, investigators have tried to replace the pro-survival effect delivered by stromal cells with single or multiple factors thought to be relevant. Among many tested, the most utilized have been: CXCL12, CD40L, and IL-4, to prolong CLL cell survival (30); anti-IgM to increase BCR and NF-kB signaling and overall activation of the leukemic cells; CpG (with or without IL-2) to induce proliferation besides prolonging survival (31).

As an evolution of the cell- or molecule-based approaches, more complex co-culture methods have recently been developed, however still in a 2D setting. Primo et al. (32) developed a novel *ex vivo* short-term culture system to potentially predict the clinical response to therapeutics including novel inhibitors (in particular PI3Kδ and BTK inhibitors) by quantifying not only the pro-apoptotic effect of conventional drugs but also the anti-proliferative effect that are characteristically exerted by kinase inhibitors. After a thorough evaluation, alone and in combination, of several cell-extrinsic factors known to affect survival and growth of CLL cells, the authors proposed an optimized co-culture setting with sustained viability and proliferation of CLL cells, resembling what may occur within the infiltrate tissues *in vivo*. The combination includes: a cellular adherent component (HS5 - BM derived stromal cells), soluble factors such as CpG + IL2 and the addition of human serum, CLL plasma and erythrocyte fraction.

However, all these systems are *per se* limited in the possibility to fully reproduce the situation and the events occurring *in vivo* due to the intrinsic limitations of a flat, 2D culture.

While in solid tumor research, the need to develop *in vitro* tissue models with a 3D structure resembling *in vivo* tumor growth was more obvious, this need was perceived much later in hematological cancers, in particular CLL, due to the circulating nature of the disease. It is now evident that the circulating cells do not represent the actual reservoir of the disease, that is composed by the cells accumulating in solid tissues, in particular in the proliferation centers within LN or the BM (11, 23). For this reason, also a 2D culture system for CLL, regardless of its complexity and efficiency, needs to be implemented if we want to study the specific mechanisms of leukemic growth and invasion.

3D Culture Strategies

For all these aforementioned motivations, the proposed concept is that more complex culture systems, including 3 dimensional

ones, should be explored in order to better mimic the physiological settings in which cells grow. Over the last few years, a number of 3D culture systems have been designed and developed and this has been evidenced by the increasing number of publications on this topic (33). The term 3D culture refers to a three-dimensional system in which cells are no longer cultured on a plastic or a glass surface but instead are allowed to proliferate, migrate, communicate and behave more physiologically.

Disease Modeling in Static *In Vitro* 3D Cultures

In the broadest sense, 3D cell cultures can be categorized into two distinct types: scaffold-based or scaffold-free. Scaffold-based models support anchorage dependent growth and supporting structures are made of polymeric hard materials or hydrogels. In contrast, scaffold-free systems enable anchorage independence cell growth such us in the context of spheroid obtained by hanging drop microplates, magnetic levitation or ultra-low attachment coatings (33).

As an example of the latter models. Farinello et al. set up a scaffold-free co-culture to form a lymphoid aggregate to study murine CLL cells and stromal cells interactions by using collagen to promote the formation of so-called *spheroids*. In detail, by a hanging drop method they obtained polymerized stromal cells-collagen drops to which they added, after 24h of culture in petri dish, murine Eμ-TCL1 leukemic cells. The latter model is well known as it appears to reproduce human CLL in particular the most aggressive form, in terms of both biological and clinical features. Exploiting this system, the authors were able to validate a previously unknown mechanism regulating cell adhesion to stroma, through the retinoid-signaling pathway (34). Setting a 3D model with murine leukemic cells is uncommon but may also provide additional advantages allowing to compare more easily cells from different compartments of a living body, to produce a higher number of replicates from individual animals while limiting the number of utilize mice and to preserve human cells (in particular from tissues) for conclusive experiments for when preliminary results have been obtained with the mouse cells. Obvious criticism to this approach lies on the potential relevant differences in terms of functional behavior of mouse leukemic cells as compared to human CLL.

In terms of scaffold-based 3D cell culture systems, the most common models can be further categorized as either biological or synthetic. The former has the advantage of containing extracellular matrix (ECM) components (e.g., sugars, amino acids, lipids, proteins) thereby more closely resembling the actual tumor microenvironment ("biomimicry of the tissue of origin"). Examples of this model include Matrigel and Collagens or decellularized tissues or organs. However, natural products have innate differences during the commercial production process leading to heterogeneity in their composition between different lots, also based on the tissues of origin, thus impeding full potential reproducibility of the experimental settings.

On the other hand, synthetic or animal-free polymers scaffolds are made of biologically compatible polymers and hydrogels (35, 36) that do not exist in the tissues but have the advantage of being highly reproducible and also provide low cost, consistent and tunable scaffolds. Examples include: gelatin, cellulose, chitosan, alginate, recombinant silk, PLA (polylactide), and PCL (polycaprolactone) (37). In this case, nutrients and other factors can be added as needed and ideally cells cultured in the scaffold could produce their own functional ECM.

Both scaffold types have been used to recreate lymphoid tissues. The BM microenvironment niche has been the most investigated for acute myeloid leukemia (AML) and multiple myeloma (MM) to study the resistance to chemotherapeutics. Aljitawi et al. designed a 3D BM-microenvironment by co-culturing AML cell lines with human bone marrow derived mesenchymal cells in a synthetic scaffold and demonstrated significant differences in leukemic cell response to chemotherapy. In particular, leukemic cells cultured in 3D were more resistant to drug-induced apoptosis compared to cells cultured in 2D (38). De la Puente et al. adopted a different approach for MM by using a biological 3D scaffold from the BM of patients. Fibrinogen present in the plasma of the supernatant of the BM was cross-linked with calcium and this process allowed the encapsulation of MM cells, stromal cells, and endothelial cells. They demonstrated that this model could mimic the native MM growth and interaction with the microenvironment as well as drug availability (39). Notably, also in the case of MM, cells grown in 3D cultures showed an increased resistance to chemotherapeutic agents thus resembling more closely the *in vivo* response to therapy.

A variant of the scaffold-based models are 3D tumor organoids, that are *in vitro* 3D cellular clusters directly derived from primary tissues, embryonic stem cells, or induced pluripotent stem cells grown on artificial ECM (e.g., matrigel). Organoids exhibit similar organ functionality as the tissue of origin, and they are widely utilized for solid tumors and drug screening (40).

Tian et al. developed a type of 3D organoid system mimicking a lymphoid tissue to culture B and T cell lymphoma cell lines by using a functionalized hydrogel that allows one to precisely define the integrin density to be tested and incorporate FDCs as supporting stroma cell subtype (41). In this way, they recreated a condition that recapitulated the natural environment of these lymphomas and was suitable for drug testing.

Innovative Dynamic In Vitro 3D Culture Models

Despite the improvements, 3D models have limitations due to the static condition of the culture system that cannot reproduce the dynamic interactions occurring *in vivo*. They are still lacking the possibility to study *ex vivo* CLL cells in a dynamic fashion with the possibility to study phenotypic or functional changes not only in time but also in space, during trafficking and homing to and from different tissue microenvironments.

In vivo cell growth is influenced by gravity, different flow regimes, shear and mechanical stresses. In addition, cells need an appropriate and continuous oxygen and nutrient transport to

really mimic the *in vivo* situation. To this end, to have a functional vasculature system becomes crucial when planning the design of a viable 3D dynamic model. Thus, an additional level of complexity is needed to recreate the natural physiology of the tissue environment through the addition of a dynamic component to the *in vitro* system.

An example has been reported by Walsby and collaborators that developed an interesting *in vitro* dynamic system that modeled circulation and allowed for detailed study of transendothelial extravasation and migration of CLL cells (42). Thanks to this model, Pasikowska et al. (43), compared the migration capacity of LN derived CLL cells with those obtained from the peripheral blood (PB) and were able to show that the latter are constitutionally primed for lymphoid tissues homing and interaction with T cells.

Achieving this further step of complexity is feasible by using bioreactors or microfluidic devices. Bioreactors are closed systems in which biological and biochemical processes develop under monitored and controlled environmental and operational conditions, such as: temperature, pH, pressure, nutrient supply, and waste removal. Precise sensors inside the bioreactor connected to control software or pumps monitor the influx and efflux of nutrients and metabolites. Mass transfer regulation is critical since the shear stress may damage the cells and must be evaluated for each 3D model and cell type (44). Bioreactors have an important role in the *ex vivo* engineering of 3D tissues based on cells and scaffolds, including cell seeding of porous scaffolds, nutrition of cells in the construct and mechanical stimulation of the developing tissues. There are different types of bioreactors, among which: 1) spinner-flask bioreactors: used for the seeding of cells into 3D scaffolds and for subsequent culture of the constructs; 2) Rotating-wall vessels (RWV) bioreactors: it provides a dynamic laminar flow generated by a rotating fluid environment and it reduces diffusional limitations of nutrients and wastes, while producing low levels of shear flow; 3) direct perfusion bioreactors: here the culture medium is perfused directly through the pores of the cell-seeded 3D scaffold. During seeding, cells are transported directly into the scaffold pores, resulting in a uniform cell distribution (44). There are some examples of 3D culture coupled with dynamic growth in the case of B cell malignancies. To study MM, Belloni et al. (45) exploited a 3D Rotary Cell Culture System bioreactor using gelatin scaffolds. This particular type of bioreactor provides a balance between increased mass transfer and reduced shear stress, thus generating optimal conditions for long term *ex vivo* maintenance of tissue explants. This model was initially validated using a co-culture system where MM cell lines were placed in contact with stromal and endothelial cells. Next, the same system was then successfully applied to primary co-cultures of MM cells and BM stromal cells from patients together with endothelial cells (Huvec cell line), allowing the development of functional myeloma-stroma interactions and MM cell long-term survival and a more precise determination of the impact of the proteasome-inhibitor bortezomib on MM cells and on the microenvironment to predict actual responses. We recently adapted this model to study the interactions that CLL cells may engage with the BM tissue microenvironment (46). We co-cultured primary CLL cells and BM derived stromal cells on gelatin scaffolds maintained in the Rotary Cell Culture System bioreactor. This allowed for the parallel analysis of both CLL cells retained inside the scaffold (i.e., an environment that mimics the cells resident in the tissues) and those released in the external environment of the scaffold (to mimic circulating cells) in the presence or the absence of pharmacological agents. In particular, we used a BTK inhibitor, ibrutinib, that has the effect of mobilizing leukemic cells from the tissues, an effect that cannot be otherwise assessed and studied in a traditional 2D culture system. Thanks to the optimization of this model, we first observed that not all CLL cells are mobilized with the same efficiency from the scaffold; in particular primary CLL cells that express the protein HS1 in its inactive form (47) more frequently remain inside the scaffold. This somehow parallels an observation made *in vivo*, where patients treated with the kinase inhibitor show increased lymphocyte count due to mobilization of the leukemic cells from the tissues into the PB, thus underlining that this model may reliably reflect the *in vivo* situation. In addition to bioreactors, microfluidic techniques can also be used where 3D structures are connected to microchannels, made of polymers, to achieve a spatial control over nutrients and fluids (48). Microfluidics can be applied to different cancer models including hematological cancer [e.g., Acute lymphoblastic leukemia (49)], for phenotypic screening and personalized medicine. Many models recently developed include vasculature components that allow angiogenesis and migration (48). Interestingly, Cancer-on-chip models for lymphoid malignancies have recently been developed for drug discovery and mechanistic studies: a lymphoma-on-chip model for diffuse large B-cell lymphoma (DLBCL) was obtained by seeding tumor cells in a vascularized hyaluronic acid hydrogel placed on a chip and used to study the cross-talk between tumor cells, immune cells and endothelial cells and the response to drug treatment (50). These models serve also as the basis for the creation a LN-like structure. The most elaborate prototype of human artificial lymph node currently available was realized thanks to a miniaturized, membrane-based perfusion bioreactor, hosting a hydrogel matrix preloaded with dendritic cells through which T and B lymphocytes recirculated continuously and a set of microporous hollow fibres provided nutrient and gas exchange (51).

PERSPECTIVE/DISCUSSION

Here, we reviewed *in vitro* models as crucial tools to study the pathophysiology of lymphoid malignancies, focusing in particular on the importance of exploring alternative models to study leukemia B cells in their tissue microenvironment. We believe that innovative 3D-models will be essential to improve our knowledge on the pathogenic mechanisms leading to the homing and dissemination of leukemia cells and consequently the identification of potentially important therapeutic targets. In our view, it is fundamental to combine cell growth in 3D with a dynamic system in order to obtain a more sophisticated and

more biomimetic preclinical cancer model and 3D models combined with dynamic culture techniques show a great potential to accurately emulate the tumor microenviroment. Last but not least, it is necessary to develop suitable computational models (52, 53), to explore and predict in advance the best culture system for our disease modeling, the reason being the numerous variables that one has to consider by scaling up into a complex 3D co-culture dynamic *in vitro* system.

To conclude, we are just at the dawn of the next era where 3D culture system will become an indispensable tool for research and drug response assessment in B cell malignancies including CLL. For the latter, the 3D model in bioreactors is just the first step on a long path towards more complex 3D *in vitro* models that can better reproduce the interactions between leukemic cells and the tissue microenvironment in a dynamic fashion. That goes along with a deeper knowledge of the different cellular and molecular components that are fundamental to mimic the *in vivo* situation that will then become essential components of a more physiological structure that one day could even be envisioned to replace, at least in part, animal models. One has also to consider the specificities of the different microenvironments through which CLL cells recirculate implying the design and set-up of tissue-specific models that could eventually be connected to fully assess *in vitro* the complexity of the dynamic interactions occurring *in vivo*. The final goal will be to generate a 3D multi-organ system that could represent a new and versatile tool to understand leukemic cells behavior but also to test the specific effects of novel drugs or target therapies before embarking in complicated and expensive clinical trials.

AUTHOR CONTRIBUTIONS

CS and PG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Telomere Dysfunction in Chronic Lymphocytic Leukemia

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Telomeres are nucleprotein structures that cap the chromosomal ends, conferring genomic stability. Alterations in telomere maintenance and function are associated with tumorigenesis. In chronic lymphocytic leukemia (CLL), telomere length is an independent prognostic factor and short telomeres are associated with adverse outcome. Though telomere length associations have been suggested to be only a passive reflection of the cell's replication history, here, based on published findings, we suggest a more dynamic role of telomere dysfunction in shaping the disease course. Different members of the shelterin complex, which form the telomere structure have deregulated expression and *POT1* is recurrently mutated in about 3.5% of CLL. In addition, cases with short telomeres have higher telomerase (TERT) expression and activity. TERT activation and shelterin deregulation thus may be pivotal in maintaining the minimal telomere length necessary to sustain survival and proliferation of CLL cells. On the other hand, activation of DNA damage response and repair signaling at dysfunctional telomeres coupled with checkpoint deregulation, leads to terminal fusions and genomic complexity. In summary, multiple components of the telomere system are affected and they play an important role in CLL pathogenesis, progression, and clonal evolution. However, processes leading to shelterin deregulation as well as cell intrinsic and microenvironmental factors underlying TERT activation are poorly understood. The present review comprehensively summarizes the complex interplay of telomere dysfunction in CLL and underline the mechanisms that are yet to be deciphered.

Keywords: chronic lymphocytic leukemia, telomere dysfunction, telomerase activation, genomic complexity, prognostic factor, clonal evolution

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INTRODUCTION

Telomeres are repetitive DNA sequences at the ends of the chromosomes that play a pivotal role in maintaining genomic stability by capping and protecting the ends from degradation and fusions. Maintenance of telomere length is a key for immortalization in cancers. In chronic lymphocytic leukemia (CLL), telomere length has been identified as an independent prognostic factor in various studies. In addition, the deregulation of different telomere components has a profound influence on the CLL pathomechanisms. The present review is thus aimed at summarizing the clinical and biological aspects of telomere shortening, mutations and deregulated expression of telomere

associated genes, and mechanisms that are important for telomerase activation in CLL, to pave way for a deeper understanding of telomere dysfunction in CLL pathogenesis.

TELOMERES—STRUCTURE AND FUNCTION

All eukaryotic chromosomes have specialized nucleo-protein structures called telomeres which cap the ends. The nucleic acid component of the telomeres comprises of long tracts of DNA repeat sequences, ending with a 3' single stranded DNA overhang. In mammals, the telomere sequences consist of TTAGGG hexamers, repeated many kilo bases in length (1). In somatic cells, a part of the DNA sequence is lost at the ends of the chromosomes during each cell division due to the end-replication problem (2, 3). The telomeres at the chromosomal ends thus serve as buffer preventing loss of vital genetic information. The telomeric repeats are associated with a six-subunit protein complex called shelterin consisting of TRF1, TRF2, TIN2, TPP1, POT1, and RAP1. TRF1 and TRF2 bind directly to the double stranded telomere sequence and POT1 binds to the 3' single stranded overhang. TIN2 and TPP1 link TRF1 and TRF2 and POT1 while RAP1 binds solely to TRF2 (4). The 3' telomere overhang at the chromosomal ends loops to form the T-loop by strand invasion. The T-loop structure along with the shelterin prevents the chromosomal ends from being recognized as DNA damage, conferring genomic stability (5).

In stem cells, germ cells, and in various cancers, the telomere length is maintained, most commonly by the reverse transcriptase enzyme, telomerase (TERT). It is an RNA-dependent DNA polymerase that uses the telomerase RNA component (TERC) as a template to synthesize the telomeric DNA (1). Thus in somatic cells that lack telomerase expression, telomere shortening beyond a critical length activate the senescence checkpoints, beyond which the cells cannot proliferate in the absence of an active telomere length maintenance mechanism. Activation of telomerase is considered as one of the hallmarks of malignant transformation (6). In addition, certain neoplasms undergo telomerase-independent alternative lengthening of telomeres (ALT), a recombination dependent pathway that utilizes telomeres of adjacent chromosomes as template for elongation and maintenance of critical telomere length (7, 8). In CLL, deregulations of various components of the telomere machinery such as length of telomeres, telomerase, and shelterin expression, and recurrent, activating *POT1* mutations point to a global telomere dysfunction that plays an important role in disease pathogenesis and evolution.

TELOMERE DYSFUNCTION AND TUMORIGENESIS

The primary role of telomeres is to confer genomic stability. The shelterin complex shields the telomeres from activation of the DNA damage response signaling at the telomeres. In particular, TRF2 of the sheltein complex is important to prevent activation of the ATM (9) and subsequently non-homologous end joining (NHEJ) (10, 11) while POT1 suppresses ATR signaling (12) activation at the telomeres. Critical telomere shortening leads to uncapping of the ends and activation of senescence checkpoints. This is an important tumor suppressor mechanism that functions to eliminate potentially harmful, pre-malignant clones.

Progressive shortening of telomeres by knocking out *Terc* and crossing through generations G1 to G6 by knocking out *Terc* led to increased incidences of spontaneous malignancies and decreased stress response and survival (13). Dysfunctional telomeres lead to intra or inter chromosomal end fusions resulting in the formation of dicentric chromosomes that undergo breakage at the anaphase. This phenomenon is known as breakage-fusion-bridge (BFB) cycle which leads to genomic complexity. Evidences of such BFB events were found in many different cancer types (14, 15). Using murine models it was further demonstrated that loss of checkpoint genes such as *TP53* along with telomere dysfunction led to development of cancers due to non-reciprocal translocations caused by BFB events (16).

Of note, length of telomeres within a cell substantially varies between the different chromosomes and it was identified that the presence of one or more critically short telomeres and not the average telomere length dictates cellular senescence versus proliferation (17). Though the activation of telomerase or ALT mediated telomere maintenance is important for cellular immortalization and cancer, a large study with 18,430 samples from tumor and normal tissues from 31 different cancer types identified telomere length of the tumor tissue to be shorter than the corresponding normal tissue in majority of the cancer types (18). In line with this, numerous studies on telomere length associations have shown that CLL tumors have significantly shorter telomere length but higher telomerase expression and activity compared to normal B-cells. Thus in cancers, the genomic instability associated with telomere dysfunction may promote selection of fit clones which bypass the senescence checkpoints promoting tumorigenesis while activation of telomerase or ALT serves to maintain the minimal telomere length to overcome senescence and sustain cell survival.

METHODOLOGY FOR ANALYSIS OF TELOMERE LENGTH IN CLINICAL SAMPLES

Various techniques have been used for the assessment of telomere length in CLL. Telomere length analyzed by telomere restriction fragment (TRF) analysis is considered to be the gold standard. The method includes the process of using a restriction enzyme that does not detect the telomere repeat sequence to digest the non-telomeric DNA, followed by resolution on a gel and southern hybridization (19, 20). Even though the method is highly reproducible, TRF analysis of telomere length has many limitations. Telomere length analyzed using TRF may

substantially vary depending on the restriction enzymes used to digest the non-telomeric DNA (21). Additionally, TRF method is not capable of reliably analyzing very short telomeres due to the requirement of hybridization with a probe. The method is low throughput and requires micrograms of DNA. Since the restriction enzymes might not effectively digest the telomere-associated sequences (TAS) that are adjacent to the telomeres, the method usually overestimates the telomere length of a sample (22). Over the years, newer and high-throughput methods for estimation of telomere length were developed, which made analysis of larger patient cohorts easier.

Fluorescence *in situ* hybridization (FISH) using fluorescence labelled $(CCCTAA)_n$ telomere binding probes are used for analyzing telomere length, where the intensity of the signal directly corresponds to the length of the telomere sequence in a given sample. The method when coupled with chromosomal banding is a valuable tool for analyzing telomere length of individual chromosomes. FISH based telomere length measurements could be made high-throughput by using flow cytometry (flow-FISH) (23). Another advantage of flow-FISH is that it can be used to analyze telomere lengths of different cell sub-populations within a given sample by using cell-type specific antibodies.

However, the most widely used technique for telomere length measurement is by qPCR, based on a method devised by Cawthon et al. (24). In brief, qPCR technology is used to detect the amount of telomere sequences per sample (T) by using a telomere specific primer and normalizing it with a single copy gene (S) to obtain the average telomere length per cell. The method could be used for relative estimation (T/S ratio) or for absolute telomere length analysis when used with telomere and single copy gene standards (22). The drawbacks of the TRF, flow-FISH, and qPCR based methods is that they provide a mean telomere length of the sample under analysis and not the chromosome specific telomere length. Therefore, to understand telomere length of specific chromosomes with high resolution, the single telomere length amplification (STELA) assay was developed (25). This PCR based method includes ligation of a linker sequence called telorette to the 5' end of the complementary C-rich strand, followed by amplification of the telomere of a specific strand using telorette and chromosome or allele specific primers. The PCR products are analyzed by Southern blotting or qPCR.

In addition to the above methods that were used for telomere length analysis in CLL, newer techniques have been developed for analyzing different aspects of telomere length. The STELA PCR is capable of analyzing critically short telomeres only on a subset of chromosomes such as XpYp that have unique subtelomeric sequences suitable for designing chromosome specific primers. This limitation was overcome by the universal STELA method (U-STELA) (26). The technique involves digesting the DNA using the enzymes *MseI* and *NdeI* that do not digest the telomeric repeats, followed by ligating adapters complementary to the overhangs created by these enzymes. The non-telomeric parts of the genome that have these adapters on both the ends form a pan-handle like structure due to complementarity between the ends, suppressing PCR amplification. On the other hand, the telomeic sequences have a digested 5' end and a 3' G rich overhang that is not processed by the enzymes. Ligation of telorette to the 3' overhang allows specific amplification of telomeres of all chromosomes. This method is useful for genome-wide analysis of the distribution of critically short telomeres.

The STELA and U-STELA, though highly sensitive, they are biased towards detection of short telomeres (<8kb). The method was further improved and the telomere shortest length assay (TeSLA) was developed (27). In TeSLA, an adapter (TeSLA-T) is first added to the G rich 3' overhang, followed by the use of restriction enzymes *BfaI, CviAII, MseI, NdeI* to digest the non-telomeric DNA as well as the non-canonical sub-telomeric DNA and to generate 5' AT and TA overhangs. The 5' ends of the digested DNA are then dephosphorylated to prevent re-ligation of the ends. Double stranded DNA adapters with phosphorylated 5' AT and TA overhangs containing C3 spacers are tagged to the digested ends. Telomeres are then amplified using a primer pair specific for the TeSLA-T and 5' AT/TA adapters. TeSLA allows high resolution analysis of the distribution of <1 to 18 kb long telomeres.

Novel approaches for telomere assessment such as using CRISPR/Cas9 RNA-directed nickase system to specifically label telomeres followed by high throughput imaging using nano channel array have also been developed. This technique permits mapping and analysis of individual telomeres based on subtelomere repeat elements (SRE) and unique sequences in the chromosomes. Recently, another method for telomere length measurement by molecular combing or DNA fiber analysis was reported (28) where cells were embedded in agarose plugs followed by protein digestion to obtain unsheared DNA. The DNA was then solubilized and stretched on cover slips with a constant stretching factor of 2kb/μM. Telomeres were analyzed using a telomere specific PNA probe and the DNA is counterstained to validate the terminal location of the telomeres in the chromosomes. Fluorescence microscopy is used to obtain the distribution of telomere lengths within a sample. The method is reported to be sensitive for estimation of telomere lengths of <1 to >80 kb. In CLL, the dynamics of telomere length distribution in cases with stable and progressive disease is not well defined. The above mentioned novel methods may be valuable in monitoring changes in telomere length landscape within a given case over time and its contribution to clonal diversification, genomic complexity, and disease evolution.

Due to the wide range of methods used for telomere length analysis, the comparability of telomere lengths analyzed in different CLL studies are limited. Moreover, while TRF and STELA based methods have greater reproducibility, qPCR and FISH based methods need to be very carefully and extensively optimized to limit batch effects (29). One of the methods to improve the use of telomere length as comparable biomarker would be to have a standardized set of control samples with telomere length estimated by TRF, included in every batch of FISH or qPCR based analyses to detect and normalize for batch variations and to convert the measured relative (T/S ratios or

relative fluorescence units) telomere length as absolute (TRF) values in kilo bases (kb).

TELOMERE LENGTH ASSOCIATIONS AND PROGNOSTIC IMPACT OF TELOMERE LENGTH IN CHRONIC LYMPHOCYTIC LEUKEMIA

Early studies on telomere length associations in CLL using TRF analysis of relatively small patient cohorts (n = 58 and n = 61) (30, 31) suggested an association of short telomere length with advanced disease stages, presence of the poor prognostic unmutated IGHV and inferior overall survival (30). Subsequent studies using TRF (31, 32), flow-FISH (33), and q-PCR (34) identified associations of short telomere length with other adverse disease features such as CD38 and ZAP70 expression (35) or lymphocyte doubling time (33). Analysis of telomere length associations with genomic aberration subgroups consistently showed significant association of short telomeres with the poor prognostic, deletion 17p (17p-) and deletion 11q (11q-) while long telomere length was found in cases with deletion 13q (13q-) (36–42). Of note, *TP53* and *ATM* which are critical checkpoint genes activated upon telomere shortening and dysfunction are found in the minimally deleted regions of 17p- and 11q-, respectively. Deletion of these genes therefore permits these tumor cell clones to undergo further telomere shortening compared to non-17p-/11q-, without activating cell death pathways. In line with this, short telomere length was found to be associated with the presence of mutations in *TP53* (37, 40, 41, 43) and *ATM* (41, 43, 44). Cases with 17p- or *TP53* mutation but long telomere length were found to have mutated IGHV (40, 43).

Among the recurrently mutated genes in CLL, *SF3B1* was found to be associated with short telomere length across different studies (37, 40, 43, 45). For *NOTCH1* mutations, some reports suggested an association (37) while others found no association (40, 43) with telomere length. Additionally, Beta-2 microglobulin (ß2M) and serum thymidine kinase (s-TK) levels were also found to be significantly associated with telomere length in CLL. Overall, the presence of short telomere length was found to be significantly associated with various other poor prognostic clinical and genetic characteristics in CLL which translates into an inferior survival compared to those with longer telomere length. Despite this strong association with other disease features, telomere length was found to be an independent prognostic factor in different patient cohorts (35, 36, 39, 40, 42, 43, 46). Accordingly, telomere length was shown to identify poor or favorable risk patients within established prognostic subgroups defined by e.g. IGHV, 17p- and 11q-. Overall, the findings suggest telomere length to be a very important prognostic factor in CLL that could be instrumental for risk stratification as well as monitoring and early detection of changes in clonality. The prognostic impact of telomere length in CLL has so far been established only in chemo or chemo-immunotherapy based trials and it would be interesting to study the telomere length associations in the context of novel therapy.

TELOMERE LENGTH AND GENOMIC COMPLEXITY

Critical shortening of telomere length, de-protection at telomeres along with loss of checkpoint genes leads to development of genetic lesions and tumorigenesis (16). In CLL, various studies have analyzed the impact of telomere dysfunction on genomic complexity. Early indicators of telomere dysfunction is the formation of DNA damage foci at the telomeres called telomere dysfunction induced foci (TIF) (47). CLL cells were found to exhibit TIF as detected by the localization of gamma H2AX and 53BP1 at the telomeres. In addition, an increase in abnormalities such as telomere deletion/doublets and terminal duplications were observed in TIF+ CLLs (48).

Activation of DNA damage response and DNA repair signaling at the telomeres lead to telomeric fusions. In CLL using STELA method, frequency of telomeric fusion events were found to increase with advancing disease stage and 58% of the Binet C stage had critically eroded telomeres and fusions. Cases having telomeric fusions also showed large scale genomic rearrangements at the telomeric regions (49), reminiscent of genomic complexity due to BFB cycles in telomeres at crisis (16). Subsequently, by analysis of large patient cohort (n = 321), the XpYp telomere length of 2.26 kb was defined as the mean length at which fusions occur (50).

Different studies analyzed the correlation of telomere length with genomic complexity, either by conventional FISH or by SNP array analysis. The analyses showed significant association of short telomeres with presence of two or more aberrations (FISH) (36, 38, 51) or with higher number of copy number alterations (CNAs) (37, 40). Of interest, we observed progressive shortening of telomere length with increase in number of copy number variations (CNVs) (40). Additionally, short telomeres in CLL were also found to be associated with increase in uni-parental disomy (UDP) and chromothripsis (52). The strong association of telomere shortening with terminal fusions and genomic complexity highlights the central role played by telomere dysfunction in clonal diversification and disease evolution in CLL.

TELOMERE LENGTH ASSOCIATIONS—CAUSE OR CONSEQUENCE?

The associations of short telomeres with various adverse prognostic markers such as unmutated IGHV, and *TP53/ATM* mutations, 17p-, 11q- could be explained as a direct outcome or "consequence" of increased proliferation of the cells harboring these high risk features (53). This is supported by the fact that telomere length in serially sampled CLL samples show

shortening, despite the presence of active telomerase (33, 40). In addition, Röth et al. identified shorter telomere length of naïve and memory T-cells from patients with more aggressive ZAP-70^{+}/CD38^{+} CLL which may be due to increased proliferation and expansion of T-cells in this CLL subtype (54). These findings show that at least in part, the distribution of telomere length among the different CLL subgroups is a direct consequence of their proliferation capacity (**Figure 1**).

On the other hand, telomere length could be considered to play a more active biological role in CLL by being a "cause" for clonal diversification and disease progression. The strong association of telomere length with mutation status of IGHV has been documented across all the studies, owing to differences in the cell of origin. Mutated IGHV CLLs are considered to develop from CD5(+), CD27(+), post-germinal center (GC) B-cell subsets (55), where a robust telomerase activation and elongation of telomere length is known to occur during the GC reaction (56). The non-GC origin of the unmutated IGHV CLL thus may explain the strong association of this subtype with short telomere length.

Telomere shortening has been shown to be a tumor suppressive mechanism, where cells with telomere length shorter than a threshold undergo DNA damage checkpoint activation, stalling further telomere shortening and controlling cell proliferation (17). In CLL cells with unmutated IGHV, the presence of short telomere length may exert a strong selection pressure for loss of checkpoint genes such as *TP53* or *ATM* which would eventually allow for further telomere shortening

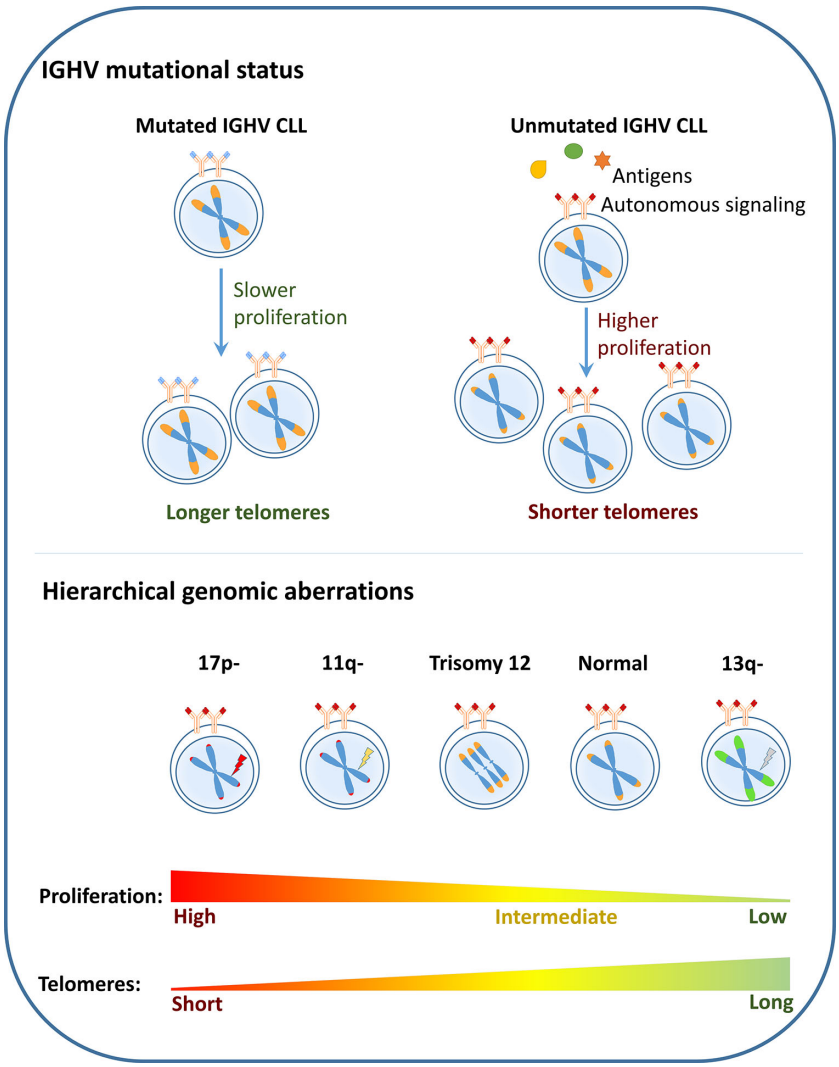

FIGURE 1 | Telomere dysfunction as a consequence: In CLL, the poor risk disease features such as unmutated IGHV, presence of deletion 17p (17p-), deletion 11q (11q-) are shown to be associated with short telomere length while the favorable prognostic subgroups such as mutated IGHV and deletion 13q (13q-) are associated with longer telomeres. It could therefore be considered that telomere length associations are a direct outcome of the proliferation capacity of the different CLL subgroups.

and cell proliferation. This notion is supported by study on temporal association of genomic alterations in CLL, where 17p-/*TP53* mutations and 11q-/*ATM* mutations were found to be later events in CLL pathogenesis (57). Moreover, we observed in a large clinical trial cohort (n = 620) that cases with 17p- and 11q- had the shortest telomere length across the different genomic aberration subgroups and interestingly, these cases had very short telomeres even when these aberrations were observed in only a small fraction of the tumor bulk. The finding suggested that critical telomere shortening in these cases could precede acquisition of these high-risk aberrations (40). High resolution analysis of genomic fusions in cases with dysfunctional telomeres showed complex inter/intra chromosomal and terminal fusions involving the telomere loci in all of the samples analyzed (n = 9). Strikingly, the telomere fusions also included the loci recurrently altered in CLL (58).

Therefore, even though telomere shortening and its association with poor prognostic features could be a consequence or outcome of these poor risk characteristics, recent findings indicate a dynamic role of dysfunctional telomeres in shaping the disease course. Critical telomere shortening confers selection pressure to acquire poor-risk variants and increases disease heterogeneity due to genomic fusion events involving dysfunctional telomeres thereby promoting disease progression and treatment resistance in conjunction with clonal evolution (**Figure 2**).

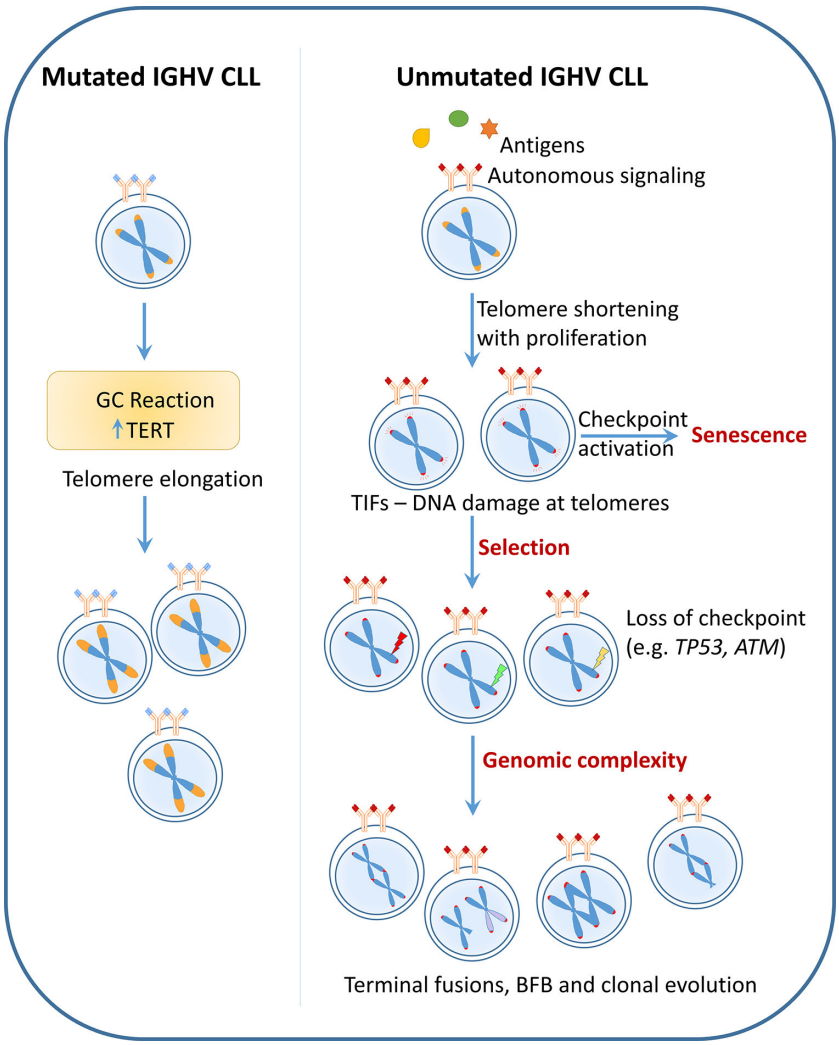

FIGURE 2 | Telomere dysfunction as a cause: CLL with mutated IGHV undergo telomerase activation during the germinal center (GC) reaction leading to telomere elongation. These long telomere length cases follow an indolent disease course and rarely acquire poor-risk features. On the contrary, unmutated IGHV CLL which have poly reactive BCRs undergo progressive telomere shortening with increasing cell proliferation. Critical telomere shortening leads to activation of DNA damage signaling at the telomeres indicated by the presence of telomere dysfunction induced foci (TIF). Persistent DNA damage at the telomeres may lead to selection of clones with dysfunctional checkpoints (e.g. TP53 or ATM loss). The presence of very short telomere length together with absence of checkpoint genes causes telomere fusions, breakage-fusion-bridge (BFB) cycles, eventually leading to heterogeneity and clonal evolution. Thus according to this hypothesis, telomere length which is defined very early in pathogenesis based on cell of origin plays an active role in disease evolution and progression.

TELOMERASE EXPRESSION AND ITS RELATION TO DISEASE FEATURES

Activation of the enzyme telomerase is considered as one of the hallmarks of malignant transformation (6) and is pivotal for sustaining cell proliferation. The predominant mechanism of TERT activation in human cancers is by acquisition of *TERT* promoter mutations. In contrast, such mutations are rarely reported in CLL. Ten percent of cancers that do not depend on telomerase depend on ALT mechanism (59). However, a study on the presence of C-Circles and extra chromosomal telomeric repeats (ECTR) which are hallmarks of ALT did not reveal the presence of ALT driven telomere maintenance in CLL (60).

Telomerase activity and/or expression in CLL has been studied across various cohorts. Initially, higher telomerase activity was found to be associated with advanced disease stages and progressive disease (30, 61). Telomerase activity was found to have an inverse correlation with telomere length (33, 62) and higher telomerase expression was associated with other poor-risk disease features and was described as a prognostic factor in CLL (42, 63, 64). Thus, intriguingly, unmutated IGHV CLLs, despite the absence of GC mediated TERT activation and telomere lengthening, these cases have short telomeres but high telomerase expression and activity (65). This indicates that the high TERT expression in unmutated IGHV CLL is therefore crucial for the maintaining the critical telomere length to ensure cell survival and proliferation. However, in contrast to mutated IGHV CLL, processes underlying the high telomerase expression and activity in unmutated IGHV CLL are not well defined.

TUMOR MICROENVIRONMENT AND TELOMERE DYSFUNCTION

With the absence of the classical oncogenic promoter *TERT* mutations in CLL, the mechanisms underlying its activation are poorly understood. Genome wide association studies repeatedly identified *TERT* as one among the susceptibility loci for risk of CLL (66, 67). Studies to identify SNPs in *TERT* and *TERC* associated with CLL identified the minor rs35033501 *TERT* variant (68), as well as the SNPs rs10936599 in *TERC* and rs2736100 in *TERT* (69) and presence of longer telomere length to be associated with CLL. Though shortening of telomere length in CLL is well characterized to be an adverse prognostic factor it should therefore be noted that telomerase activation and telomere lengthening constitute an important phase in malignant transformation. Also, in cases with poor risk features and rapid disease progression, constant lengthening of telomeres by telomerase is the key to sustain cell survival to counteract telomere loss due to proliferation.

CLLs with unmutated IGHV are known to have a poly-reactive/auto-reactive BCR in contrast to that of mutated IGHV. Apart from this, the CLL BCRs can also signal through cell-autonomous signaling (70, 71). These findings, along with the clinical success of the BCR signaling inhibitors such as ibrutinib and acalabrutinib (72, 73), highlight the importance of BCR signaling for survival and proliferation of CLL cells. BCR along with activation of co-receptors, drives various downstream mechanisms such as activation of PI3K/AKT, NF-kB (74) and MAPK (75) that dictate proliferation, homing and guide interaction with other cells in the microenvironment. Of importance, Damle et al. showed (76) that stimulation of BCR using multivalent BCR ligand, dextran conjugated anti-μ mAb HB57 (HB57-dex) or bivalent $F(ab')_2$ goat anti-μ antibody led to an increase in telomerase activity, predominantly in CLLs with unmutated IGHV. This BCR driven activation of TERT was accompanied by an induction of cell proliferation. They also identified that the TERT activation was mediated by PI3K/AKT signaling, as the use of a PI3K inhibitor abrogated the BCR mediated TERT activation. Another study identified higher *TERT* and *TERC* expression and activity in *SF3B1* mutated CLL, however the underlying mechanism is not well understood (77).

The tumor microenvironment mediated signaling are known to contribute to activation of TERT in different cancers. In breast cancer, STAT3 was found to activate telomerase expression by binding to the TERT promotor (78). In CLL, a constitutive activation of JAK2/STAT3 signaling has been reported (79) and it would therefore be interesting to understand its role in the regulation of TERT in CLL. Another factor that may be of interest for driving TERT activation in CLL is hypoxia. HIF-1α plays an important role in interaction of CLL cells and the microenvironment (80). HIF-1α (81) as well as the levels of hypoxia (82) are known to regulate the expression and activity of telomerase and impact telomere length. Similarly, the Wnt/ß-catenin pathway is a direct regulator of TERT (83) which could be of relevance in the context of CLL. Overall, various pathways that are active in CLL are described to play a role in TERT activation and investigations on the relevance of these mechanisms in regulation of telomerase in CLL may therefore have therapeutic relevance.

MUTATIONS AND DEREGULATED EXPRESSION OF TELOMERE-RELATED GENES IN CHRONIC LYMPHOCYTIC LEUKEMIA

Different components of the telomere system are found to be mutated or deregulated in CLL. Among the recurrently mutated genes, *POT1* mutations have been reported in about 3.5% of the cases. It is the first telomere structural component known to be mutated in human cancers. *POT1* mutations in CLL occur in the OB1 and OB2 domains that alters its binding to the 3' telomeric tail, leading to de-protection of the ends and genomic instability. In cell line models, loss of POT1 function led to aberrant lengthening of telomeres (84). Thus *POT1* mutations were associated with complex karyotype and are independent prognostic factors for overall survival in CLL (85).

Whole exome sequencing of 66 familial CLLs revealed the presence of germline deactivating *POT1* mutations in four families as well as in the sheltering components adrenocortical

dysplasia homolog (ACD, in two families) and telomeric repeat binding factor 2 (TERF2IP, three families) (86). These telomere component mutations are therefore important pre-disposing factors for CLL, highlighting the important role of telomere dysfunction in CLL pathogenesis. In addition, expression analysis of telomere related genes in different CLL cohorts have identified deregulation of various telomere components. One study identified a significant downregulation of Dyskerin, *TRF1, hRAP1, POT1, hEST1A, MRE11, RAD50*, and *KU80* while *TPP1* and *RPA1* were upregulated compared to normal B-cells (87). Another study reported a downregulation of *TIN2* and *ACD* in a subset of CLLs which correlated with increase in TIF, indicating telomere dysfunction (88). Also, downregulation of the telomere components *POT1*, *TIN2*, *TPP1*, and high *TERT* were found to be associated with adverse outcome (89). The shelterin components play a very important role by tightly regulating access of telomerase to the telomeres. Though the mechanisms underlying deregulation of the shelterin components in CLL is unknown, it could be presumed that the downregulation of these genes would promote access of TERT to the telomeres, which would be crucial in maintaining the critical telomere length to sustain cell survival. However, this deregulated expression of the shelterin components also result in uncapping of the ends and increase in DNA damage signaling and DNA repair, leading to fusion and genomic complexity.

TELOMERES AND TELOMERASE TARGETED CANCER THERAPIES

Since telomere maintenance is one of the key features of cancers, the telomere system has been considered an attractive target for cancer therapy. Accordingly, therapeutic agents targeting various components of telomeres and the different maintenance mechanisms have been developed and studied across cancers. One of the first inhibitors of telomerase to have progressed to clinical trials is imetelstat. It is a synthetic lipid conjugated 13-mer oligonucleotide that competitively binds to hTR, thereby inhibiting telomerase function (90). In vitro analysis showed that the drug sensitized primary CLL cells to fludarabine (91). Imetelstat is currently being investigated in phase 2 and 3 trials for various solid tumors and hematological malignancies as a single agent or in combination therapies. Small molecule inhibitors of telomerase such as BIBR1532 are currently under pre-clinical evolution (92). Recently, a covalent telomerase inhibitor (NU-1) that targets the catalytic active site of telomerase has been developed (93). The main disadvantage of telomerase inhibitors is the necessity for continuous long term treatment to impede telomere maintenance and critically shorten the telomere length. Moreover, long term treatment with telomerase inhibitors may additionally affect the function of germ cells and stem cells that express telomerase.

Another class of molecules that affect telomerase activity include nucleoside analogs such as 6-thio-2'-deoxyguanosine (6dG), didanosine (ddITP), azidothymidine (AZT-TP), and 5-fluro-2'deoxyuridine (5-FdU). These compounds when incorporated at the telomeric ends by telomerase leads to chain termination and uncapping of the telomeric ends (94). Uncapping by nucleoside analogs prevents binding of the shelterin complex, thereby activating DDR. Unlike telomerase inhibitors, treatment with nucleoside analogs leads to rapid induction of cell death irrespective of the telomere length. Similarly, compounds such as telomestatin which are G-quadruplex stabilizers lead to impaired telomere maintenance by telomerase thereby inducing DDR and cell death (95, 96).

Though limited clinical progress has been achieved with inhibitors of telomerase, various telomere based immunotherapies are successfully being evaluated in clinical trials for different malignancies. Since telomerase is one of the most commonly expressed tumor associated antigen, different methods are being employed to activate adaptive immune responses against telomerase. TERT peptide vaccines such as INO-1400 (NCT02960594—solid tumors), GV1001 (NCT04032067—Benign Prostatic Hyperplasia), UCPVax (NCT04263051—non-small cell lung cancer), and GX301 (97) are currently being tested in clinical trials for cancer therapy. Of note, DNA vaccine encoding hTERT is being evaluated in a phase 2 study for CLL (NCT03265717). Additionally, adoptive transfer of dendritic cells expressing TERT mRNA (GRNVAC1—NCT00510133) is being studied for the treatment of AML. Another interesting therapeutic approach includes the use of oncolytic adenovirus that replicates under the control of hTERT promoter thereby specifically targeting the tumor cells. The oncolytic adenovirus based therapy telomelysin (OBP-301) is currently being studied for the treatment of a wide range of solid cancers across 6 different clinical trials.

In summary, though the direct inhibition of telomerase has shown limited success, hTERT based immunotherapy are rapidly gaining importance for the treatment of a wide range of tumor entities. In CLL, the novel agents such as ibrutinib and venetoclax have achieved tremendous clinical success however, treatment of Richter transformation has still proved to be challenging. Since Richter syndrome is a highly proliferative tumor type, they might have a greater dependency on telomerase than CLL and hence the novel TERT based immunotherapies either as single agents or in combination with checkpoint inhibitors maybe of interest.

CONCLUSION

The relation between telomeres and CLL is complex. Though a large amount of effort has been put forward in understanding the prognostic relevance of telomere length and telomerase, various other aspects such as mechanisms underlying telomerase activation and molecular alterations leading to deregulation of telomere maintenance system still needs to be understood. In summary, deregulations of the different components of the telomere system play important roles at specific phases of CLL pathogenesis and progression. A deeper understanding of these mechanisms is vital for the development of therapeutics options for targeting these disease features, especially in patients that turn refractory to novel agents, or as combination treatments to improve efficacy or in the treatment of Richter transformation.

AUTHOR CONTRIBUTIONS

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MINI REVIEW
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Germinal Center Cells Turning to the Dark Side: Neoplasms of B Cells, Follicular Helper T Cells, and Follicular Dendritic Cells

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Gaining knowledge of the neoplastic side of the three main cells—B cells, Follicular Helper T (Tfh) cells, and follicular dendritic cells (FDCs) —involved in the germinal center (GC) reaction can shed light toward further understanding the microuniverse that is the GC, opening the possibility of better treatments. This paper gives a review of the more complex underlying mechanisms involved in the malignant transformations that take place in the GC. Whilst our understanding of the biology of the GC-related B cell lymphomas has increased—this is not reviewed in detail here—the dark side involving neoplasms of Tfh cells and FDCs are poorly studied, in great part, due to their low incidence. The aggressive behavior of Tfh lymphomas and the metastatic potential of FDCs sarcomas make them clinically relevant, merit further attention and are the main focus of this review. Tfh cells and FDCs malignancies can often be misdiagnosed. The better understanding of these entities linked to their molecular and genetic characterization will lead to prediction of high-risk patients, better diagnosis, prognosis, and treatments based on molecular profiles.

Keywords: peripheral T-cell lymphomas, angioimmunoblastic T cell lymphoma, follicular T-cell lymphoma, follicular dendritic cell sarcomas, follicular lymphoma, Burkitt lymphoma, diffuse large B cell lymphoma

INTRODUCTION

The germinal center (GC), a specialized microstructure with a high rate of cell division, is the site where antigen-driven somatic hypermutation (SHM) occurs (1, 2), a process that ultimately will produce high-affinity antibodies during adaptive immune responses (3). Over weeks, memory B cells and high-affinity antibody producing plasma cells will generate from GCs, which are necessary to protect against invading microorganisms (4). However, the more potent the immune response, the greater the risk of autoreactivity or malignancy. This is particularly relevant for the GC, where B cells may have an unfavorable outcome driving to lymphomagenesis. Importantly, most of B–cell lymphomas originate from GC B cells (5–7).

To succeed during GC reactions, B cells need the help of other crucial cells, such as Follicular Helper T (Tfh) cells and follicular dendritic cells (FDCs). Here, we focus on one dark side of GCs: malignancies derived from their aforementioned three players, B-cells, Tfh cells, and FDCs (**Figure 1**), with greater emphasis on Tfh lymphomas and FDC sarcomas (**Table 1**).

THE GERMINAL CENTER

GCs arise from proliferating B cells in the follicles of peripheral lymphoid tissues during T cell-dependent antibody responses. Naïve B cells encountering their antigen migrate to the T-B border, where they become fully activated during interaction with cognate CD4+ T cells (3, 4, 8–12). The engagement of CD40 by CD40L (CD154) represents the major component of the T cell help. Activated B cells can then either differentiate rapidly into antibody-secreting plasma cells in specialized extra-follicular niches or mature their affinity for the antigen into GC reactions, a microstructure of B cells, in a high-rate of cell division, Tfh cells and a network of FDCs (3, 13, 14). There, B cells begin to proliferate rapidly giving rise to the distinctive structure of the GC: a dark zone (DZ) of centroblast proliferating B cells and a light zone (LZ) with higher frequencies of smaller, non-dividing centrocytes. GC DZ B cells undergo SHM, and those cells that improved the affinity for the antigen are selected in the LZ to eventually differentiate into memory B cells or plasma cells (3, 4, 15, 16).

THE DARK SIDE OF GC B CELLS

The GC response, beneficial for the host during immune responses against invading pathogens, may have a detrimental role, the development of malignancies. B cells inside GC reactions are mutating at much higher rates than in any other site in the body (17), these mutations might turn B cells into a dark side, B cell lymphomas. Except the relatively rare lymphoblastic and mantle-cell lymphoma subtypes, B cell non-Hodgkin lymphomas (B–NHLs)—including diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL) and Burkitt lymphoma (BL)—are derived from GC B cells. This can be demonstrated by the presence of SHM in the immunoglobulin genes, together with histological, immunophenotypic, and gene expression characteristics (5, 18–23).

Our understanding of the molecular mechanisms driving GC lymphomas has increased due to next-generation sequencing (24). Gene translocations targeting MYC, BCL2 and BCL6, as

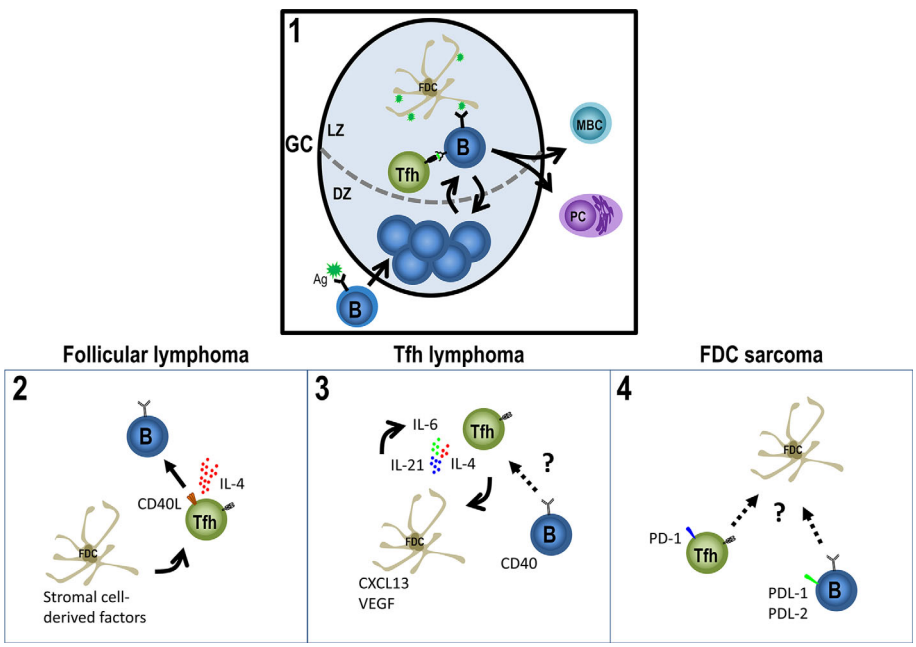

FIGURE 1 | Germinal Center-derived malignancies' players. The GC (1) is the site for B cell affinity maturation for the antigen through somatic hypermutation (in the DZ) and for antigen-driven selection of B cells which have improved their affinity (in the LZ). To success the reaction, the help from Tfh cells is fundamental, as well as, the presence of the antigen on iccosomes on FDCs. Eventually, GC B cells will differentiate to PCs or MBCs. However, the GC response may have a detrimental role, the development of malignancies from their three main players: B-cells, Tfh cells and FDCs. Follicular lymphoma cells (2) derive from follicles partly resembling normal GCs and depend on Tfh cells and FDCs to survive, while Tfh cells provide a high production of IL-4 and CD40L, FDCs provide a scaffold attracting FL cells and Tfh cells around them. (3) Infiltration of different immune cells and the proliferation of FDCs and HEV in AITL are probably caused by a stimulatory niche, secreting IL-21, IL-4 or/and IL-6, CXLC13 and VEGF, promoting a loop of Tfh cell generation and FDCs growth. (4) Tfh and Treg cells seem to be enriched in FDC sarcomas with high levels of PD-1 and its ligands PD-L1 and PD-L2 and the B/T cells mixed with the neoplastic population, altogether supporting the neoplastic niche and the evasion of effector immune cells. See text for further details.

TABLE 1 | Lymphomas of Follicular Helper T (Tfh) cells and sarcomas of follicular dendritic cells (FDCs).

	Incidence and OS	Clinical features	Morphology	Immunophenotype	Genetic profile	Treatment
Malignancies of Tfh cells origin						
AITL	15-30% of non-cutaneous T-cell lymphomas and 1-2% of all non-Hodgkin lymphomas. Poor prognosis overall. Median survival < 3 years.	Systemic symptoms, lymphadenopathy, hepatosplenomegaly, polyclonal hypergammaglobulinemia. Frequent skin rash. Hemolytic anemia, pleural effusion, arthritis, and ascites are also common.	Polymorphous infiltrate with small- to medium-size neoplastic cells. **-Pattern 1.** Neoplastic cells surrounding follicles. **Pattern 2.** Neoplastic cells in the expanded paracortex. **Pattern 3.** Diffuse with nearly total LN architecture altered. Expanded FDCs meshworks. Prominent vascularity.	Pan-T cell markers (CD2, CD3, and CD5) with Tfh cell markers: CD4, CD10, CXCL13, ICOS, BCL6, PD1 (60-100% of cases).	Trisomies of chromosomes 3, 5, and 21; gain of X; and loss of 6q (90% cases). Mutations of *IDH2* (20-30%), *TET2* (50——80%), and *DNMT3A* (20——30%), *RHOA* (60——70%).	Multiagent chemotherapy regimen, CHOP. Steroids. Stem cell transplantation.
FTCL	Unknow incidence, accounts for < 1% of all T cell neoplasms. Aggressive course. OS not well characterized. 50% patients dye within 24 months of diagnosis.	See AITL clinical symptoms. Reports of few patients with localized disease and/or no B cell- related symptoms.	Monotonous lymphoid cells with abundant pale cytoplasm and round nuclei. Nodular/follicular proliferation. **Pattern 1.** GC-like growth. **Pattern 2.** nodular FL-like growth. No expanded FDCs meshworks nor HEV proliferation.	Tfh cell markers. See above.	t(5;9) (q33;q22) (ITK/SYK) (20% cases). *TET2, RHOA and DNMT3A* mutations (75, 60 and 25% of cases, respectively. Not largely studied).	See AITL.
Nodal PTCL with Tfh cell phenotype	—————	Overlaps with AITL.	Diffuse infiltration without vascular proliferation or expansion of FDCs meshwork.	CD4+ T cells with two (preferred three) Tfh cell markers.	Mutations shared with AITL	See AITL.
Malignancies of FDCs						
FDC sarcoma	Unknow. Rare disorder. Constitutes <0.4% of soft tissue sarcomas. 2-year survival rates: Early disease: 82% Local advanced disease: 80% Distant metastatic: 42%	Systemic symptoms are uncommon. Often large tumors (mean size of 7cm). Painless, slow-growing mass lesion.	Spindled to ovoid cells forming different patterns (storiform arrays, fascicles, whorls, diffuse sheets, or vague nodules).	One or more FDCs markers: CD21, CD23, CD35. CXCL13 and podoplanin (not specific). Clusterin (strongly positive). FDCSP, serglycin (SRGN) and PD-L1.	Limited studies. BRAF V600E mutation (0-19% of cases). Alterations in tumor suppressor genes.	Complete surgical excision. No clear benefit of radiotherapy and chemotherapy.

AITL, angioimmunoblastic T cell lymphoma; LN, Lymph node; FDCs, Follicular Dendritic cells; Tfh, T follicular helper; FTCL, Follicular T cell lymphoma; GC, germinal center; FL, Follicular lymphoma; PTCL, peripheral T cell lymphoma. CHOP; cyclophosphamide, doxorubicin (or hydroxydaunorubicin), vincristine (also known as Oncovin®) and prednisolone.

well as the disruption of the epigenome, predominantly driven by somatic mutations within KMT2D, CREBBP, EZH2, and linker histones, have been well-established (25–29). The molecular and genetic characterization of these diseases will lead to prediction of high-risk patients and treatments based on molecular profiles (24).

DIFFUSE LARGE B CELL LYMPHOMA

DLBCL and FL are the two most common forms of GC NHLs. With a greater degree of genetic heterogeneity than FL, DLBCL can be divided into at least two major subtypes: GC B cell (GCB)-like and activated B cell (ABC)-like DLBCL (24, 25, 30). Whole

exome sequencing has allowed the study of recurrent mutations and the characterization of new genetic DLBCL subtypes. A recent study identified five genomic clusters based on the enriched genetic feature of each group (31). MYD88 cluster [with MYD88 (L265P), PIM1, CD79B and ETV6 mutations] were strongly associated with ABC subtype. Three clusters were associated to GCB subtype (BCL2, SOCS1/SGK1, and TET2/SGK1). The BCL2 cluster showed mutations of EZH2, BCL2, CREBBP, TNFRSF14, KMT2D, and MEF2B. The SOCS1/SGK1 cluster with mutations in SOCS1, CD83, SGK1, NFKBIA, HIST1H1E, and STAT3; and the TET2/SGK1 cluster characterized by mutations including TET2, SGK1, KLHL6, ZFP36L1, BRAF, MAP2K1, and KRAS. A NOTCH2 cluster with mutations on NOTCH2, BCL10, TNFAIP3, CCND3, SPEN, TMEM30A FAS, and CD70 showed a mixture of ABC, GCB and unclassified DLBCL. This study correlated with two recent studies classifying the disease (32, 33). Importantly, patient outcome was evaluated with the worst prognosis in the MYD88 group (42% 5-year overall survival, OS). Patients within the GCB-associated clusters had better 5-year OS (> 60%) while NOTCH2 cluster had intermediate survival (53.6% 5-year OS). Patient outcome correlated with previous studies (24, 34–38).

FOLLICULAR LYMPHOMA

FL is the most frequent indolent and incurable NHL. Over time FL may progress to DLBCL, with a more aggressive clinical course requiring more aggressive treatment (39, 40). Malignant cells morphologically resemble the two B cell subsets found in reactive GCs (centrocytes and centroblasts). Low-grade FL cases (grade 1–2) contain <15 centroblasts per high-power microscopic field (40 x objective, 0.159 mm^2) while grade 3 contains >15 centroblasts, evaluated in 10 different follicles. Grade 3 FL is further separated in 3A with a background of centrocytes present or grade 3B with follicles composed entirely of centroblasts. A diffuse pattern of 3B grade cells is compatible with DLBCL diagnosis (41, 42). The molecular pathogenesis of FL includes the high recurrence of two mutations: chromosomal translocations that lead to the ectopic expression of BCL2 and somatic mutations in the histone methyltransferase MLL2 (also known as KMT2D) (43). Also, histone modifiers such as EZH2, CREBBP and EP300 are frequently altered in FL (44–46). While BCL2 translocation is thought to occur in B-cell precursors in the bone marrow, the translocation is found also in healthy humans (40%) (47). This supports the hypothesis that the translocation is necessary, but not sufficient for FL and probably lymphomagenesis is consequence of antigen stimulation (48, 49).

FL derives from follicles partly resembling normal GCs, with the FL cells depending on Tfh cells and FDCs. While Tfh cells provide a high production of IL-4 and CD40L as survival factors of FL cells, FDCs provide a scaffold attracting FL cells and Tfh cells around them. FDCs also contributes with a positive feedback through the overexpression of stromal cell-derived factors, supporting the abnormal production of IL-4 (50, 51).

BURKITT LYMPHOMA

BL is a highly aggressive NHL associated with Epstein-Barr virus (EBV), human immunodeficiency virus (HIV) or Plasmodium infection. Three clinical variants of BL are recognized: endemic BL, sporadic BL and immunodeficiency-associated BL. Whilst EBV and Plasmodium are associated to endemic and sporadic BL, HIV is associated to the immunodeficiency-related variant (52, 53). BL derives from DZ GC B cells, as indicated by its genetic profile (20, 23, 54). Although the potential pathological role of EBV in BL it is still controversial, the virus is present in all BL cases. Aberrant expression of MYC and the BCR-induced PI3K signalling pathway activation are genetic alterations that are common in BL (23, 55, 56).

FOLLICULAR HELPER T CELLS

Fundamental studies by Mitchison in the 1970's established the essential role of T helper cells in antibody responses. Hapten-protein carrier conjugates revealed that carrier-specific T cells were necessary for the maturation of hapten-specific B cells [(57) and reviewed in Ref (10)]. Then, it was described that this help from T cells consisted in co-stimulatory signals through CD40 ligand (CD40L) to B cells leading them to proliferate, differentiate, and antibody class-switching (10, 58). T cells in the T-B border that have undergone T-B interactions can migrate inside the follicles as Tfh cells, afterwards making cognate interactions with GC B cells within the GC reaction. T cell help into GCs are needed to maintain the reaction (59–63).

At present, a combination of markers is needed to identify Tfh cells as a distinct population. Tfh cells differentiate from the classical CD4+ T cell subpopulation and share plastic characteristics with other CD4+ helper T cells until they engaged in GC reactions. Inside GCs, the expression of typically described Tfh cells associated-molecules—CXCR5, PD-1, BCL6, BTLA4, ICOS (inducible T cell costimulator) and SAP—is upregulated whilst CD127, PSGL1, and EBI2 are downregulated (64–66). When these molecules are low-intermediate expressed, particularly CXCR5, PD-1, ICOS and SAP, define stage known as pre-GC Tfh cells (10, 66–69).

FOLLICULAR HELPER T CELLS IN MALIGNANCY

Peripheral T-cell lymphomas (PTCLs) are generally described as diverse and aggressive malignancies with unfavorable therapeutic outcomes (70). Nodal T-cell lymphomas with Tfh-cell phenotype are classified into three diseases: angioimmunoblastic T cell lymphoma (AITL), follicular T-cell lymphoma (FTCL) and nodal PTCL with Tfh cell phenotype. AITL is an aggressive rare tumor with a 5-year survival of only 33%, first described as a distinct clinic-pathologic entity in the 1970s and is the best well-established subtype of mature PTCL (71, 72). The tumors contain neoplastic

Tfh cells expressing BCL-6, CD10, CXCL13, PD-1, ICOS, SAP, and CXCR5 (10, 73–75). TET2, DNMT3A and IDH2 mutations have been detected in about 80% (76–78), 20–30% (76, 79, 80), and 20–30% of the cases (81), respectively. The mechanism of action described for these molecules is by dysregulating DNA methylation (70). A missense mutation in RHOA GTPase is detected in 50–70% AITL patients (77, 78, 82, 83). It has been described that some primary cutaneous T cell lymphomas also originate from neoplastic cells that express Tfh cells markers, and can also induce the typical rosettes found in AITL (84).

Representative clinical symptoms of AITL are generalized lymphadenopathy, hepatosplenomegaly, fever, effusion/ascites and skin rash. The incidence is low without sex predilection, affecting advanced-age individuals (median age of diagnosis 65 years) (72). Characteristically, lymph nodes acquire an effaced architecture, with only a few benign follicles been retained. A typical feature is to find infiltration beyond the capsule of the lymph node, with a preserved but enlarged subcapsular sinus; also high endothelial venules (HEV) and FDCs proliferate (71). Infiltration of other cells include: B cells, plasma cells, eosinophils, histiocytes and epithelioid cells. Active EBV infection can be found in most large B cells, whilst the malignant Tfh cells do not (72).

Cytological diagnosis of AITL is usually difficult and both reactive and lymphomatous processes need to be discarded. Combination of conventional cytology, immunocytochemistry and flow cytometry is needed to make an accurate diagnosis (85).

AITL, a lymphoma with poor prognosis, is often refractory to chemotherapy or relapses. Due to the unfavorable outcomes for PTCL patients treated with chemotherapy alone, autologous stem cell transplantation (SCT), as a consolidation treatment for first-line therapy or salvage therapy for relapse/refractory PTCL patients, may be an option. On the other hand, some relapse/refractory AITL patients may benefit from allogenic-SCT, presumably because of graft-versus lymphoma effects (74).

FTCL presents clinical and immunophenotype features of AITL but differs histologically. Two patterns have been described, one shows a GC-like growth with IgD+ B cells surrounding the neoplastic cells and the second resembles a FL-like pattern with malignant cells forming nodules. Another difference with AITL is the absence of proliferation of HEVs and FDCs (86).

While TET2, RHOA and DNMT3A mutations have been shown in both, AITL and FTCL, there is no evidence of IDH2 mutation in FTCL (87). Also, 20% of cases show a t(5;9) (q33; q22) (ITK/SYK) translocation but studies are limited (88).

The study and characterization of normal Tfh cells phenotyping led to the recognition and classification of previously diagnosed PTCLs-NOS (Not otherwise specified) to Nodal PTCLs with Tfh cell phenotype (84). While clinical, phenotypic, pathological, and genetic features overlap with AITL, further research is needed to include this neoplasm within the spectrum of one entity. Differences with AITL include the absence of expansion of HEVs and FDCs while histological differences from FTCL are due to the diffuse pattern of Nodal PTCLs with Tfh cell phenotype (86, 89).

TFH LYMPHOMAS AND THE INTERACTION WITH THEIR NICHES

The interaction of neoplastic Tfh cells and their niches has not been extensively studied. The reported infiltration of different immune cells and the proliferation of FDCs and HEV in AITL are probably caused by a stimulatory niche but the underlying mechanisms are still unknown. Some signals present in a normal counterpart niche like IL-21, IL-4, or/and IL-6 are over-expressed in AITL creating a loop of Tfh cell generation and FDCs growth (84, 90, 91). Also, this microenvironment could explain in part the depletion of Treg cells in AITL, an important population for suppressing Tfh cells in immune responses (92). Regulatory CAR T cells therapy might be a potential treatment to re-establish a favorable microenvironment.

Although neoplastic cells in FTCL show a GC- or follicle-growth pattern, the low incidence of this malignancy has made difficult the in-depth study of their interaction with resident cells. Understanding the crosstalk between neoplastic cells and their niche would definitely potentiate the development of more rationale treatments.

FOLLICULAR DENDRITIC CELLS: ORIGIN AND FUNCTION

Originally discovered by Alexander Maximow and subsequently termed FDCs by Steinman et al. in 1978, FDCs are critical participants in the GC reaction (93–96).

FDCs are stromal cells residing exclusively in B cell follicles, where they play a key role supporting B cell homeostasis and maintaining the follicular architecture. They are essential promoting robust humoral immune responses through the retention of antigens within immune complexes (ICs) over long periods. For this, FDCs express complement receptors (CRs)-1 and -2 and can be induced to express Fc-gamma receptor (FcyR) IIb (93, 97). Lymphoid organs lacking B cells or tumor necrosis factor (TNF) or lymphotoxin (LT) are devoid of FDCs (98–100). Mice lacking stromal CR1 and CR2 have reduced T-dependent antibody responses (93, 98, 101). ICs are released in FDCs-derived iccosomes, then cognate GC B cells can acquire antigen and present it to Tfh cells. FDCs also support the proliferation of GC B cells enhancing antibody production (93, 98).

FDCs are a subset very different from conventional DCs (cDCs). FDCs originate from stromal cells: it has been shown that in the spleen, FDCs come from vascular mural cells but in the lymph nodes, FDCs come from marginal reticular cells (MRCs) (102, 103). Nowadays, it is suggested that different stromal cells of secondary lymphoid organs—including FDCs and MRCs—are generated from one and the same precursor (93).

Functionally, whereas cDCs activate naïve T cells by presentation of processed antigens *via* major histocompatibility complex (MHC) molecules, FDCs show unprocessed antigens, trapped in ICs, to GC B cells. In addition, FDCs secrete the signalling molecule Mfge8 which has been shown to be essential in

controlling the removal of apoptotic GC B cells. It has been suggested that FDC-mediated phagocytosis of apoptotic GC B cells might play an important role in avoiding autoimmunity (93).

FOLLICULAR DENDRITIC CELLS TURNING TO THE DARK SIDE: FDC SARCOMA

Termed FDC sarcomas, the first reported cases of tumors derived from FDCs occurred in cervical lymph nodes. FDC sarcoma is classified as a distinct entity by the World Health Organization (WHO) under histiocytic and DC neoplasms Classification of Tumours. It is described as a neoplastic proliferation of spindled to ovoid cells with morphologic and immunophenotypic characteristics similar to those of normal FDCs. Despite the fact that their histopathological, morphological and clinical features have been described relatively in detail, their clinical course is unpredictable and no specific treatment is available (95, 104).

While FDC sarcomas do not have gender predilection, it mainly occurs during adulthood (median age in the fifth decade). Interestingly, a very rare and distinct variant of FDC sarcoma consistently associated with the EBV, termed inflammatory pseudotumor-like variant of FDC sarcoma, is more prevalent in females. Approximately 10–20% of FDC sarcoma cases have presented or concur with Castleman disease, a rare and non-malignant lymphoproliferative disorder, typically the hyaline vascular variant (104).

We now know also that FDC sarcomas can involve any anatomical area besides nodal sites. FDC sarcomas generally appear as a slow growing mass, an asymptomatic and painless cervical lymphadenopathy (95, 104, 105). Nearly a third of FDC sarcoma cases arise in extranodal sites: tonsils, skin, mediastinum, gastrointestinal tract and soft tissue (104, 106, 107). Furthermore, it seems there is an association between FDC sarcoma and the autoimmune diseases, paraneoplastic pemphigus and myasthenia gravis (104, 108–111).

Histopathology and cytomorphology of FDC sarcomas are characteristic, however their identification may be difficult and additional confirmation with immunohistochemical studies is frequently necessary. FDC sarcomas generally present the immunophenotype of normal FDCs, being positive for: CD21 (CR2), CD23 (Fc epsilon RII) and CD35 (CR1) (104, 112, 113). Clusterin and podoplanin are other molecules shown to have high sensitivity for FDC sarcomas (114–116). Clusterin shows strong positivity with weak to no expression in other DC tumors (114).

Especially when arising from extranodal sites, FDC sarcoma can often be misdiagnosed (106, 117). Then, differential diagnosis is needed, including interdigitating DC sarcoma, thymoma, spindle cell carcinoma, metastatic undifferentiated carcinomas, malignant melanoma and gastrointestinal stromal tumor (GIST) (104).

Clinical courses of FDC sarcomas are not consistent and consequently, treatment schemes are variable. Complete surgical resection seems to be the treatment of choice for both primary and recurrent lesions, with unclear benefits from radiation and chemical therapies (111, 118).

FDC SARCOMA AND ITS INTERACTION WITH LYMPHOCYTES

Being a very uncommon neoplasm, the in-depth study of FDC sarcomas have been difficult and almost neglected. Although it has been described an enrichment of Tfh and Treg cells in FDC sarcomas compared to other mesenchymal tumors (119), the interaction of malignant FDCs with other lymphocytes and other resident cells has not yet been studied. High levels of PD-1 and its ligands PD-L1 and PD-L2 (119) and the B/T cells mixed with the neoplastic population (120, 121) could point to a feedback from these lymphocytes to support the neoplastic niche and the evasion of effector immune cells.

CONCLUDING REMARKS

As discussed in this review, although lymphomas from GC B cells are explored in more detail and better understood, Tfh lymphomas and FDCs sarcomas need more attention.

Tfh lymphomas diagnosis is challenging, requiring multimodality methods including conventional cytology, immunohistochemistry, and flow cytometry. Usually with a poor prognosis, treatments need to be combined, frequently with unfavorable outcomes.

On the other hand, FDC sarcomas can often be misdiagnosed and differential diagnoses are needed. With variable clinical courses and unspecific and heterogeneous treatment at present, surgical resection is the treatment of choice.

Greater knowledge of the normal GC microuniverse will undoubtedly provide insights on its neoplastic side, allowing us better diagnosis, treatment, prognosis, and monitoring, the better to improve the quality of life of patients.

IN MEMORIAM

Dedicated to the memory of LF-R, a brilliant Mexican immunologist who inspired many generations of scientists through his passion. His legacy will last forever.

AUTHOR CONTRIBUTIONS

Conceptualization, LF-R and JY-P. Writing—Original Draft Preparation, RM-F, RM-A, and JY-P. Writing—Review and Editing, RM-F, RM-A, RC-S, LF-R, and JY-P. Supervision, LF-R and JY-P. All authors contributed to the article and approved the submitted version.

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REVIEW
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Lymphoma Heterogeneity Unraveled by Single-Cell Transcriptomics

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High-definition transcriptomic studies through single-cell RNA sequencing (scRNA-Seq) have revealed the heterogeneity and functionality of the various microenvironments across numerous solid tumors. Those pioneer studies have highlighted different cellular signatures correlated with clinical response to immune checkpoint inhibitors. scRNA-Seq offers also a unique opportunity to unravel the intimate heterogeneity of the ecosystems across different lymphoma entities. In this review, we will first cover the basics and future developments of the technology, and we will discuss its input in the field of translational lymphoma research, from determination of cell-of-origin and functional diversity, to monitoring of anti-cancer targeted drugs response and toxicities, and how new improvements in both data collection and interpretation will further foster precision medicine in the upcoming years.

Keywords: single-cell RNA sequencing (scRNA-seq), lymphoma, microenvironment, bioinformatics analysis, cell of origin (COO)

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INTRODUCTION

Like any other cancer, lymphomas are heterogeneous diseases exhibiting molecular aberrations at multiple levels. Adding difficulties to the understanding of lymphomagenesis, interactions with bystander stromal and immune cells in specific, highly organized microenvironments (ME) dictate tumor cell behavior. Since 2000, bulk transcriptomic and mutational profiles have been extensively characterized from pooled, heterogeneous mixtures of both cancer and ME cells, leading to the first classifications based on *cell of origin*. In diffuse large B-cell lymphomas (DLBCL, the most common lymphoma subtype), prognosis after chemo-immunotherapy differs according to germinal center or non-germinal center transcriptomic signatures. In follicular lymphoma (FL, second most frequent entity), prognosis is better predicted by the type of ME cells (T cells *versus* macrophages), rending interpretation of data dependent of the type and abundance of cells in a single biopsy specimen.

Single-cell RNA-sequencing (scRNA-Seq), by shedding light on gene expression levels across thousands of cells mixed into a patient's biopsy without sorting, has revolutionized our understanding of normal human tissues' anatomy, ontogeny, and diseases (1–3). Thanks to this technology, dissecting tumor heterogeneity is now increasingly an achievable goal in cancer care (4, 5). Indeed, development of resistance to most recent targeted agents originates both from tumor and ME transcriptomic variability, the latter directly influencing lymphoma phenotypic

heterogeneity. According to Darwinian laws, evolution selects the fittest phenotype, not genotype. Several studies have confirmed that genetic variations are observed in distinct ecosystems within the same tumor, and that spatial distribution of cellular subsets with specified transcriptomic signatures correlates to clinical outcome (6, 7). Lymphomas are a group of lymphoid tumors with widespread body dissemination (though not considered *metastases*), invading blood and lymph vessels. They are therefore asking daily the question of how to interpret a selective biopsy of tumor sample in the light of cancer heterogeneity.

Other reviews have provided excellent historical perspectives on the emergence and development of increasingly cost and time saving scRNA-Seq protocols, and how the commercial platforms now allow dissemination of knowledge in many Centers treating patients (4, 6, 8–10). After going through technological considerations about advantages/disadvantages of scRNA-Seq as compared to other techniques, our goals are to inform readers about latest insights in basic and translational lymphoma research about:

-*Cell of origin* of tumor cells
-Functional and phenotypic heterogeneity of lymphomas
-Inputs in clinical research: monitoring the response to therapy, and defining markers of early progression/toxicity (with an emphasis on the latest anti-lymphoma armamentarium: cellular therapies (CAR T-cells), and immune checkpoint blockers)

BULK RNA ANALYSES: WHAT HAVE WE LEARNED ABOUT INTRA- AND EXTRA-TUMOR HETEROGENEITY IN LYMPHOMA OVER 20 YEARS?

Malignant lymphomas mirror the complexity of immune system by many aspects. Since the advent of whole transcriptome profiling by Affymetrix-based microarrays, transcriptomics of tumor samples has enabled the identification of various molecular subsets of cancer cells, as originally the differential profiles of germinal center (GC)-like and activated B cell type (ABC) diffuse large B-cell lymphoma (DLBCL) defining cell (11). This has led to a better characterization of entities (>90 in the WHO2018 classification).

The genuine technology consisted in capturing each mRNA from a cellular lysate thanks to arrays of thousands oligonucleotide probes, each specific for a defined gene, and quantifying the captured mRNA by fluorescence signals (11). This allowed to quantify quite precisely the expression level of each gene taken individually, an information which once paralleled across the ~20,000 human genes, provided a global view of most cellular hallmarks of the cell types within the analyzed sample. Further direct sequencing of the mRNAs (RNA-seq) from bulk cell samples improved the sensitivity and precision of transcriptomes over the former microarrays, but did not revolutionize significantly the quality of the results: the microarray and RNA-seq based transcriptomes of a same sample give highly superimposable results. Various other declinations of the hardware part of this technology have emerged, such as to analyze more than just mRNA (*e.g.*, lcRNA, miRNA, …) in samples. Likewise, various transcriptomics computing algorithms have allowed to determine the genes differentially expressed (DEG) between two samples, the functional significance of DEGs by gene set enrichment analyses (GSEA) (12), as well as the inference of leukocyte cell composition of a tissue sample by deconvolution of its bulk transcriptome (*e.g.*, CIBERSORT) (13, 14), to quote a few. Since two decades, thousands of transcriptomics studies have been produced and were made freely available on public repositories (NCBI Gene Expression Omnibus (GEO), https://www.ncbi.nlm.nih.gov/gds, European Bioinformatics Institute's Array Express https://www.ebi.ac.uk/arrayexpress/). Despite such significant improvements however, transcriptomics remained bulk and, by lacking the ultimate resolution of its single cells taken individually, was the mere arithmetic mean of its cell constituents.

Bulk RNA studies in lymphoma patients proved that TME signatures strongly impacted prognosis in (15, 16), besides genetic lesions. Deconvolution of publicly available gene expression profiles (GEP) datasets from 480 DLBCL patients treated with R-CHOP (standard therapy frontline) has allowed to draw maps of TME in 2015 (CIBERSORT), through characterization of cell composition from their GEP (this method has been applied to 18,000 human tumors and correlated leukocytes subsets to survival across various cancers) (14). However bulk transcriptomic analyses that rely on RNA extraction from pooled cell populations from tumor tissue cannot identify low abundant population or rare cell subtypes and could be not able to differentiate cells with similar expression patterns. The characterization of the complex relationship between tumor and bystander cells requires a correct transcriptional characterization at cellular level. Digital cytometry (CIBERSORTx) establishes molecular profiles and specific gene matrices from single cell or sorted cell transcriptomes, and uses them to "barcode" other samples with bulk RNA available to evaluate cellular abundance and GEP. This method has been applied to isolate 49 distinct transcriptional states across 13 signatures including macrophages, neutrophils, fibroblasts, and T cells. An atlas of ecosystems in DLBCL correlating to somatic mutations and distinct from tumor-based GEP classification has been built. Though more applicable to large datasets, this interesting approach remains less effective than genuine scRNA-Seq for understanding of the function and/or phenotype of individual cell types, even rare or unknown cellular subsets without signature matrix known.

IMPLEMENTATION OF scRNA-SEQ: TECHNICAL CONSIDERATIONS

How we can tackle the obstacles discussed above with bulk RNA analyses? Among the huge development of single-cell technologies and computational advances to understand single cell transcriptomic profiling, the most commonly used technique remains scRNA-seq. This technology allows for precise

determination of cell counts (with the limitation sometimes that across samples, in a heterogenous cell mixture, certain populations might be lost), cell types, and fine identification of the transcriptomic hallmarks of each cell cluster present in the initial sample. Whether based on microfluidics or on micro-wells, each single cell from a sample of thousand cells is processed individually. Each single cell from cell suspensions is successively lysed, all its mRNAs are captured through their 3' end by an oligonucleotide carrying a cell-identifying tag, cut in smaller fragments (~100 nt), converted to cDNA, and amplified to produce a single cell library (capture can be done also by 5' end, but this technique is not commonly used yet). All the single cell-derived and tagged libraries are pooled together to constitute the sample library. The library is then separately sequenced, each initial mRNA fragment becomes a "read," yielding for the whole sample a file typically composed of 400 million reads, each corresponding to each initial mRNA fragment. In a further bioinformatics preprocessing of the sample, each read from this file is aligned *1)* on the sequence of the species transcriptome to identify its gene and *2)* to the cell-specific tag to identify its originating cell. This procedure is reiterated for all reads of the library such as to count how many reads are measured for each gene from each cell, yielding the so-called *(cell, gene)* matrix from the sample. Typically, a single cell RNA sequencing (scRNA-seq) matrix result comprises thousands of cells and about ten thousands of genes (since not all genes are detected and each cell does not express all the genes). Today, current scRNA-Seq technologies measure about <2,000 genes per cell. Further standard pre-processing of the data includes a normalization of all read counts and a quality control (QC) in which cells with too few genes, genes in too few cells, dead cells, and cell doublets are discarded from the dataset. A first step of data processing consists in clustering cells according to their gene expression profile, providing the most coherent and data-driven analysis of a mixed sample. To this aim, a principal component analysis is first performed to reduce the large dimensionality of all transcriptomes to their first principle components (PC). Once these fewer dimensions are selected upon user's decision based on the wanted precision, clusters of cells with similar profiles are delineated under the same user's criteria: low granularity makes less clusters of very different cells while more granularity means more clusters of more closely related cell types. Finally, the entire dataset is represented on bi-dimensional maps of cells, in which the above *n* first principle components are dimensionally reduced to two dimensions by sophisticated unsupervised algorithms such as t-distributed stochastic neighbor embedding (t-SNE). More recently, a superior method for both PCA and dimension reduction called uniform manifold approximation and projection (UMAP) has gained a large popularity for processing and visualization of scRNA-seq datasets (17). Within t-SNE or UMAP representations of those datasets, clusters are typically shown with colors and define cell subsets with high fidelity to the data: all cells alike are plotted next to each other while different cells form separate clusters with or without continuity to the former (**Figure 1**). Those steps are now performed by skilled bioinformatics users applying either

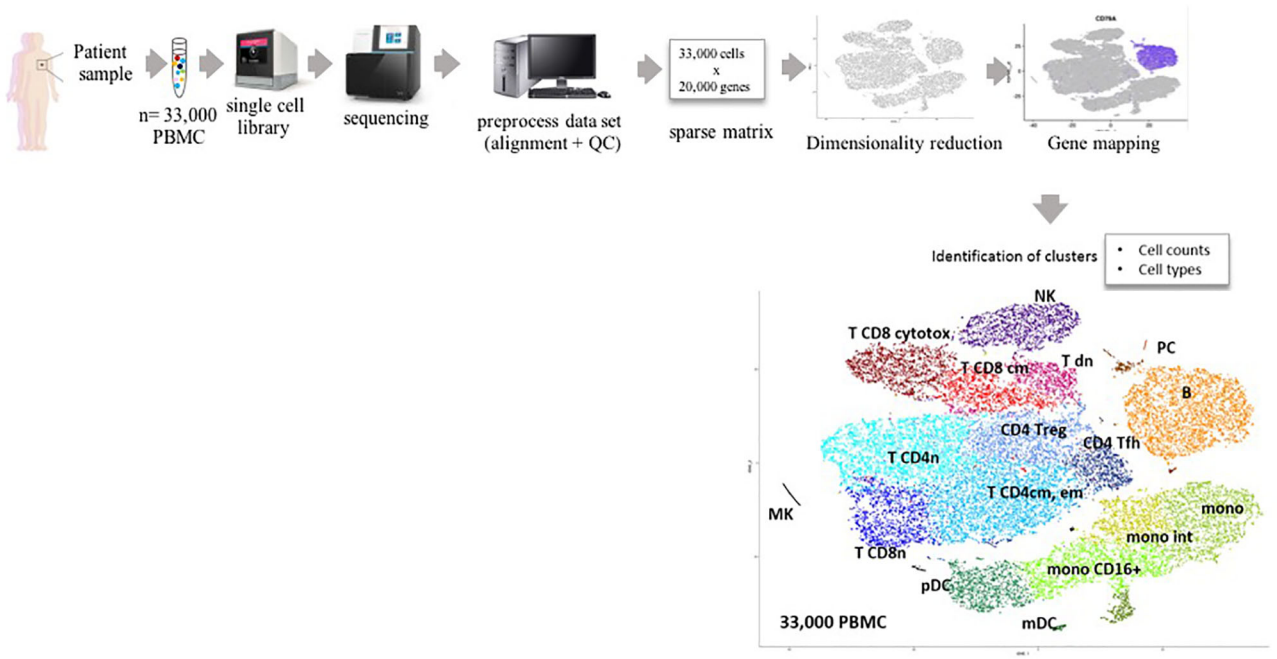

FIGURE 1 | Sample flow chart for single cell RNAseq analysis. PBMC, peripheral blood mononuclear cells; PCA, principal component analysis; QC, quality control; t-SNE, t-distributed stochastic neighbor embedding. Legend for the t-SNE map:MK megakaryocytes, mono, monocytes; int, intermediate; m/pDC, myeloid/plasmacytic dendritic cells; NK, natural killer; PC, plasma cells, and for T cells: cytotox cytotoxic, dn, double negative; cm, central memory; em, effector memory; fh, follicular helper; n, naïve; reg, regulatory.

R scripts from algorithms such as Seurat (18), or the proprietary Chromium™ (https://www.10xgenomics.com/) tools. Nevertheless, methods to infer cell types from scRNA-seq results are manifold, but still face challenges. Indeed, to identify a cell type, screening the dataset for cells expressing a specific marker gene is currently the most straightforward method. Data comprise many single cells in which many genes are often undetected for either biological (low expression level in the cell) or technical (read not sequenced or not captured) reasons.

APPLICATIONS OF scRNA-SEQ: IN SEARCH OF THE CELL OF ORIGIN AND SUBCLONAL DIVERSITY

The first demonstration of a true insight of scRNA-Seq approaches has been a pioneer study in a germinal center (GC)-derived tumor, called follicular lymphoma (FL), which shed light on the ontogeny of FL cells (19). By comparing single-cell expression of a panel of 91 pre-selected genes in cancer *versus* normal B-cells, the authors demonstrated a clustering between FL, normal GC, memory, and antibody producing cells (plasma cells). They developed an original pseudo-time inference algorithm which suggested a developmental ordering of gene expression in the latter cells (from plasmablasts to mature plasma cell). Such modeling of maturation inferred from a panel of genes expression was then applied to phenotypically defined GC cells subsets (light zone LZ $CXCR4^{lo}CD83^{hi}$ and dark zone DZ $CXCR4^{hi}CD83^{lo}$), this algorithm indicated that 26% of the cells were misclassified, with an intermediate CXCR4/CD83 phenotype, proving that our vision of GC maturation was incorrect. Normal GC B cells spanned over a continuum of gene expression, not fully captured by the two states model, as they cyclically transitioned from LZ to DZ, with about one third of cells being in an intermediate state sharing LZ/DZ markers. Furthermore, correlations (and anti-correlations) between discrete clusters of genes allowed to demonstrate a synchronized gene expression program defining identity of normal GC cells. Avoiding averaging effects of mRNA bulk analysis, scRNA-Seq study of five FL patients at diagnosis clearly showed heterogeneity of gene expression patterns within and between patients' samples, not seen in normal GC B cells. By comparing the 91 genes expression profiles, FL cells clustered separately from GC or memory cells, and with a major desynchronization of the GC-specific program, suggesting that despite sharing a common ancestral signature FL cells are genetically different from their putative cell of origin. Sample origin was a major source of genetic heterogeneity across the 5 FL patients, but intra-patient heterogeneity was not linked to subclonal genomic diversity, since *IGH* subclones tracking found that a given subclone could be found in different states (at least in the 91-gene panel). A second study (20) applied scRNA profiling to dissect the heterogeneity of GC tumors cell-of-origin (sc-COO). Given inter-donor consistency, in terms of gene signature associated to specific subsets of GC cells, they identified multiple functionally linked subpopulations (with some cells showing intermediate level of GC differentiation process between DZ/LZ), as well as the precursors of both memory B cells and antibody secreting cells (based on expression of *CCR6* and *PRDM1*). Moreover, a gene classifier (selecting the 50 up- and down-regulated genes in the sc signatures associated with these GC subpopulations) was built and applied on bulk RNA-Seq expression profiles from two published DLBCL panels (481 fro NCI and 230 from BCA). Non-GC cases were scattered across the sc-RNA Seq classes (but mostly in late GC stages) while the majority of GC cases were related with normal GC B cells in the LZ (like early of intermediate stages). Interestingly, 12% of DLBCL cases in both datasets displayed DZ gene signatures despite distributing across GC/non-GC/unclassified tumors. Together with an enrichment in double-hit (MYC/BCL2) cases, these results suggest a different ontogeny for this high-risk subset of DLBCL. Progression-free survival after R-CHOP was found to be dependent across the 5 prognostic categories isolated from the baseline 13 sc-*coo* signatures, proving that the latter identified clinically relevant subgroups of DLBCL patients.

To explain how these states are defined, cell-intrinsic genetic events and/or interactions with the microenvironment are possible. Mutations of epigenetic regulating genes are seen in 100% of FL cases (21–23) because they are key in the processes of initiation, apoptosis resistance, and progression of the disease, such as loss-of-function *KMT2D* (23) or *CREBBP* mutations (24, 25), or gain-of-function *EZH2* mutations (26). But besides those genes, genomic landscape of FL dramatically influences the nature of the ecosystem, as recently demonstrated with *CREBBP* (25), cathepsin S (*CTSS)* (27), *EZH2* (28), and *TNFRSF14/HVEM* (29) mutations. On the one hand, activation of *EZH2* gene attenuates T follicular helper (T_{FH}) cells' help for proliferation (indispensable to normal GC B cells differentiation) in FL cells, and drives slow expansion of centrocytes the characteristic niche of the tumor, embedded within the Follicular Dendritic Cells meshwork (30). On the other hand, loss of HVEM leads to FL proliferation by inducing tumor supportive ME with increased stroma activation and T_{FH} cells recruitment (31, 32). Lastly, *CREBBP* loss-of-function mutations (genocopied by *TET2* loss-of-function mutation) contribute to immune evasion *via* a decrease of class II trans-activator (CIITA)-dependent MHC class II expression (of both transcript and protein), leading in the tumor bed to a decrease of infiltration of both CD4+ helper and CD8+ memory cytotoxic T cells (25). Very seemingly, *CTSS* gain-of-functions mutations or amplifications allow for an enhanced MHC class II-restricted antigen presentation to CD4+ T cells, with better prognosis for patients.

But detecting genetic variants, such as mutations and copy number alterations, in scRNA-Seq reads is not an easy task. Tools have now been developed to study subclonal complexity of a tumor. Mutations can now be identified at the single cell level, to cluster tumor from normal cells, derive mutation-specific gene signatures, identify cell surface markers, and build phylogenetic trees of subclonal driver genes evolution, as shown in acute myeloid leukemia (31) (this has never been done in lymphomas). This latter study acquired transcriptional and mutational data in 16 AML and 5 normal bone marrows to profile AML tumor

ecosystems changes with therapy, and demonstrate for the first time that differentiated AML had immune-modulating properties against T cells. There is no such study to date in lymphomas, the scRNA-Seq profiling is being extensively used to build an atlas of tumor *versus* normal immune cells within specific micro-environments.

Still, indirect evaluation of oncogenes mutational status can be inferred from scRNA-seq approaches. In another study in six FL patients and five control specimens (32), authors managed to assign cells to eight different lineages, or immune subsets, in concordance with a 13-parameter FACS analysis for B, T, and NK cells but not monocytes (it is likely that development of CITE-Seq will tackle this kind of discordance). As expected from the study by Milpied et al. (19), evolutionary paths (different subclonal somatic mutations) followed by pre-malignant B cells to FL cells could be inferred from transcriptomic heterogeneity (normal B cells on the other hand clustered together). Differential expression analysis revealed transcription of genes specific to the tumor *versus* the normal B cells within a patient's sample. But most interestingly, samples with CREBBP mutation indeed had lower expression of *HLA-DR* gene than wild-type samples, but also lower expression by two separated clusters of quiescent subpopulations within a single tumor, suggesting scRNA-Seq captured transcriptomic consequences of genomic alterations inter- and intra-patients. In this study, coding somatic mutations deeply modified the expression of sets of genes, structuring the tumor into various subclones based on their genetic disparities (4–5 per sample), the sizes of which were similar regarding on the method used to quantify them (scRNA-Seq and exome-Seq). Therefore, gene expression heterogeneity, at least in part, was also attributable to subclonal genomic heterogeneity, even if other drivers of phenotypic diversity are stronger as proposed by the preceding study. Lastly, authors investigated T cells subsets and gene expression in double immune checkpoint expressing cells, confirming expression of genes inhibiting T-cell activation in CD4+ memory T cells co-expressing *TNFRSF18* and *TNFRSF4*. Overall, droplet-based scRNA-Seq with 10,000 cells only demonstrated its power to analyze tumor heterogeneity and infiltrating T cells phenotype.

APPLICATIONS OF scRNA-SEQ: DECIPHERING FUNCTIONAL DIVERSITY WITHIN ECOSYSTEMS

The tumor microenvironment (TME) is constituted by heterogeneous cellular populations including tumor cells and the surrounding non-malignant cells, such as numerous and distinct immune cells and stromal cells. Beyond the heterogeneity of the tumor cells (see above COO), the diversity and plasticity of the microenvironment also contributes to the intra-tumor heterogeneity (7, 33, 34). Strong evidences show that diverse immune subsets and their interactions within the tumor microenvironment are critical to diverse aspects of tumor biology, treatment response, and prognosis (35, 36). To avoid the need for manual annotation of cell types to existing data after unsupervised clustering, a probabilistic model [CellAssign (37)] has been developed, able to statistically frame the analyses of TME across samples and cancers by assigning cells to both known and *de novo* cell types in scRNA-seq data.

The TME is always a mix of lymphoma and normal cells [with Hodgkin's disease (HD) even as a paradigm of TME cells largely overwhelming the number of Reed-Sternberg cells] (38). Thus, a perfect isolation of the malignant cell population from a surgical biopsy is a significant challenge especially when tumor cells are low abundant. TME composition could be extremely variable according to the invaded sites (blood, bone marrow, or lymphoid secondary organs). It could be composed of extra-cellular matrix and stromal cells (shaping the architecture of the lymph node and T-B cells contacts), innate (myeloid and lymphoid) and adaptive immune cells, and vascular (blood and lymphatics for homing/egress from niches) cells (39, 40).

To date, the most detailed functional and spatial profiling of TME cells at the single cell resolution has been published in HD (41). Authors performed scRNA-Seq characterization of immune cells and assessed their spatial sub-localization out of 22 HD patients and five reactive lymph nodes. Transcriptome data from >100,000 cells and 1,200 genes in median identified 22 clusters, all being assignable to a cell type based on the published transcriptomes of sorted immune cells. No tumor specific cluster was found. But three regulatory T cells (Treg) clusters dominated the TME of HD. The cluster in HD cases with the highest proportion of immune cells was also enriched in LAG3 and CTLA transcripts, as opposed to controls where B cells and CD8+ T cells were enriched. Non-Treg CD4+ clusters also enriched in HD included Th2 and Th17 subsets. Treg CD4+ subsets in HD cases expressed GITR, CD25, not FOXP3 (and thus endowed with a type 1 Tr1 phenotype). Inhibitory receptor-mediated immune tolerance of HD cells is further reinforced by the co-localization of LAG3+ Treg near MHC class II-negative tumor cells by multicolor immunohistochemistry. Since FOXP3+ Treg were significantly reduced in the later samples (increased only in CMH class-II positive tumors), LAG3+ Treg are thus considered a disease-specific subset. In an independent series of 166 patients treated with standard ABVD regimen, IHC confirmed that expression of LAG3+ T cells correlated with the loss of CMH-class II by tumor cells, with no impact on prognosis.

Besides HD, ecosystem of cutaneous T-cell lymphomas (CTCL) has been evaluated in a study applying scRNA-Seq focusing on 14,000 CD3+ T lymphocytes (and a median of 1,200 genes) from four healthy skin donors and five advanced stage patients. The results revealed large inter-patient heterogeneity and no overlap with normal skin samples (42). Twenty-six clusters were identified and cell types annotated with normal dermis signatures. Greater heterogeneity was found at the level of lymphocytes, macrophages, keratinocytes, and fibroblasts. A gene expression signature identifies highly proliferative T cells, among which 17 genes were shared by all five patients' samples, including two markers assessable by IHC to diagnose aggressive CTCL. Over-expression of TIGIT, LAG3 and TIM3 by CD8+ and CD4+ T cells (exhaustion signatures) indicated strong rationale for

immunotherapies in this disease. Presence of TIGIT+ Treg correlated with lack of granzyme B and perforin in infiltrating CD8+ T cells. Such differences in malignant and reactive T lymphocytes in CTCL have been also reported in circulating form of a CTCL subtype (mycosis fungoides), called Sézary syndrome. Another group investigated this latter condition using scRNA-Seq and unraveled the importance for disease evolution of transitioning from *FOXP3*+ malignant T cells to *GATA3*+ or *IKZF2*+ cells. This transcriptional heterogeneity could be used to inform on prognosis, with FOXP3 and another set of 19 genes being involved in early progression in CTCL cases (43).

Beyond investigating heterogeneity, scRNA-seq has been also used to study putative cell-to-cell communications, inferred from the correlation of expression levels of paired ligand and receptor of individual cells (without knowing spatial proximity) (44–46). These original approaches have been extended thanks to an algorithm called NicheNet (47). One single example of the application of this computational approach in nine lymphoma patients has been published (48). This analysis suggested that cancer B cells could receive signals from all four major subsets of T cells (especially T follicular helper subset as the major source for IL-4, a putative resistance mechanism against ibrutinib). These dynamic stromal niches, as already reported in solid cancers (49), fully support outgrowth of lymphoma cells.

Other studies using scRNA-seq have also focused on depicting differentiation single-cell states, pro and antitumor function of immune cell and their distributions in cancer (50). Furthermore, the ability to define the TCR sequence at a single-cell level enabled to analyze the association between therapeutic response and activation states of specific T cell clusters (51, 52). Then, the development of single-cell transcriptomic technology enables to analyze the heterogeneity of immune subsets within the TME and emerges as powerful tools to screen immune-related signatures and identify potential biomarker which may as prognostic factors or therapeutic targets (53–55). International efforts attempting to set up a cancer human atlas at the single-cell resolution have been fruitful (3, 56), delineating immune contexture and activation state, for prognosis prediction and immunotherapy guidance in solid cancers. But data in lymphomas are still quite scarce (57).

scRNA-SEQ AND CLINICAL RESEARCH: MONITORING RESPONSE TO THERAPY AND UNDERSTAND RESISTANCE/ TOXICITIES

Most personalized therapies do not take into account heterogeneity of nodal lymphomas. A previously discussed paper elegantly investigated both malignant and non-malignant lymphocytes in 12 donors (nine with lymphoma and three reactive lymph nodes) (48). Authors have found coexistence of up to 4 transcriptionally distinct subpopulations of lymphoma cells, responding differently to treatments *in vitro* and *in vivo* in an example assessed at the time of relapse. This scRNA-Seq molecular profiling of transcriptomic signatures of resistant subclones will undoubtedly help tailor better therapies in each patient, and how resistance subclones evolve over time (clonal competition).

In relapsed/refractory DLBCL patients receiving CD19 chimeric antigen receptor T-cells (CAR T-cells), scRNA-Seq has been used to investigates biomarkers of early progression, but also of toxicities [cytokine release syndrome (CRS), immune cell activation neurologic syndrome (ICANS)]. Previous studies had highlighted the implications of T-cells subsets in the success of therapy. By doing scRNA-Seq analysis of CAR products in 24 patients, authors of a very recently published paper identified that exhausted T cell phenotype was more abundant in patients not entering complete response (CR) (58). But they also identified a very small subset of IL-1β+ and IL-8+ myeloid cells (<300) associated with more severe ICANS. These results, together with the explanation of scRNA-Seq curated data showing expression of CD19 by mural cells maintain brain-blood barrier integrity (therefore targeted by CARs) (59), elegantly demonstrated that efficacy and toxicity of immunotherapies can both be optimized.

Though not yet with an obvious application in the therapy of lymphoma, scRNA-Seq studies have been extensively used to predict immunotherapy responses (especially immune checkpoint inhibitors, or ICIs) in various solid cancers, based on T-cell infiltrating lymphocytes (TILs)'s characteristics in the TME, that target neo-antigens [review in (60)]. TILs in different cancers have proved to share common signatures, but also possess specific characteristics in line with the organized TME they reside in. After ICIs exposure, two studies published in 2018 (61, 62) have defined melanoma-specific and TILs-specific transcriptome signatures associated with outcomes. By pairing scRNA-Seq with TCR sequencing, two other groups showed that ICI induced expansion of T cells with different clonotypes (meaning recruitment of peripheral CD8+ T cells inside the tumor bed), rather than boosted the pre-existing TILs (63, 64). Paucity of myeloid cells subsets were also correlated to outcomes in another study (41). With the development of more and more ICIs strategies, in many cancers, and with the help of single cell-based technologies, this a revolution surging in the field of immunology, not only onco-immunology (65, 66).

UPCOMING IMPROVEMENT TO UNRAVEL TME FROM scRNA-SEQ APPROACHES

A major improvement has recently been brought by implementing scRNA-seq with use of DNA oligonucleotide-tagged antibodies (ADT), such as to integrate cell surface proteins together with transcriptome measurements. The resulting technique, called cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), analyses ADT-labeled cells by scRNA-seq to provide simultaneously both immuno-profiling and transcriptomics of the same single cells (67). ADT-labeling enhances the identification of each cellular population present in the samples, and avoid further misinterpretation of results. Data can be processed with dedicated

software, such as Single-Cell_Signature_Explorer and single cell virtual cytometer, the first published method for qualitative and scoring of single gene or gene-set based signatures (68). This method could thus provide informations about: cellular heterogeneity and evolution (our data, *in press*), clues of cell maturation through pseudo-temporal trajectory analysis (74) or cellular dynamics by RNA velocity determination (70), or definition of GC differentiation state based on CXCR4 and CD86 proteins expression levels, thus confirming sc transcriptomics data (20).

By its compatibility with various declinations of the scRNA-seq technology, the results of such multimodal analyses allow to reach an unapproached level of multi-omics characterization. On the other hand, detecting genetic variants, such as mutations and copy number alterations, in scRNA-Seq reads is not an easy task. Tools have now been developed to study subclonal complexity of a tumor. Mutations can now be identified at the single cell level, to cluster tumor from normal cells, derive mutation-specific gene signatures, identify cell surface markers, and build phylogenetic trees of subclonal driver genes evolution (31). More recently multi-omics single cell strategies are focused to study genetic, epigenetic, phenotypic, and transcriptomic profiles within the same cell. As examples, SCI-seq (single cell combination marker) provides cell copy number variation (71), scATAC-seq could identifies specific chromatin motifs (72), RAGE-Seq (repertoire and gene expression by sequencing) identifies B or T cell repertoire (73), scNGS could provide information about somatic mutations (1). All these approaches combined to new computational software should give huge knowledge of cellular heterogeneity, evolution, dynamics of lymphoma cells within their TME. Multi-omics (three techniques) evaluation of seven patients with chronic lymphocytic leukemia (CLL) exposed to ibrutinib has unraveled a consistent regulatory program of treatment-induced changes, but at a pace that varied among patients. First events were signs of NF-κB inhibition, followed by reduce activity of lineage transcription factors, resulting in erosion of CLL cells' identity and after a few months, acquisition of a dormant, quiescence-like gene signature (72). This important study is the first to suggest that multi-omics approaches, combined with multi-timepoints analyses, are able to predict the molecular response to a kinase inhibitor in lymphoma patients.

CONCLUSION

To advance cancer research and resolve heterogeneity, we need integrated single cell multi-omics platforms to study cell-by-cell (tumor, immune, stroma) transcriptomes, proteomes, methylomes, cell surface proteins, localization within the tumor, and even future evolution through (pseudo-time analysis), across various patients' datasets. The information of the precise localization of normal immune *versus* tumor cells will be resolved in part through spatial transcriptomics. Thanks to newer bioinformatics tools, we need to understand the functional significance of the different clusters, better visualize them, and aggregate our data, from labs to labs all together, in a global effort of a multi-modal integration across generated datasets of tumors worldwide. These efforts will be paid back by enhanced risk stratification, disease monitoring, and personalized therapy in lymphomas, like a few studies in multiple myeloma and acute myeloid leukemia have already demonstrated.

AUTHOR CONTRIBUTIONS

LY, AQ-M, CL, MT, FP, and J-JF wrote the manuscript and analyzed the data. All authors contributed to the article and approved the submitted version.

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REVIEW
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AID in Chronic Lymphocytic Leukemia: Induction and Action During Disease Progression

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The enzyme activation-induced cytidine deaminase (AID) initiates somatic hypermutation (SHM) and class switch recombination (CSR) of immunoglobulin (Ig) genes, critical actions for an effective adaptive immune response. However, in addition to the benefits generated by its physiological roles, AID is an etiological factor for the development of human and murine leukemias and lymphomas. This review highlights the pathological role of AID and the consequences of its actions on the development, progression, and therapeutic refractoriness of chronic lymphocytic leukemia (CLL) as a model disease for mature lymphoid malignancies. First, we summarize pertinent aspects of the expression and function of AID in normal B lymphocytes. Then, we assess putative causes for AID expression in leukemic cells emphasizing the role of an activated microenvironment. Thirdly, we discuss the role of AID in lymphomagenesis, in light of recent data obtained by NGS analyses on the genomic landscape of leukemia and lymphomas, concentrating on the frequency of AID signatures in these cancers and correlating previously described tumor-gene drivers with the presence of AID off-target mutations. Finally, we discuss how these changes could affect tumor suppressor and proto-oncogene targets and how they could be associated with disease progression. Collectively, we hope that these sections will help to better understand the complex paradox between the physiological role of AID in adaptive immunity and its potential causative activity in B-cell malignancies.

Keywords: AID, CLL, microenvironment, off-target mutations, SHM

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INTRODUCTION

In general, cancers progress by the emergence of subclones with additional, distinct genomic aberrations not recognized in the initial tumor. These subclones possess advantages in cell survival and/or growth. It is still debated whether these more aggressive cell variants are present from the beginning of the neoplasm, develop afterwards, or are induced or selected by therapy. Regardless, the key concept is that these variants must be generated to lead to disease progression and therapeutic resistance.

DNA abnormalities can result from several processes, either inherent to the cell type being considered and/or induced by environmental insults. In this document, we concentrate on the ability of activation-induced deaminase (AID) to generate such genetic variants, focusing on the specific leukemic subsets in which AID protein is upregulated. Finally, we approach the pathological role of AID and the consequences of its actions on the development, progression, and

therapeutic refractoriness of chronic lymphocytic leukemia (CLL) as a model disease for mature lymphoid malignancies.

ACTIVATION-INDUCED DEAMINASE IN NORMAL B LYMPHOCYTES AND ITS EFFECTS ON NORMAL B-CELL BIOLOGY

AID as a Key Molecule in the Adaptive Immune Response

The immune system of vertebrates is unique because the antigen-specific receptor expressed by lymphocytes, which initiates cascades leading to activation of the adaptive immune system, is not the product of a single germline inherited gene. Rather, receptors are generated somatically during cell ontogeny from genes scattered at a particular locus (1). Specifically, an individual B lymphocyte acquires the capacity to respond to external antigens (Ags) but not to self-antigens by a complex and regulated tolerance mechanism. Humoral immunity depends on the production of immunoglobulins (Igs) capable of recognizing the full range of these Ags with high affinity. The generation of this diversity is linked to three different modifications in the genes encoding the Igs: (a) *genetic recombination*, which takes place on the genes encoding the variable (V), diversity (D) and junction (J) regions. This recombination gives rise to the formation of the variable domain of an IG, which will then be associated with the constant region Cμ to establish the first repertoire of IgM-type Ig (2). This event occurs in the fetal liver and in the bone marrow and is independent of the interactions of the B cell with Ag and/or T lymphocytes.

The following two steps take place when B cells meet Ag through the B-cell receptor (BCR). After this exposure, B lymphocytes accumulate in secondary lymphoid organs (SLO) in which two other genome modifications take place: (b) *somatic hypermutation (SHM)* occurs in the variable domains of Ig heavy chains (VH) and light chains (VL) introducing point mutations mainly in the complementary determining regions (CDRs) of VH and VL domains (3), and (c) *class switch recombination (CSR)*, which is also dependent on the Ag and takes place at "pre-switch" regions causing the deletion of portions of the IgH (switch regions) located upstream of each Ig isotype locus, thereby permitting the assembly of the variable domain (VDJ) to the constant domain of a heavy chain (CH) located downstream. Through this process, the Ig effector function exerted by the CH domain is altered, giving rise to the expression of different Ig isotypes (IgG, IgA, and IgE) (4). Isotype-switched Igs more readily leave the circulation and enter solid tissues and eliminate foreign insults. Both SHM and CSR take place in SLO and are Ag- and T cell-dependent (5).

AID Structure and Function

AID is coded by the *activation-induced cytidine deaminase* (*aicda*) gene that in humans is located on chromosome 12p13 and is a member of the APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide) family of deaminases. Because the two genes, APOBEC-1 and AID, are found at the same portion of the same chromosome, it is assumed that they came about from a duplication event (6). AID is a molecule of 198 amino acids with a relative molecular size of 24 kDa and has a 34% identity with APOBEC-1 at the amino acid level (7).

The AID gene (*Aicda*) was discovered by a subtraction technique using Ig isotype switch-induced and uninduced derivatives of a murine B-cell line (7). The fact that AID has a catalytic cytosine deamination domain highly conserved among all members of the APOBEC family led to the recognition that the physiological role of AID in B lymphocytes is the induction of mutations and deletions of segments of DNA (8–11). AID can deaminate deoxycytidine in a single-stranded DNA, thus converting it to deoxyuridine (12). This is already a mutagenic lesion causing a C:G to T:A base change after replication. Processing of the uracil by base-excision repair (BER) and mismatch repair (MMR) enzymes leads to the broader spectrum of point mutations characterizing SHM, and to DNA double strand breaks, which are necessary intermediates in CSR [**Figure 1** and reviewed in (14, 15)].

Similar to APOBEC-1, AID has cytidine deaminase activity *in vitro* that is inhibited by tetrahydro-uridine, a Zinc ion chelator (7). Despite the fact that, by analogy with APOBEC-1, initially AID was proposed to deaminate cytidines in specific RNAs, no evidence showing this action was documented. However, AID does display a mutational preference for small RNA genes suggesting a putative role for RNA in its recruitment (16, 17). Rather, the DNA deamination model proposing that AID promotes antibody diversity by deaminating deoxycytidine (dC) to deoxyuridine (dU) within Ig genes is currently accepted (18). AID acts on single strand DNA (ssDNA) by inducing multiple deaminations per binding event while remaining bound to the same ssDNA (19, 20). A detailed review of the mechanisms explaining AID activity and the biochemical, biophysical, and structural characteristics of AID activity are reviewed in Feng et al. (15) and Methot and Di Noia (21).

Structure–function relationships of AID have been probed by different *in vitro* experiments focused on natural and engineered mutations in the protein. Interestingly, these data underline differential roles for the N-terminus of AID, which appears to be involved in SHM but not in CSR, whereas the C-terminus appears to selectively initiate CSR (22–24).

Four splice variants of *Aicda* have been identified that remove portions of the C and N termini. Detailed analyses of the expression of these variants have indicated that human centroblasts, the cells that are actively producing AID and undergoing SHM, express only full-length *Aicda* messenger, whereas centrocytes, which are re-expressing membrane Ig and undergoing antigen selection to identify and preserve high-affinity antigen binders, produce full-length and spliced forms of the mRNA (25). Since AID functions as a dimer, these findings suggest a potential inhibitory and amplificatory role for the splice variants, since they could heterodimerize with the full-length form and alter its functional capacities.

Finally, an experiment of nature highlights the importance of AID in CSR and SHM. Hyper-IgM syndrome, type 2, is an autosomal recessive disease caused by mutations in *aicda* (11). People with this syndrome have elevated levels of serum IgM and

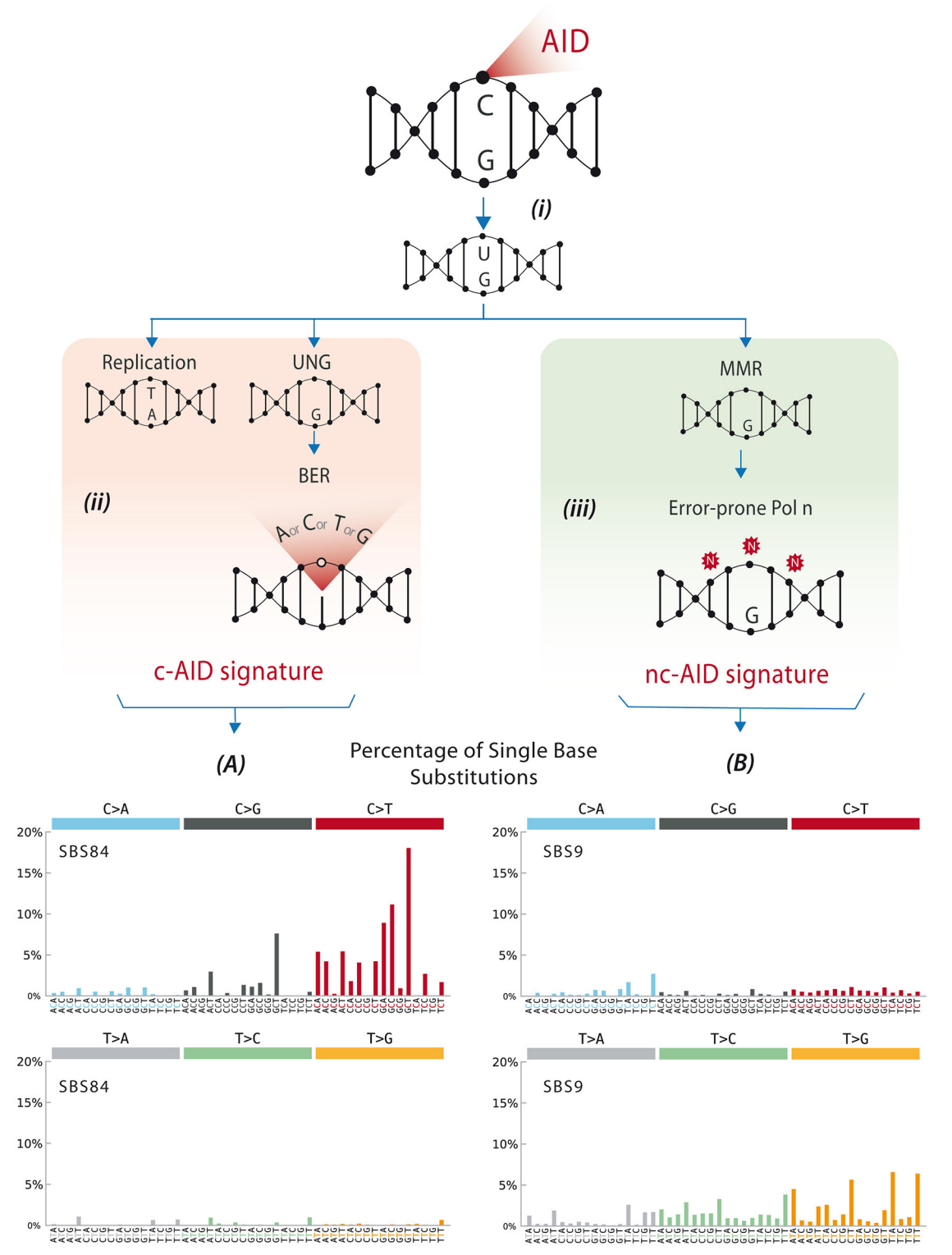

FIGURE 1 | Differential processing of AID lesions. **(i)** AID deaminates cytosine residues on single-stranded DNA that is exposed during transcription, converting C (cytosine) into U (uracil). **(ii)** The U-G (guanine) mismatch can be processed through different pathways. Either by replication that will result in a C>T transition or by

(Continued)

FIGURE 1 | uracil DNA glycosylase (UNG) followed by base excision repair (BER) resulting in C>T/G/A substitutions or homologous repair. This will often result in a mutational profile known as canonical AID signature (c-AID). **(iii)** On the other hand, mismatch-repair proteins (MMR) can also recognize and process AID-induced lesions. Exonucleases resect the abasic sites, which are followed by error-prone polymerase repair. This processing often results in a mutational profile similar to the non-canonical AID signature. Lower panels **(A,B)** depict mutational profiles using the conventional 96 mutation type classification. This classification is based on the six substitution subtypes: C>A, C>G, C>T, T>A, T>C, and T>G (all substitutions are referred to by the pyrimidine of the mutated Watson–Crick base pair). Each of the substitutions is examined by incorporating information on the bases immediately 5′ and 3′ to each mutated base generating 96 possible mutation types (6 types of substitution × 4 types of 5′ base × 4 types of 3′ base). Mutational signatures are displayed and reported based on the observed trinucleotide frequency of the human genome, i.e., representing the relative proportions of mutations generated by each signature based on the actual trinucleotide frequencies of the reference human genome version GRCh37. **(A)** SBS84 is found in clustered mutations in the immunoglobulin gene and other regions in lymphoid cancers. **(B)** SBS9 may be due in part to mutations induced during replication by polymerase eta. Mutation frequencies were retrieved from the Comic Catalog v3.1 (cancer.sanger.ac.uk) (13).

markedly diminished levels of switched Ig isotypes, consistent with a defect in CSR. Additionally, their Ig molecules do not develop V region mutations, indicating the inability to carry out SHM. Inactivating *aicda* in the murine B cell line used to identify AID leads to similar functional defects (8).

Molecular Actions and Targeting of AID

AID is a highly efficient DNA mutator, based on the much greater frequency that it alters Ig loci ($\sim 10^{-4}$-10^{-3}/base pair/generation) compared to mutations occurring spontaneously in the genome ($\sim 10^{-9}$) (26). Because of its potency, AID's actions need to be tightly regulated. This is accomplished at the transcriptional, post-transcriptional, and post-translational (27–35) levels as well as by its location, which is predominantly cytoplasmic (36, 37).

Additionally, its actions are normally rigorously restricted to Ig loci of B lymphocytes. This is done in a lineage specific and a stage-specific (activated B cell) manner. Restriction to B lymphocytes is controlled primarily by lymphoid-specific transcription factors (38–42). Once directed to the Ig loci of B cells, AID directs its action to the variable (V) and switch (S) regions. At the V region, mutations start shortly downstream of the promoter and proceed for ∼2 kB (43). At S regions, mutations are found downstream of the intronic promoter and extend ∼4–6 Kb beyond (44). Of note, AID-mediated mutations are rarely found in the C region (45, 46).

AID activity is characterized by cytidine deamination, and repair of U:G lesions may result in mutations within defined trinucleotide signatures. Direct replication over the AID-induced lesions or removal of the uracil by UNG (uracil DNA glycosylase) followed by replication accounts for the mutations of the canonical AID signature (c-AID) (C to T/G mutation at RCY motifs, R = purine, Y = pyrimidine) (47). Processing of the AID-induced lesions by the error-prone DNA polymerase η may result in A>C transversions at WA motifs (W: A or T, A), earlier described as the non-canonical AID signature (nc-AID) (48) (**Figure 1**). The signature generated by polymerase η repair could reflect either an initial AID-induced lesion or the consequence of other mutagenic events, and therefore the nc-AID signature may be less informative of AID activity than the RCY motif (47, 49, 50). COSMIC analyses on tumor samples in general also demonstrate that this signature is less representative than the classical c-AID signature (**Figure 1**). A third pattern, recently described in association with AID-mediated CpG-methylation dependent mutagenesis, is characterized by C>T transitions at RCG motifs (49, 51).

AID preferentially targets cytidines (C) at WRC (W = A/T, R = A/G) hotspots (52–54), known targets of SHM (55–60). The resulting mutations are the consequences of the enzyme's direct action as well as from the repair mechanisms (BER or MMR) that follow to correct or not the change. These mutations are primarily transitions rather than transversions (∼2:1) (**Figure 1**). Additionally, the overlapping AID hotspot, WGCW, has a markedly enhanced propensity to be deaminated (61). If overlapping hotspots in CDRs are not altered, there is a significant decrease in the mutation frequency throughout the V region (61, 62).

SHM is focused to DNA sequences that are being transcribed (63–65), occurring most effectively at pause sites (66). Consistent with AID acting on ssDNA is the finding that highly transcribed, non-Ig genes can be mutated (*Bcl-6, Bcl7, Myc, Pax-5, Pim-1, Rhoh, S1pr2, Socs1, CD95,* and *mir142*) (26, 67). This occurs in normal B cells (68–79) as well as in B-cell lymphomas (80–82).

Thus, in some instances, restricting AID's mutational and deletional functions to the Ig loci is comprised, resulting in DNA alterations at other sites in the genome. This aberrant action has been found in cancers not only of the B-lymphocyte lineage (83, 84) but also in other lymphoid (85) and non-lymphoid types (86, 87). Moreover, we (88) have reported data consistent with this occurring in CLL, which will be discussed in detail below.

Normal B Lymphocytes Expressing AID

AID is expressed in activated B lymphocytes, specifically in cells undergoing germinal center activation (centroblasts) (7, 89). Based on finding the gene upregulated upon immunization and finding it in B lymphocytes localized in germinal centers, AID was shown to be crucial in murine and human germinal center B cells, although it can be found in extrafollicular B cells (38). AID can be upregulated in mature B lymphocytes in T-cell-dependent and T-cell-independent manners. The former is carried out by CD40–CD40L interactions in the presence of IL-4 (90). T-cell-independent stimulation can be achieved with TLR7 + IL-4 (91) and by dual engagement of TLRs and the BCR (92–94). Both stimuli upregulate AID and lead to CSR and/or SHM. Moreover, a combination of T-dependent and T-independent stimuli do the same. Notably, in this setting, the combination can reduce the level of signal needed to accomplish AID expression and action (95).

However, the effects of BCR engagement on the production of AID by mature murine B cells stimulated *in vitro* appear to be time dependent. For example, B cells stimulated T-independently

by LPS + IL-4 upregulate AID levels dramatically by 48 h. However, engaging the BCR at that point reduces AID levels to essentially baseline within ∼ 4 h (96). This fall is due to a block in transcription. The rapidity and efficiency of BCR engagement in downregulating AID is probably aided by the very short half-life of the enzyme. This fall in production is consistent with a negative feedback loop initiated in an activated cell that has achieved enhanced affinity for the stimulating antigen through V region mutations induced by AID.

Finally, although in general AID is found in activated mature B lymphocytes, several reports suggest that it can be found in less mature, developing (immature/transitional 1) B cells (93, 97–100). These young cells can undergo CSR to produce non-IgM antibodies (93, 101, 102) and SHM to generate higher-affinity antibodies (97). Expression of AID in these cells can be constitutive (93, 97–99) or induced by CD40 (103, 104) and TLR (93, 94) signaling.

THE ROLE OF AID IN CANCER

AID-generated uracils are recognized by the uracil-DNA glycosylase (UNG) or the MMR heterodimer MSH2/MSH6 (105). The processing of these uracils produces double-strand breaks, which are the substrates of the end-joining mechanisms that complete CSR by joining two separate S regions (106). Although AID was originally believed to specifically target Ig gene V and S regions, it has become evident that AID also deaminates other genes, which, unless faithfully repaired, can be oncogenic (107). UNG and MSH2/MSH6 modulate the mutagenic capacity of AID either by initiating error-free BER and MMR, respectively, or by triggering mutagenic repair (26).

The first evidence about the oncogenic role of AID was supported by mice models in which constitutive *Aicda* gene expression was forced (108). AID-transgenic mice show tumors developed in various cells, in which mutations accumulate in non-Ig genes, including proto-oncogenes (108, 109). However, the number of gene AID that actually targets is not clear (18). Using AID chromatin immunoprecipitation (ChIP), Yamane et al. (67) showed that AID binds to thousands of genes in activated B cells but mutates only a fraction, although these data have been contested (110). So, although the number of non-Ig genes targeted by AID remains elusive, it is clear that AID can initiate chromosomal translocations or point mutations, some of which can be oncogenic (74, 111).

In a normal setting, DSBs are promptly repaired; for instance, homologous recombination prevents widespread DNA breaks by AID (112). Still, since the main factor influencing the rate of translocations is the formation of DSBs, continual localized DNA damage by AID probably favors recurrent translocations (74). A prime example is the *IgH/c-Myc* translocation typical of mouse plasmacytoma models and a hallmark of Burkitt's lymphoma in humans (72, 113). C-myc transgenic animals develop pre-B lymphomas or B lymphomas without SHM, whereas ubiquitous AID transgenic overexpression is sufficient to cause T cell lymphomas, lung adenomas, and adenocarcinomas (108).

Endogenous levels of AID are sufficient to predispose B cells for transformation. This has been demonstrated in IL-6 transgenic or pristane-induced plasmacytomas, in which AID is crucial for the creation of the *IgH/c-Myc* translocation (114, 115). Additionally, similar experiments indicate the importance of AID for diffuse large-cell lymphoma (DLBCL)-like malignancies in the Iμ-BCL6 transgenic mouse model (31). Interestingly, AID deficiency reduces the incidence of B lymphomas, but not pre-B lymphomas, whereas this deficiency also prevents GC- and post-GC-derived lymphomas, but not marginal zone lymphoma development, in Iμ Bcl6 transgenic mice (109). These results indicate that AID is mainly involved in tumorigenesis in mature, activated B cells (18).

AID is expressed physiologically in GC B cells (8) and accordingly in GC-derived human B-cell lymphomas, such as diffuse large B-cell lymphoma (DLBCL), follicular B-cell lymphoma (FL), and Burkitt lymphoma (BL), which express AID constitutively (116–119). While aberrant SHM in normal and lymphoma B cells affects many proto-oncogenes and tumor suppressors (*Myc, Ig alpha, Pax-5, Bcl-6, Rhoh,* and *Pim-1*) (80, 120–122), presently no direct evidence has been published relating an AID DNA mutation signature with these off-target mutations.

AID is consistently expressed not only in neoplastic cells in Hodgkin's lymphoma but also in many other human hematological malignancies including CLL (123–125), B-cell acute lymphoblastic leukemia (126), mantle-cell lymphoma (127), and in some cases of MALT lymphoma, which derives from marginal zone B cells in mucosa-associated lymphoid tissue, of immunocytoma, which is derived from plasma cells (117, 119), and of hairy cell leukemia, which derives from memory cells (128). These studies indicate that AID can be expressed not only in GC-derived B-cell lymphomas but also in leukemias and lymphomas originating from B cells at various stages of differentiation (18). In addition, AID expression can be found in a number of non-B cell malignancies including epithelial cancers such as *H. pylori*-associated gastric cancer (129), hepatocellular carcinomas (130, 131), and lung carcinomas (132). AID expression can be especially problematic in chronic diseases, where even a small but continuous level of AID activity could lead to selectable genetic mutations over time, giving rise to more aggressive tumors and treatment resistance.

CHRONIC LYMPHOCYTIC LEUKEMIA: THE ROLE OF MICROENVIRONMENTAL SIGNALING ON INTRACLONAL CLL FRACTIONS

CLL is characterized by progressive accumulation of monoclonal B-lymphocytes expressing CD5 and CD23 molecules and characteristic low amounts of surface membrane Ig and CD79b molecules (133). Interestingly, in this leukemia, the mutational profile of VH immunoglobulin genes (IgHV) divides patients into two categories (134, 135) that differ dramatically in prognosis (136, 137). Patients expressing mutated IgHV (M-CLL)

develop a more indolent disease, whereas IgHV unmutated (U-CLL) patients display a more aggressive disease that is often unresponsive to treatment. Although CLL remains an incurable disease, very important progress in this area of knowledge has been recently achieved. The accumulation of mature B-cells, which have escaped programmed cell death and have undergone cell cycle arrest in the G0/G1 phase, is characteristic of CLL. In contrast with these *in vivo* features, apoptosis occurs after *in vitro* culture, suggesting an important role of the microenvironment on CLL cell survival (138, 139). CLL is the prototype of a cancer where both genetic and microenvironmental factors promote the onset, expansion, and progression of the disease (140, 141). Although classically CLL was considered a disease of accumulation, new data indicate that CLL expansion is a dynamic process in which cell proliferation compensates for the cell loss from death (142, 143) and that accumulation of the malignant cells reflects a balance between the effects of cell proliferation and death (144). Moreover, the balance between cell proliferation and cell loss appears to vary in different subsets of the disease, which have been defined based on the cell genotypic and phenotypic features (143, 145). The seminal hypothesis postulating that phenotypic cell heterogeneity could exist within the tumor clone of the same CLL patient was first explored two decades ago. The notion that the leukemic clone could hold CLL cells either phenotypically activated or kinetically resting leads to key questions in the CLL biology: (a) Are there distinct subpopulations within the tumor clone? (b) Does the microenvironment favor the development of proliferative CLL subpopulations? (c) Which microenvironmental elements influence the malignant clone and which molecular pathways do they utilize? (d) Is the microenvironment affecting the relationships between proliferation and extended survival?

Evidence for the important role of the BCR in CLL pathogenesis is given by the fact that the mutational status observed in BCR sequences divides CLL cases into two subsets (134, 135), and this is one of the strongest predictors of disease outcome (136, 137). In addition, BCR signaling has been postulated to play a role for CLL trafficking and interaction with the stromal microenvironment (146). Increasing evidence suggests that CLL cells in lymph nodes (LN) and bone marrow (BM) interact with stromal cells and thereby receive proliferative and survival signals. Disease prognosis and evolution is probably related to the consequence of this interaction. In line with this hypothesis, CLL cells residing in LN show increased proliferation when compared to leukemic cells in the bone marrow (BM) and peripheral blood (PB) (147) and have a gene expression profile compatible with activated B cells (148).

Pseudofollicular proliferation centers (PCs) are classical anatomical structures in CLL patients similar to those observed in inflamed tissues of patients with chronic autoimmune/inflammatory disorders. These PCs are composed of a complex mixture of T cells, monocyte-derived cells, and stromal cells that provide pro-survival signals and form suitable niches for tumor growth (149, 150). CLL cells in PCs can have higher levels of the proliferative marker Ki-67 (151). Microenvironmental signals in additional to BCR that appear to be delivered in tissues important for the crosstalk between CLL cells and their microenvironments involve CD40 (138), TLR (152), B-cell activating factor receptor (BAFFr), and transmembrane activator CAML interactor (TACI) (153). However, the relative, individual pathogenetic influences of each molecule are unclear, as it is unknown to what extent they cooperate with the BCR stimulation in different patients.

During recent years, a variety of novel kinase inhibitors targeting various components of the BCR signaling pathway have been designed. These mainly inhibit phosphoinositide 3'kinase (PI3K), Bruton's tyrosine kinase (BTK), and spleen tyrosine kinase (SYK). Each of these shares a pattern of response resulting in nodal reduction and increased lymphocytosis, thus reflecting microenvironmental modulation (154). These new drugs affecting the signaling activation pathway have generated significant promise by targeting the proliferative pools existing in BM and LN and inducing release from and preventing re-entry to survival niches, thus bringing us closer to curing the disease.

Intraclonal CLL Fractions as a Marker of Disease Progression

The presence of proliferating and accumulating clonal CLL cells inside of the tumor clone in the same patient was postulated by Caligaris-Cappio (155), and this was confirmed in 2005 by Messmer et al. (143). In the latter, CLL patients drank deuterated water (^2H_2O), and ^2H incorporation into the DNA of newly divided CLL cells was measured. Collection and analysis of these data indicated that the leukemic cells of each patient had definable and substantial birth rates, varying from 0.1 to 1.0% of the entire clone per day. These findings were confirmed and extended by others (156, 157). More importantly, the former suggested that those patients with higher proliferation rates experienced a more progressive disease than those with lower birth rates (143), and this was confirmed in a larger, independent study (158).

Based on these observations, two key concepts in the CLL biology were established: (1) CLL is not a static disease but a disease of proliferating and over-surviving pools, and (2) the balance between these subsets explains, at least in part, the heterogeneous clinical outcome of CLL patients (144, 146). These two concepts are exemplified in **Figure 2**.

In the ensuing years, efforts have been made to characterize the proliferative compartment of the leukemic clone considering that the study of this fraction could lead to new therapeutic targets in CLL. Initial studies focused on leukemic cells expressing CD38 ($CD38^+$), a molecule involved in signaling and activation that also serves as a prognostic marker in the disease. This identified a close association between the expression of CD38 expression and of increased Ki-67 and ZAP-70 levels, suggesting that the $CD38^+$ fraction contains more activated members and could more frequently enter the cell cycle than the counterpart $CD38^-$ fraction (159). However, and despite these activation/proliferation differences, both $CD38^+$ and $CD38^-$ fractions have similar telomere lengths, suggesting that CD38 expression is a temporal and dynamic marker of an activated B-cell status instead of a specific marker of the proliferative fraction in CLL. As previously mentioned, CD38 expression is

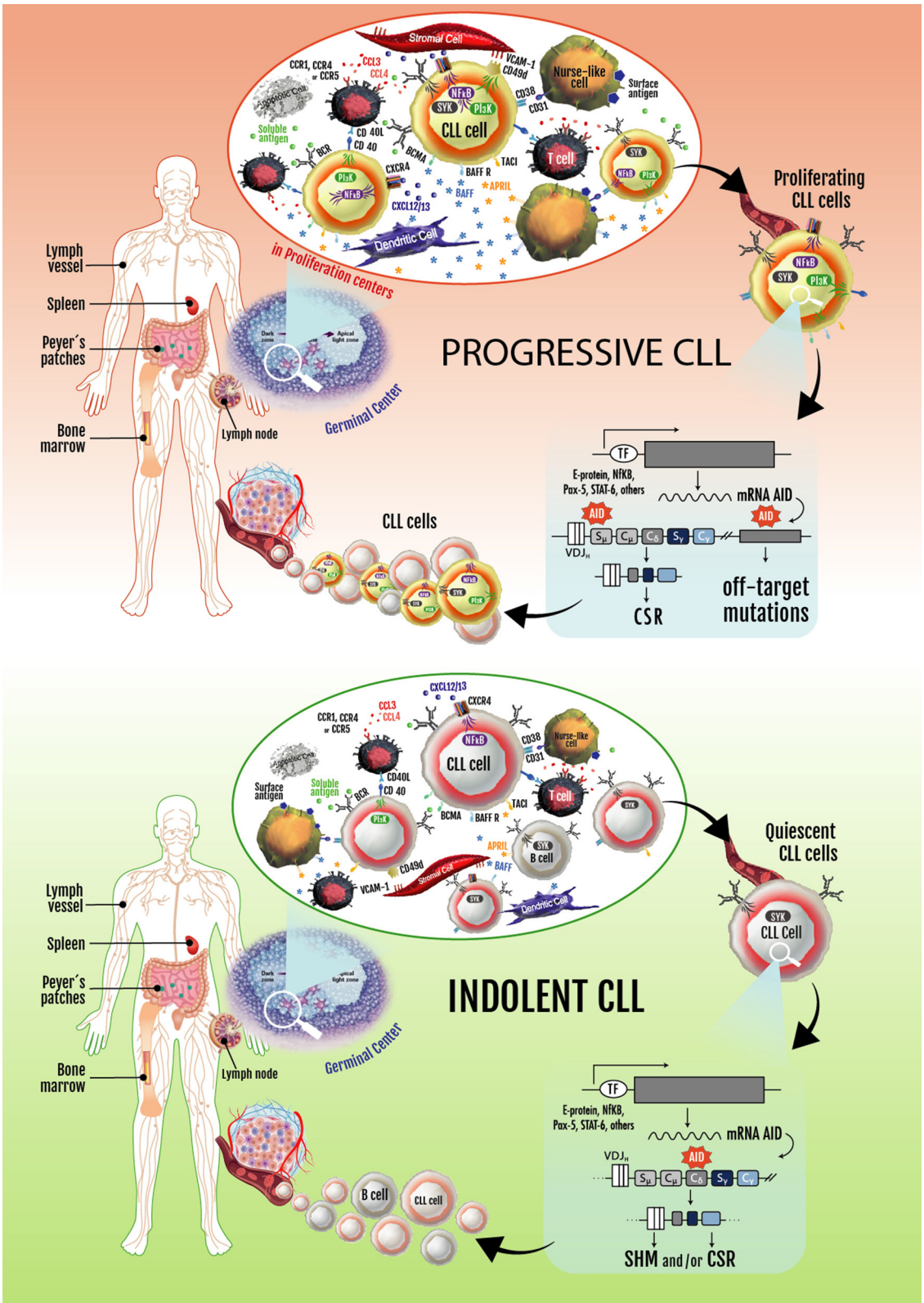

FIGURE 2 | Progressive and indolent CLL landscapes based on microenvironmental influences. In patients with progressive disease, an activated microenvironment continuously nourishes leukemic cells by maintaining proliferative fractions that can express AID. Proliferating cells overexpressing AID are susceptible to novel DNA

(Continued)

FIGURE 2 | lesions (many of them in non-Ig genes), establishing clonal and subclonal entities before and/or after treatment. These can lead to CLL progression and/or therapy resistance. Some of the leukemic cells dividing in proliferation centers leave the tissues and move into the blood. These circulating cells must return to lymphoid tissues to receive survival signals. If not, they eventually die. Cycles of these two events overtime lead to increased numbers of circulating AID$^+$ leukemic B cells, which is a hallmark of progressive CLL. In patients with indolent disease, microenvironmental signaling is similar to that taking place in normal GCs, with CLL cells becoming physiologically activated and AID expression and non-Ig genes mutations being better controlled.

also a prognostic marker in CLL in which a correlation with poor prognosis was described for those patients with higher percentages of CD38$^+$ cells (>/=30% of the leukemic clone) (136). The fact that CD38 is expressed in a high percentage of leukemic cells in IgHV-unmutated (U-CLL) patients indicates that CD38$^+$ leukemic cells constitute a heterogeneous population including a small fraction of cells with an increased proliferative potential, ranging from 0.08 to 1.7% (143), suggesting that not all CD38$^+$ cells are actively proliferating.

Pepper et al. also characterized the CD38$^+$ fraction showing that CD38$^+$ CLL cells overexpressed vascular endothelial growth factor and its expression associated with increased expression of the anti-apoptotic protein MCL-1 (160). Overall, these data describe an interesting subset of proliferative CLL cells with overexpression of different molecules that suggest microenvironment signaling activation but are not enough to specifically identify the proliferative subset in CLL.

In a similar research line, Palacios et al. described a different, clonally related CLL subset with ongoing CSR to IgG (IgM/IgG), mainly found in progressive and U-CLL patients (161). This subpopulation exhibited increased levels of Ki-67 and Survivin proteins and decreased levels of p27^{-Kip1}, which underline a clear proliferative behavior for these cells (145). Other molecular markers associated with tumor proliferation and microenvironment activation such as high expression levels of mRNA *of c-myc*, *Bcl-2*, *CD49d*, *Ccl3*, and *Ccl4* were found in this subset. Interestingly, Palacios et al. also found that AID, a molecule responsible for SHM and CSR of Ig genes in B lymphocytes, is expressed mainly in this proliferative subpopulation of the CLL clone (161). Since AID is a mutagenic enzyme, its expression is strictly regulated in B cells (162) and in physiological conditions limited to the centrocyte/centroblast stages in germinal centers (163). The discovery that CLL cells with ongoing CSR expressed AID in the PB of progressive CLL and that percentages of these subset ranged between 0.1 and 5% leads researchers to speculate that these cells could also represent a proliferative compartment of the CLL clone (145).

It is difficult to determine the precise role of these highly proliferating, activated leukemic B cells expressing class switched Igs distinct from that of the parent clone. Since the presence of this subset is clearly associated to poor prognosis, it might play an adjuvant role in the maintenance of the CLL proliferative pool. However, given their increased proliferative potential, one would expect that they would eventually outnumber the IgM$^+$ cells, and this is not the case. Thus, we assume that these cells undergo apoptosis once leaving the PCs. A recent study suggesting a link between AID expression and B-cell apoptosis in GC favors this view (164). In these conditions, the IgM$^+$/IgG$^+$ subset could reflect the existence of an active microenvironment leading to permanent stimulation of the IgM$^+$ pool, which could turn on the CSR machinery maintaining this subset in the PB. Despite the fact that a clear association was demonstrated between the existence of this subset with an unmutated profile and poor clinical outcome, their roles in CLL evolution remain to be elucidated.

Subsequently, another proliferative subset that can also express AID was identified by Calissano et al. (165). They characterized the proliferative and resting compartments of CLL using differences in the densities of a surface membrane molecule upregulated after normal B-cell activation, CD5 (166), and another involved in maintaining B-cell retention in contact with stromal elements of solid lymphoid tissues, CXCR4 (165). Specifically, they postulated that high CD5 density would reflect cellular activation as in normal human B cells and low CXCR4 levels would identify cells that internalized the receptor because of an activation event and thereby moved out of a lymphoid tissue to the periphery (165).

This work proposed a life cycle for individual CLL cells representing a continuum between the CXCR4^{dim}CD5^{bright}, CXCR4^{int}CD5^{int}, and CXCR4^{bright}CD5^{dim} fractions. At one extreme is the proliferative fraction, highly enriched in young, vital cells that recently left a solid lymphoid tissue where activation and proliferation occurred. At the other end is the resting compartment, containing older, less robust cells that may have been circulating in the periphery longer and are attempting through high CXCR4 levels to migrate into a solid tissue niche to avoid death (165).

A similar approach involving the marker CXCR4 was performed by Huemer et al., using another B cell activation marker CD86 to identify the proliferative fraction (167). CD86 is upregulated in B lymphocytes undergoing cell division in the light and dark zones of the germinal centers (168) and, in line with this idea, they found that expression of the proliferation associated antigen Ki67 was higher in CD86^{high}CXCR4^{low} CLL cells than in CD86^{low} CLL cells (167). This proliferative fraction also expressed AID and overlapped with the CXCR4^{dim}CD5^{bright} subset previously described (165).

There is clear evidence that a fraction of the CLL clone is generated each day. These results and those of Herishanu et al., describing that the LN constitute a privileged site of tumor proliferation (148), underlining the importance of the proliferating fractions inside the tumor clone of different CLL patients. Being aware that AID overexpression is a hallmark of these subsets and is associated with loss of target specificity resulting in mutations in non-Ig genes (*Bcl-6, Myc, Pax-5*, and *Rhoh*) (169), it is logical to assume that progressive disease could be related to clonal evolution of these proliferating cells. The constitutive expression of AID in the leukemic

clone history could be a key event in disease progression (**Figure 2**).

ACTIVATION-INDUCED DEAMINASE IN CLL CELLS AND ITS EFFECTS ON CLL-CELL BIOLOGY

AID Molecular and Structural Considerations in CLL

There are no reports suggesting that the *aicda* gene is mutated in CLL, as is the case in Hyper-IgM Syndrome, type 2. However, the splice variants mentioned above that have been found in normal B lymphocytes have also been detected in CLL by our (124, 125) and other (25, 123, 170–172) laboratories. Furthermore, like normal human B lymphocytes, CLL B cells express only a single variant form of AID mRNA (25), although this might change with environmental input. Thus, it appeared that splice variants lacking the CSR domain were better able to carry out SHM than full-length protein. This was consistent with the finding that normal germinal center B lymphocytes express full-length AID mRNA, whereas the CSR-deficient, SHM-enhanced spliced variant was present at higher levels in CLL B cells. Interestingly, AID transcript levels have been associated with the occurrence of CLL founding events. In a cohort of 149 patients, higher levels of alternatively spliced transcripts of AID (AIDΔE4a, AIDΔE4, AIDivs3, and AIDΔE3E4) were associated with trisomy of chromosome 12. Functional analysis of AID splice variants revealed loss of their activity with respect to SHM, CSR, and induction of double-strand DNA breaks (25, 172).

Despite these findings, a recent study suggested that AID splice variant proteins are not functional (172), which might be expected since AID splice variant patterns are not different at the time of diagnosis, nor do they appear to have an impact on progression-free and overall survival (171). Favoring this hypothesis, the work of Rebhandl et al. proposed that despite the presence of alternatively spliced AID transcripts, only full-length AID was detected at the protein level (173). Interestingly, when analyzed under limiting dilution conditions, it became clear that AID was expressed in a very minor subpopulation of the CLL clone (125). As has been previously demonstrated, the expression of AID in CLL is restricted to the proliferative fractions, which can be visualized in the peripheral blood of the most progressive cases (88, 174).

Molecular Actions and Targeting of AID in CLL

AID efficiently mutates the V and S regions of CLL cells. Since some CLL cells do not contain any or only a few mutations (U-CLL) (134, 135) but can express isotype-class switched Igs (175, 176), we correlated AID levels with V and S region mutations in CLL patients (124). This revealed that U-CLL cells express AID, and this expression associates with mutations in the S region. The cells of these patients also contained clonally related, isotype-switched transcripts (124), as previously reported (176–178).

Next, we focused on mutations occurring in the IgHV gene to determine those that could be attributed to the action of AID (158). Moreover, replacement (R) mutations segregated to complementarity-determining region 1 (CDR1) and CDR2 and silent mutations concentrated more in framework regions, FR (CDR R:S = 4.60 vs. FR R:S = 1.72). These results were consistent with the IgHV mutations resulting from an AID-mediated process and being selected for altered but structurally sound BCRs. Similar findings were reported by others (179), who also investigated expression of DNA polymerases zeta and eta that can be involved in repair of DNA altered by AID.

Because overlapping AID hotspots are critical sites for V region diversification (61) and key evolutionary components of human IgHV genes (62), Yuan et al. analyzed those IgHVs most used in CLL patients and in the clinically relevant U-CLL and M-CLL subgroups for such hotspots (180). This revealed a highly significant, but surprisingly inverse relationship between the number of WGCW hotspots in the germline IgHV and the observed mutation frequency in patients. This correlation was not observed in sequences from the B-cell repertoires of normal individuals and from those patients with autoimmune diseases. The relevance of these observations to the development of CLL and to patient outcome is not clear at this point.

Relationship of AID Expression to CLL Cell Activation

There is consensus that AID activity is upregulated in activated B lymphocytes in G1 or the G1-S phase transition and is maximally expressed in the highly proliferative centroblasts in the germinal center. Thus, proliferating leukemic cells resemble this situation and upregulate or maintain AID expression (50). When analyzing PCs in lymph nodes for the presence of AID protein, we found that only Ki-67$^+$ leukemic B cells contained the enzyme (88). Moreover, when examining CLL cells in the blood, we (124) and others (123, 170) found that AID is present in subsets of CLL clones, in particular those of the U-CLL type. However, expression was restricted to a minor subfraction of the clone, ∼0.1% (125, 161).

Based on the latter observation and the requirement for cell cycle entry to upregulate AID, we tested if the AID$^+$ fraction in CLL clones correlated with those few CLL cells in the blood that contain ^{2}H-DNA after ingestion of ^2H_2O (165, 181). Cells containing ^{2}H-DNA have recently completed the cell cycle and duplicated their DNA (182). In CLL, this fraction is small, ranging from 0.1 to ∼4.0% of the CLL clone on a daily basis (156, 157, 165, 181). Indeed, we found that recently divided cells ("proliferative fraction"), identified by a CXCR4^{Dim}CD5^{Bright} (165) or CLL cells undergoing CSR to IgG (IgM$^+$/IgG$^+$ phenotype) (161), were the only circulating leukemic cells that expressed AID (88, 145, 161). Cells that had divided earlier did not. Thus, those few cells in the blood of CLL patients that express AID are recent emigrants from tissue niches where cell division occurred and where AID was upregulated. Therefore, although circulating CLL B cells are unlike normal B lymphocytes from healthy people in that they express AID, this expression is a function of upregulation as a consequence of proliferation in tissue niches and not an aberrant function of a neoplastic cell (**Figure 2**).

Moreover, AID expression is dynamic, with its levels and the cells making it changing over time in individual patients (88, 125). This recognition led us to follow the clinical course of >100 patients over several years (88). The presence of circulating AID^+ cells presaged significantly shorter time to first treatment and to decreased overall survival. This was the case, not only for U-CLL patients who had higher levels of AID, but also for the fraction of M-CLL patients with increased AID amounts. Moreover, U-CLL patients with high AID levels had a shorter overall survival than U-CLL patients with lower levels. Finally, AID levels correlated with cytogenetic abnormalities that associate with poor clinical outcome, and together, these foretold shorter time to treatment (88). The latter findings are reminiscent of other studies associating AID and AID^+ cells with genomic abnormalities and clinical poor outcome (167, 170, 183–185).

Ibrutinib, Which Blocks CLL Cell Replication, Inhibits AID Expression

Ibrutinib has emerged as a potent treatment for CLL (186). Although the drug alone does not appear to be curative, it clearly delays disease progression and markedly improves patient quality of life (186). Because ibrutinib has been shown to rapidly inhibit CLL B cell proliferation *in vitro* (187, 188) and *in vivo* in mouse (188) and man (189), we tested if it would affect the size of the recently divided, proliferative fraction and thereby block AID expression (190).

In this way, we found that CLL proliferative fractions, defined as cells bearing a surface membrane phenotype of $CXCR4^{Dim}CD5^{Bright}$ (165) or IgM^+IgG^+ (161), were significantly decreased by ibrutinib. We recently demonstrated that ibrutinib downregulated AID in treated CLL patients and that, interestingly, this downregulation correlated with reduced AKT pathway and Janus Kinase 1 signaling (190). These findings also had important clinical implications since they showed that ibrutinib did not lead to increased levels of AID and thus would likely not result in genomic instability, as had been reported in a preclinical study (191).

Additionally, we studied the effects of ibrutinib on CLL cell growth directly in patients, using the ^2H_2O ingestion technique to label leukemic cells dividing *in vivo* (189). Treatment-naive patients with progressive disease, who were deemed to require treatment within 6 months, drank ^2H_2O before starting therapy, allowing the direct determination of birth rates of their leukemic clones and calculation of death rates based on blood counts.

Upon starting ibrutinib, birth rates decreased to negligible levels and death rates increased. This was direct documentation in patients that ibrutinib blocks CLL cell proliferation and that it promotes CLL cell death by inhibiting trafficking to tissue survival niches (189). Although AID levels were not measured in these samples, based on our data above and the knowledge that AID requires cell proliferation to be expressed, it is highly likely that AID's synthesis and its on- and off-target actions were aborted.

Collectively, therefore, these findings indicate several key features of AID expression in CLL. First, like normal B lymphocytes, AID is regulated by the stage of cell activation of the leukemic B cell, and those CLL cells in the blood expressing AID have recently divided and emigrated from lymphoid tissues, most likely lymph nodes based on the documented higher rate of cell division at that site (147). Second, the fraction of activated, AID^+ cells is small and changes over time. Finally, the presence and levels of AID^+ intraclonal members correlate directly with worse clinical course and survival.

Thus, the small AID^+ fraction, which is contained almost exclusively in the recently divided proliferative fraction of a CLL clone and that just replicated its DNA, has the highest likelihood of having just developed a new genetic lesion that could lead to disease progression. Hence, this fraction is especially dangerous. This supposition is supported by our finding that the daily rate of CLL-cell division, which correlates with the extent of AID expression, correlates with poor prognostic markers (unmutated IgHV, levels of ZAP70 and CD38) (143, 192) and predicts time-to-first treatment (192). This helps explain the underpinnings that allow ibrutinib and other small molecules that inhibit the activation of CLL cells to extend and improve the quality of life of CLL patients (193). Another promising possibility to accomplish this is the use a bispecific antibody that in preclinical studies preferentially targets this small intraclonal fraction (194).

Other interesting drugs targeting PF cells expressing AID are the inhibitors of HSP90. Chaperon HSP90 regulates numerous signaling proteins and pathways helping the cancer survive environmental stresses (195). Results reported by Ortehwein et al. propose AID as a novel HSP90 client, and consequently, treatment with HSP90 inhibitors could inhibit AID nuclear import or induce AID degradation (196). Preliminary results in collaboration with Di-Noia lab allowed us to provide proof-of-concept that HSP90 inhibitors target human AID in primary CLL cells (197). Other studies have also proposed the use of HSP90 inhibitors as candidate drugs in CLL to achieve a multi-targeting effect by inhibition of AKT and different kinases signaling (198). Currently, clinical trials using Hsp90 inhibitors in CLL are under development. For example, SNX-5422 (a highly selective, small molecule inhibitor of the HSP90) in combination with Ibrutinib is being tested in a phase I clinical trial in CLL patients with residual disease (NCT02973399, by Esanex Inc.).

Thus, targeting the proliferative fraction appears to have therapeutic value in CLL, but more time is required to corroborate the success of this idea.

Microenvironmental Signals That Induce AID Expression in CLL B Cells *in vitro*

Like normal B lymphocytes, CLL cells upregulate AID in response to T-cell-dependent and T-cell-independent stimulation. Specifically, culturing CLL B cells with activated T lymphocytes (199); C3d-coated Ag, IL-4, and BAFF (200); CD40L + IL-4 (88, 124, 201); and TLR9 agonist + IL-15 (202) can lead to AID expression. Consistent with TLR signaling being relevant to this disease are gene expression analyses indicating that CLL cells in LNs express a profile consistent with TLR activation (203).

Specifically, we have provided T-cell-dependent stimulation in the form of intact activated T cells or T-cell-derived signals (CD40L + IL-4) to stimulate upregulation of AID and induce CSR (124) and SHM (88, 199) *in vitro*. In this way, we found that

cultured CLL samples expressed AID mRNA and that production of AID protein varied considerably on a patient basis with the percentages of AID^+ cells ranging from ∼1 to ∼80% (88). As expected, production of AID followed the extent of cell divisions undergone by a given sample. Furthermore, the cells in these cultures exhibited features consistent with the protein being biologically active, in that phospho-histone H2A.X (pH2A.X) that binds to dsDNA breaks, mRNA transcripts for IgG along with surface membrane IgG, and new DNA mutations in IgHV-D-J DNA sequences were found. Strikingly, there was no major difference between U-CLL and M-CLL cases for these findings. Finally, we investigated the association of AID expression with time to first treatment over an 8 year period. This revealed that patients with AID^+ cells in the blood had significantly shorter time to treatment and overall survival (88).

Consistent with this, when primary CLL cells were transferred into lymphoid mice, a technique that leads to leukemic B-cell engraftment and growth (204), a relatively large fraction of transferred leukemic B cells synthesized AID and underwent CSR and SHM (205). Thus, the production of AID in response to T-cell co-stimulation can occur *in vivo* as well. Notably, the ability to express AID and undergo CSR and SHM *in vitro* and *in vivo* is as efficient for U-CLL clones as M-CLL clones (88, 205). This suggests strongly that the absence of IgHV-D-J mutations in U-CLL patients is not inherent but influenced by the microenvironment (205).

After Leukemic Transformation, AID Continues to Act on Ig Variable and Switch Regions in Individual Members of CLL Clones

Early studies analyzing molecular clones by Sanger sequencing suggested that CSR (201) and SHM (199, 206) occurred after leukemic transformation in individual, clonally related members of the leukemic CLL cell. This finding has been confirmed using next-generation, deep DNA sequencing (207).

Collectively, these data indicate that the AID mutational process continues after leukemic transformation within individual members of CLL clones, including those in which the clinically defined clone is classified as U-CLL. Thus, it is reasonable to expect that non-Ig genes could also be affected by this ongoing mutational process. The contributions of AID-mediated and non-AID-mediated mutations to genomic instability and disease progression are discussed in detail below.

THE CLL GENOME

The CLL genome is characterized by the presence of structural alterations and a wide range of mutations that depicts a heterogeneous genomic landscape. IgHV mutation status and the presence of chromosome abnormalities are among the strongest predictors of clinical outcome (208). Approximately 80% of CLL patients carry at least one of four common chromosomal alterations, and the average CLL mutation rate ranges between 0.4 and 2.1 alterations per Megabase (48, 209). Overall, a typical CLL genome carries ∼2,500 molecular lesions. M-CLL patients carry a higher mutational load (3,000 mutations/Mb) than U-CLL patients (2,000 mutations/Mb) (210).

Structural Lesions

The most recurrent alterations in CLL are chromosome abnormalities, and the most frequent lesions are deletions of chromosome 13q (55% of cases), 17p (7%), 11q (6% to 18%), and trisomy 12 (12 to 16%) (211).

Structural rearrangements and SNV occur at similar frequencies when compared with other indolent B-cell lymphomas such as follicular lymphoma (212); however, their frequency is substantially lower than in most solid tumors. Initial studies of somatic copy number variations using karyotyping, fluorescence *in situ* hybridization, or single-nucleotide polymorphism arrays are extensively reviewed elsewhere (213).

In the clinical setting, these prevalent chromosomal aberrations are used in the widely accepted cytogenetic classification proposed by Dohner et al. (211). A hierarchical model based on five risk categories was established by correlating FISH lesions with clinical outcome. CLL cases with the 17p13 deletion (prevalence 7%) had the worst prognosis, independent of the presence of concomitant abnormalities, with a median survival of 32 months. Cases carrying the 11q22-q23 deletion (prevalence 18%) had a median survival of 79 months. Longer survival rates were associated with trisomy 12 (prevalence 16%, median survival 114 months), normal karyotype (prevalence 18%, median survival 111 months), and 13q14 monoallelic deletion (prevalence 55%, median survival 133 months) (211).

Cytogenetic lesions, however, do not entirely explain the genetic basis of the heterogeneous clinical outcome of CLL. The wide availability of next-generation genome sequencing has enabled the identification of new recurrent structural and single-nucleotide lesions. New recurrent genomic aberrations include trisomy 19, amplifications at 2p and 8q, and deletions at 8p, 6q21, 18p, and 20p (209, 214). A recent study supports the existence of multiple recurrent focal copy number alterations and of copy number neutral losses of heterozygosity affecting key oncogenic pathways, associated with higher proliferative capacity, shorter survival, and altered gene expression. Therefore, focal structural changes may also play a relevant role in CLL pathogenesis (215). Genome sequencing has also enabled the definition of the molecular correlates of CLL chromosomal aberrations (216, 217), in particular *Tp53*, the tumor suppressor gene affected by 17p13 deletion, and *Atm*, the gene targeted by 11q22-q23 deletion (218).

Recurrent Oncogenic Mutations

Although recurrent chromosomal abnormalities can be found in most CLL patients, only very few single-nucleotide variants show a recurrence higher than 5% across patients. Additionally, a large number of biologically and clinically uncharacterized genes are mutated at a lower frequency.

Similar to the inherent clinical heterogeneity, the genetic landscape of CLL is markedly complex with a rapidly growing list of genes mutated at a low frequency. Most gene mutations still require rigorous validation in large, prospective patient studies, and only a few genes have been implied to have diagnostic and prognostic impact.

TABLE 1 | Recurrently Mutated Genes in Different Clinical Settings.

	Early (Binet A) (222) (%)	Advanced (Binet B/C) (222) (%)	Mutated (223) (%)	Unmutated (223) (%)	Refractory Relapsed (224) (%)
NOTCH1	6	13	7.0	20.4	14.9
SF3B1	6	18			28.1
TP53	8	17			22.8
BIRC3	1.9	4.5			
MYD88	2	2.5	5.6	0.8	2.6
XPO1			0	4.6	14.9
KLHL6			4.5	0	
ATM					26.3

Within this heterogeneous landscape, there is a set of shared genetic lesions among B-cell malignancies, affecting similar mechanisms and processes such as DNA repair and antigen receptor signaling indicating certain degrees of shared pathways involved in lymphomagenesis (219, 220).

The most frequent recurrent mutations in CLL affect *Notch1, Sf3b1,* and *Birc3* and have been reported to occur in approximately 2–10% of patients within a general practice setting (221). The frequency of mutations of candidate driver genes, with the exception of *Myd88* and *Igll5*, has been consistently associated with progressive, high-risk disease or U-CLL (**Table 1**) (225, 226).

The Role of AID in CLL Mutagenesis

SHM represents an endogenous mutator mechanism in B lymphocytes initiated by AID, and its mutagenesis has been associated with lymphomagenesis in B-cell neoplasms (195, 220). The availability of next-generation sequencing and the development of modern machine learning algorithms to deconvolute underlying mutagenic processes has enabled identification of putative mechanisms driving genetic lesions in cancer cells (227). In CLL, the main mechanisms consistently identified in patients are aging, enzymatic deamination, and defects in DNA repair (48, 227–229). Whereas, aging-induced deamination may account for up to three quarters of the single-variant substitutions, the remaining lesions can be linked to endogenous deamination and defects in DNA repair (48).

One intriguing issue regarding AID activity in lymphomagenesis is the apparent decoupling between AID expression and SHM, not only in CLL (124) but also in other B-cell malignancies such as follicular lymphoma (230, 231). In line with these results, the ongoing AID activity is enriched in higher risk U-CLL cases (228), and the contribution of subclonal aberrations to CLL pathogenesis is being increasingly recognized (232). The analysis of clonal and subclonal mutations has allowed the reconstruction of tumor phylogeny (233). Clonal lesions, which encompass mostly structural changes, generally correspond to earlier driving events, while subclonal lesions in driver genes (e.g., *Notch1* and *Sf3b1*) are acquired later over the course of the disease (222, 234, 235) (**Table 1**).

Considering all this evidence, it can be hypothesized that AID first plays a broader role at early stages of leukemogenesis contributing to the induction of founding events and later preferentially acts in proliferating fractions contributing to mutagenesis, facilitating the emergence of new clones involved in tumor progression.

An interesting study by Kasar et al. showed that in a cohort of 30 indolent CLL cases, c-AID and nc-AID signatures accounted for 25% (5 and 20% respectively) of somatic mutations (210, 236). Our preliminary data analyzing an unbiased CLL cohort (237) also shows a proportion of c-AID signature distribution similar to those described by Kasar and Brown (236). However, when follicular lymphoma (FL) cells (a germinal center B-cell malignancy with constitutive AID expression) are analyzed, our results revealed a c-AID contribution up to 9% (237).

Given that from a biological point of view CLL can be separated into two broad subsets according to the IgHV mutation status (208), we speculate that c-AIDl and nc-AID signature contribution could be different between both entities. U-CLL and progressive patients might have a more important ongoing c-AID signature similar to those presented in FL cells, whereas indolent and mutated CLL cases might exhibit a lower ongoing c-AID signature. At present, the real rate of the c-AID contribution in those progressive CLL patients in which a functional AID was established (25, 124) or in the CLL proliferative fractions that express AID in these patients (88, 161) remains unknown. Indeed, how this ongoing c-AID activity influences the mutational status of the CLL genome and, in consequence, in the long term, the disease outcome remains an important and unanswered question.

To move deep into this hypothesis, we recently developed an *in vivo* model by inducing constitutive AID expression in Eμ-TCL1 mice (named DT-AID). In this CLL-like model, we observed that DT-AID mice showed altered disease kinetics and higher percentages of CLL-cell proliferation, and these resulted in a more rapid progression of the disease compared with their TCL1 counterparts. Interestingly, a comparison of c-AID and nc-AID contributions between DT-AID and TCL1 revealed an increased ongoing c-AID signature in many non-Ig, cancer-related genes also described in human neoplasms (238).

In summary, cumulative evidence demonstrates that AID is involved in leukemogenesis and tumor progression. We speculate that AID is involved in mutagenesis of CLL clones at very early stages, thereby participating in founding events leaving a genomic footprint readily sizable by somatic signature analysis. At later stages or after cytotoxic treatment, AID could further

act at the subclonal level, facilitating the emergence of additional, mutated malignant cells involved in progression.

AUTHOR CONTRIBUTIONS

PO and NC: conception, design, and writing the manuscript. PO, MN, and NC: writing and/or revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Absence of Non-Canonical, Inhibitory *MYD88* Splice Variants in B Cell Lymphomas Correlates With Sustained NF-κB Signaling

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Gain-of-function mutations of the TLR adaptor and oncoprotein MyD88 drive B cell lymphomagenesis *via* sustained NF-κB activation. In myeloid cells, both short and sustained TLR activation and NF-κB activation lead to the induction of inhibitory *MYD88* splice variants that restrain prolonged NF-κB activation. We therefore sought to investigate whether such a negative feedback loop exists in B cells. Analyzing *MYD88* splice variants in normal B cells and different primary B cell malignancies, we observed that *MYD88* splice variants in transformed B cells are dominated by the canonical, strongly NF-κB-activating isoform of *MYD88* and contain at least three novel, so far uncharacterized signaling-competent splice isoforms. Sustained TLR stimulation in B cells unexpectedly reinforces splicing of NF-κB-promoting, canonical isoforms rather than the 'MyD88s', a negative regulatory isoform reported to be typically induced by TLRs in myeloid cells. This suggests that an essential negative feedback loop restricting TLR signaling in myeloid cells at the level of alternative splicing, is missing in B cells when they undergo proliferation, rendering B cells vulnerable to sustained NF-κB activation and eventual lymphomagenesis. Our results uncover *MYD88* alternative splicing as an unappreciated promoter of B cell lymphomagenesis and provide a rationale why oncogenic *MYD88* mutations are exclusively found in B cells.

Keywords: MYD88, B cell lymphoma, DLBCL - diffuse large B cell lymphoma, NF- kappa B, TLR - Toll-like receptor, alternative splicing, negative feedback loop

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HIGHLIGHTS

- In human B cells the TLR adaptor and oncogene, *MYD88*, can give rise to at least 8 mRNA splice variants with different signaling capabilities.
- Unlike myeloid cells, transformed B cells and cells with sustained TLR/NF-κB activation show a preference for NF-κB-promoting canonical *MYD88* splice variants.
- The negative feedback loop of inducing signaling incompetent splice variants is absent in proliferating B cells and may render them susceptible to lymphomagenesis.

INTRODUCTION

MyD88 has long been studied as an adaptor molecule for Toll-like receptor (TLR) and Interleukin-1 receptor (IL-1R) signaling in innate immunity (1). Its pivotal role is strikingly illustrated by the fact that loss-of-function mutations lead to severe immunodeficiency, whereas gain-of-function mutations promote oncogenesis: For example, rare dysfunctional alleles of *MYD88* compromise formation of the MyD88-mediated post-receptor complex (2), the so-called Myddosome (3, 4). Its assembly is a pre-requisite for effective activation of the IL-1R-associated kinases (IRAKs) 2 and 4 and eventual activation of NF-κB and mitogen activated protein (MAP) kinases (1). Patients carrying loss-of-function *MYD88* alleles consequently fail to respond to microbial TLR agonists and IL-1 and thus do not mount a sufficient innate immune response against pyogenic bacteria, leading to insufficient immunity and frequent premature death (5). Conversely, *MYD88* mutations leading to constitutive Myddosome assembly (6), most notably the mutation Leu 265 to Pro (L265P) (7), are oncogenic and associated with sustained NF-κB signaling. L265P drives lymphoproliferation in murine models (8). In humans, L265P is highly prevalent in various B cell malignancies (7) but absent in other, e.g. myeloid, hematopoietic (8) malignancies. Its strict occurrence in B cell malignancies has highlighted L265P's diagnostic, chemo- and immunotherapeutic potential (9–11) but also posed the questions why only B cells are vulnerable to *MYD88* gain-of-function mutations? Additionally, the varying frequency of the L265P mutation in different B cell malignancies has been puzzling: Although the MyD88 L265P mutation may be found in up to 90% of Waldenström's Macroglobulinemia patients (12), in diffuse large B cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL) only 30 or 4% of patients carry this or other known gain-of-function *MYD88* mutations, depending on subtype (7, 13). Thus, other mechanisms apart from mutation of *MYD88* appear to operate in L265P-negative patients, whereas a consistent "NF-κB signature" has been recognized as a unifying feature for most of these B cell malignancies (14–16).

The activation of NF-κB is also a primary outcome of MyD88-dependent signaling in myeloid cells (1). However, negative feedback on NF-κB signaling by alternative splicing seemingly operates in myeloid cells: TLR stimulation with LPS leads to the upregulation of a splice variant, then termed 'MyD88 short' (MyD88s, here also referred to as isoform 3, see **Figures 1A, B** and **Table 1**) (17). Conversely to constitutive splicing (18), alternative splice variants arise from "alternative" splice sites in pre-mRNAs, that trigger, for example, exon skipping, alternative 5' or 3' splice site usage within exon or intron sequences or intron retention. The resulting transcripts may be subject to frame shifts, premature termination codons and/or non-sense mediated decay (NMD) (18, 19). Collectively, >90% of human multi-exon genes are subject to alternative splicing which greatly expands the diversity and function of the proteome (20, 21). In eukaryotes the spliceosome, where so-called splice factors (SFs) cooperate with five small nuclear ribonucleoprotein complexes (U1, U2, U4/U6, and U5), recognizes and assembles on introns to cleave and ligate RNA molecules for intron removal, generating protein-coding mRNAs (22). The spliceosome catalyzes splicing with high precision, but also displays high flexibility to regulatory signals for rapid responses, such as alternative splicing. Such a direct link between regulatory signals and innate immunity was recently proposed for the SF3A and SF3B mRNA splicing as both factors were shown to connect TLR signaling with the regulation of MyD88s (23, 24).

MyD88s (isoform 3) represents an alternatively spliced in-frame deletion of exon 2 and thus a MyD88 variant significantly shorter than the canonical isoform 2: Whereas isoforms 1 and 2 contain the canonical N-terminal death domain (DD), central intermediate domain (ID) for IRAK recruitment, and C-terminal Toll/IL-1R (TIR) domain for TLR binding, MyD88s (isoform 3) lacks the ID. The ID has been proposed to couple activated TLRs to the IRAK-containing Myddosome and thus transduce incoming signal (25). Hence, MyD88s is signaling-incompetent. Even though its characterization has been limited to myeloid and epithelial cells, MyD88s (isoform 3) by many has been considered a primary negative regulator of this pathway and part of an essential negative feedback loop induced upon TLR signaling in myeloid cells and epithelial (26–28). Isoform 1, the first reference sequence described, represents the longest transcript and translated protein for MyD88 by taking an alternative donor splice site 24 nt downstream of exon 3, adding 8 amino acids within the TIR domain. Apart from isoforms 1-3, two additional splice isoforms of *MYD88* have since been described, namely, isoforms 4 and 5 (**Figures 1A, B** and **Table 1**), whose properties have been less studied. Additionally, whether alternative splicing and feedback regulation is operable in other, non-myeloid immune cells has not been addressed.

Abbreviations: BL, Burkitt Lymphoma; CLL, Chronic Lymphocytic Leukemia; DD, Death Domain; DLA, Dual Luciferase Assay; DLBCL, Diffuse Large B Cell Lymphoma; FL, Follicular Lymphoma; GCB, Germinal Center B Cells; HEK, Human Embryonic Kidney; ID, Intermediate Domain; IL-1R, Interleukin-1 Receptor; IRAK, IL-1R-Associated Kinase; LPS, Lipopolysaccharide; Myd88, Myeloid Differentiation 88; NF-κb, Nuclear Factor κb; TIR, Toll/Interleukin-1 Receptor; TLR, Toll-Like Receptor; TNF, Tumor Necrosis Factor; WT, Wild-Type.

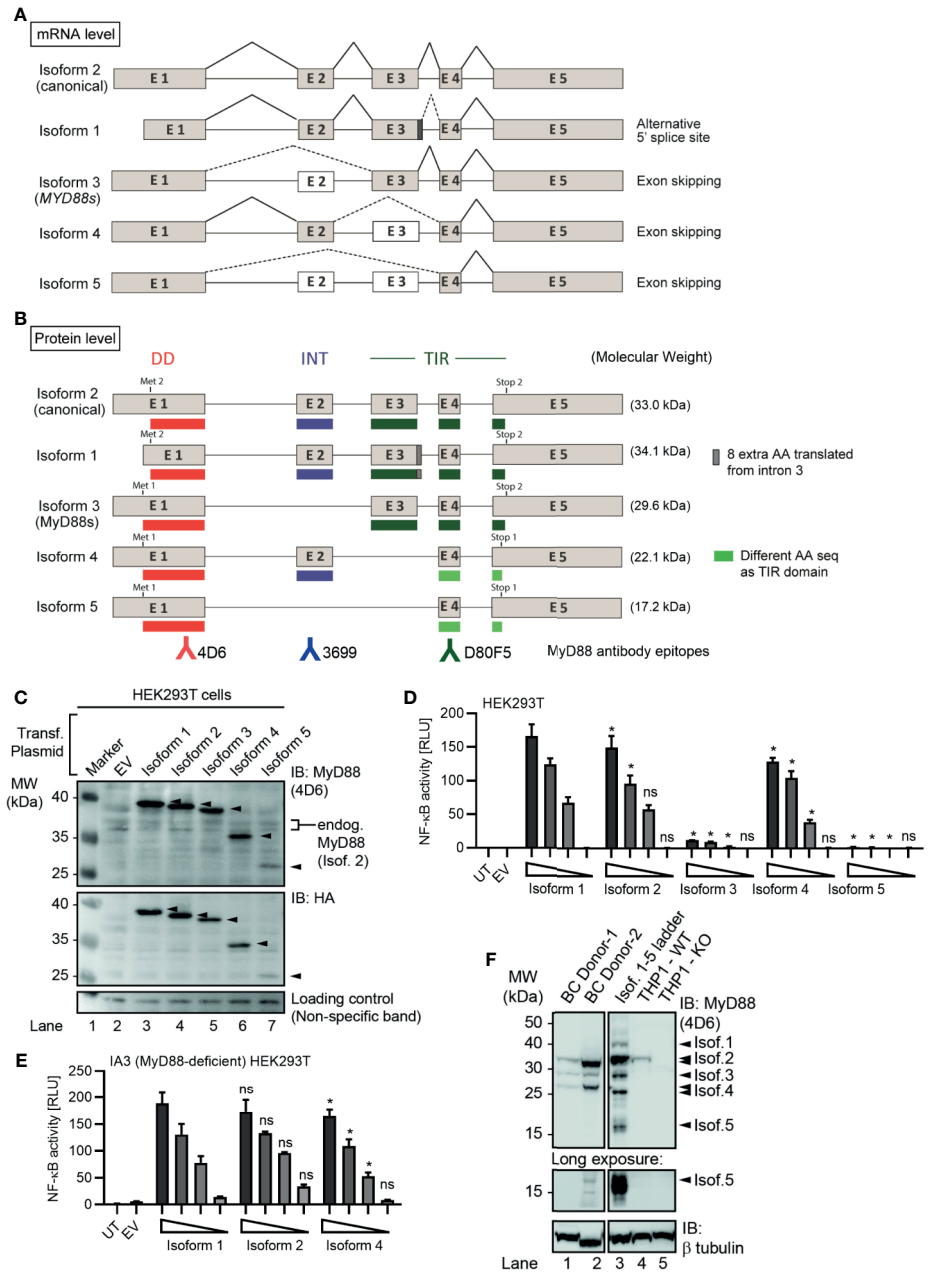

FIGURE 1 | Several alternative MyD88 isoforms support NF-κB signaling**. (A, B)** Schematic representation of *MYD88* isoforms on mRNA **(A)** and protein **(B)** level according to references in **Table 1**. **(B)** Illustration of target epitopes of the different antibodies used in this study. **(C–E)** HEK293T cells were transfected with plasmids for different *MYD88* splice isoforms and lysates analyzed for expression or pathway activation by immunoblot (**C**, n=3) or NF-κB dual luciferase assay (**D**, n=4), respectively. **(E)** as in D but using MyD88-deficient I3A cells (n=3). **(F)** Immunoblot of primary B cell lysates from two different donors, lysates of HEK293T transfected with untagged isoforms 1 to 5 ('Isof. 1-5 ladder') and MyD88-competent or deficient (KO) THP-1 reporter cells. In C-E one representative of 'n' technical replicates is shown as mean + SD from three repeats. ns, non-significant; * p<0.05 according to two-way ANOVA comparing to isoform 1 **(D, E)**.

We speculated that if a negative feedback loop existed in B cells, TLR activation should also induce MyD88s (isoform 3) and thereby limit ongoing signaling. Interestingly, we found here that B cells only transiently induce isoform 3 upon short exposure to TLR agonists, but extended TLR-MyD88 stimulation rather maintained the canonical isoform. Our data thus indicates that in B cells an isoform 3-mediated negative feedback loop does not seem to restrain NF-κB long-term; rather, extended TLR stimulation drives the canonical, i.e. NF-κB promoting, isoform and thus does not restrict extended NF-κB activation by diverting transcripts to less signaling-competent isoforms like MyD88s (isoform 3) as in myeloid cells. In line with this, primary

TABLE 1 | *MYD88* splice isoforms.

MyD88 isoforms	mRNA		Protein			Expression construct MW (incl. Strep-HA tag; kDa)*
	Reference ID	CDS (bp)	Reference ID	Length (aa)	MW (kDa)	
Isoform 1	ENST00000421516.3 NM_001172567.2	915	ENSP00000391753 NP_001166038.2	304	34.1	40.5
Isoform 2	ENST00000396334.8 NM_002468.5	891	ENSP00000379625 NP_002459.3	296	33.0	37.4
Isoform 3 (MyD88s)	ENST00000417037.7 NM_001172568.2	795	ENSP00000401399 NP_001166039.2	264	29.6	34.7
Isoform 4	ENST00000651800.1 NM_001172569.3	615	ENSP00000499012 NP_001166040.2 Uniprot: Q99836-3	204	22.1	27.2
Isoform 5	ENST00000650112.1 NM_001172566.2	480	ENSP00000497991 Uniprot: Q99836-4	159	17.2	22.8
Isoform 6	ENST00000652213 NM_001365876.1	738	ENSP00000498576 NP_001352805.1	245	27.1	34.9§
Isoform 7	NM_001365877.1	642	NP_001352806.1	213	23.5*	29.9§
Isoform 8	ENST00000652590.1	723	n/a (new)	240	26.4*	32.8§

*Reference IDs from Ensembl and NCBI. ENST: cDNA sequence, ENSP: protein sequence, NM: curated NCBI mRNA; Protein-coding transcript, NP, NCBI protein coding sequence; Strep-HA, Strep III - Hemagglutinin tag; n/a, not available. *Values were calculated using ExPASy. §Generated constructs use Met1 as start codon.*

B cell malignancies showed significantly higher degrees of the canonical *MYD88* splice isoform and include transcripts for an additional three hitherto unrecognized *MYD88* splice isoforms. Our data warrant a re-evaluation of previously assumed myeloid cell derived concepts of *MYD88* splicing and NF-κB regulation in human primary cells, especially B cells, and provide an explanation for the susceptibility of B cells to oncogenic *MYD88* mutation.

MATERIALS AND METHODS

Study Participants and Sample Acquisition

All patients and healthy blood donors included in this study provided their written informed consent before study participation. Approval for use of their biomaterials was obtained by the local ethics committee at the University Hospitals of Tübingen; Germany, in accordance with the principles laid down in the Declaration of Helsinki as well as applicable laws and regulations. Patient recruitment, sample acquisition and preparation of B cell lymphoma, CLL and ovarian cancer patients are described below. Healthy blood donors were recruited at the Interfaculty Institute of Cell Biology, Department of Immunology, University of Tübingen; Germany.

Isolation and Stimulation of Primary Human Immune Cells

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from whole blood or buffy coats (University Hospital Tübingen Transfusion Medicine) using Ficoll density gradient purification, primary B cells from PBMCs using B Cell Isolation Kit II (Miltenyi Biotec, >90% purity by anti-CD19 staining) and hMoMacs using Monocyte attachment Medium (PromoCell). B cells and hMoMacs were stimulated with 200 ng/ml LPS (from *E. coli* K12, Invivogen) or 2.5 μg/ml CpG 2006 (TIB MOLBIOL) for the indicated time periods. B cells were also stimulated with 2.5 μg/ml CpG 2006 and 5 μg/mL anti-human IgM (Fc5μ, Jackson Immuno Research) for proliferation assays. Carboxyfluorescein-succinimidyl ester (CFSE, Life Technologies) was used to track cell proliferation. Flow cytometry (BD FACSCanto II) was analyzed using FlowJo PC version 10. Further details in **Supplementary Material**.

Plasmid Constructs

N-terminally StrepIII-Hemagglutinin (HA) tagged and untagged *MYD88* isoform expression constructs were based on the reference sequences listed in **Table 1** and generated by gene synthesis (Genewiz; Germany) or PCR cloning and verified by DNA sequencing. Further details in **Supplementary Material**.

Cell Cultures

All HEK293T and DLBCL cell lines were described and cultured as previously (6). THP-1 WT and MyD88-deficient cells were a kind gift from V. Hornung, Gene Center, Munich, Germany. THP-1 WT and MyD88-KO Dual reporter cells were provided by R. Amann, University of Tübingen, Germany. Further details in **Supplementary Material**.

Dual Luciferase Assay

Dual luciferase assays (DLA) were described previously (6). Briefly, *MYD88* isoforms (1-100 ng), NF-κB firefly luciferase reporter (100 ng) and Renilla luciferase control reporter (10 ng) were transfected into HEK293T cells. 48 h after transfection cell lysates were measured using the Dual-Luciferase Reporter Assay System by Promega according to instructions. Further details in **Supplementary Material**.

Immunoprecipitation, SDS-PAGE and Immunoblot

For immunoprecipitation cell lysates (RIPA buffer with phosphatase and protease inhibitors) were incubated for 1.5 h with MyD88

D80F5 (CST) or anti-GFP G1544 as control antibody (Sigma), then protein G dynabeads (Thermo Fisher) were added for 1.5 h at 4°C. Beads were washed 3 times with RIPA buffer and the bound proteins were eluted with 2X LDS sample buffer (Thermo Fisher). For immunoblot cell lysates or elutions were separated on 10% or 4%–12% SDS-PAGE gels. Proteins blotted onto nitrocellulose membranes were probed with anti-HA H3663 (Sigma-Aldrich), MyD88 4D6 (epitope surrounding Leu77, Thermo Fisher), MyD88 D80F5 (epitope surrounding Cys233, CST) and MyD88 3699 (epitope surrounding Lys119, CST), HRP-conjugated secondary antibodies (1:8000) or HRP-conjugated anti-Mouse IgG (1:1000, Kappa light chain) and visualized using CCD-based ECL detection. Further details in **Supplementary Material**.

Quantitative PCR

Upon total RNA isolation (RNeasy Mini Kit, Qiagen) and reverse transcription, qPCR reactions (20 ng cDNA, 0.3 or 1 μM primers, 1x FastStart Universal SYBR Green Master Rox, Sigma; Germany) were performed and normalized to GAPDH expression. Primer sequences and concentrations are shown in **Table S1**. Further details in **Supplementary Material**.

Lymphoma, CLL and Ovarian Cancer Dataset Analysis

RNAseq libraries for Burkitt's Lymphoma (BL, n=20), Follicular Lymphoma (FL, n=80), Diffuse Large B cell Lymphoma (DLBCL, n=71), FL-DLBCL (n=15), naïve B cells (n=5) and germinal center B cells (n=5) were from the European genome-phenome database archive at EBI: https://www.ebi.ac.uk/ega/home. Chronic Lymphocytic Leukemia (CLL) RNAseq data (n=289) from the ICGC-CLL Consortium (https://dcc.icgc.org/releases) (29, 30). Ovarian cancer RNAseq libraries (n=85) were from the ICGC/OV-AU project (Australian Ovarian Cancer Study, https://dcc.icgc.org/projects/OV-AU) (31, 32). For RNAseq data analysis, isoform 2 abundance was calculated as 1-$\Sigma_{isoforms}$, because it has no unique splice site, intron retention (isoform 8) was calculated as relative to the flanking exons' expression and the relative usage of exon 4 acceptor splice site. For the rest of isoforms, the number of unique splice junctions divided by number of reads at the respective splice site is shown. The unique splice junctions considered for analysis are for isoform 1: exon 3 + 20nt ➔ exon 4, isoform 3: exon 1 ➔ exon 3, isoform 4: exon 2 ➔ exon 4, isoform 5: exon 1 ➔ exon 4, isoform 6 and 7: exon 3 -20nt ➔ exon 4. More details are given in **Supplementary Material**.

Statistic Analysis

Experimental data was analyzed using Excel 2010 (Microsoft) and/or GraphPad Prism 6, 7 or 8 or in *R*, flow cytometry data with FlowJo 10. Normal distribution in each group was always tested using the Shapiro-Wilk test first for the subsequent choice of a parametric (ANOVA, Student's t-test) or non-parametric (e.g. Friedman, Mann-Whitney U, Kruskal Wallis or Wilcoxon) test. p-values (α=0.05) corrected for multiple testing were then calculated in Prism. Values <0.05 were generally considered as statistically significant and denoted by * or # throughout. Comparisons were made to unstimulated control, unless indicated otherwise, denoted by brackets.

RESULTS

MYD88 Displays Comprehensive Splicing Leading to Functionally Disparate Isoforms

Given the importance that the MyD88s splice variant has been ascribed in murine myeloid cells (17, 23), we sought to conduct a systematic characterization of all known human *MYD88* splice variants. Until recently, five *MYD88* mRNA transcripts with differential splicing have been reported (**Table 1** and **Figure 1A**), giving rise to five protein isoforms with different domain structure (**Figure 1B**). Compared to the canonical isoform 2, isoform 1 features an additional 8 amino acids in frame between exon 3 and 4, i.e. in the TIR domain, due to the use of an alternative splice site (dark grey box and/or dashed lines in **Figures 1B** and **S1B**). Isoform 3 lacks the ID (exon 2) but includes both DD and TIR domain and corresponds to the aforementioned MyD88s variant. Isoform 4 and 5 both lack the TIR domain entirely, due to frame-shifts resulting from the skipping of exon 3 (**Figure S1A**). In terms of canonical MyD88 domains, isoform 4 thus is limited to a DD-ID protein followed by 36 C-terminal amino acids that bear no apparent similarity to any known proteins (**Figure S1A**). In isoform 5, exon 2 is additionally skipped, thus resulting in a DD-only variant. In order to investigate functional differences, these isoforms were cloned into StrepHA-tagged expression constructs and their expression verified in transfected HEK293T cells. Evidently, all constructs could be detected as proteins of 40, 37, 35, 27 and 23 kDa (**Figure 1C** and **Table 1**), albeit with different expressions levels. The shortest isoform, termed isoform 5, was barely detectable, indicating it may be less stable. Next, we assessed the ability of all isoforms to drive NF-κB activation using dual luciferase assays upon transfection of equal amounts of expression plasmids in HEK293T cells. Whilst this assay cannot report on the ability to transduce incoming TLR signals, it is well established to assess MyD88 downstream signaling potential (2, 6, 7, 33–35). Here, isoform 1 was the most active isoform, followed by isoform 2, the canonical MyD88 splice variant (**Figure 1D**). Isoform 4 was also able to induce NF-κB activity, at slightly lower levels. Isoform 3 and 5 were not able to induce NF-κB activity, consistent with a lack of ID, which is required to assemble into a Myddosome and recruit IRAK4 (4, 34). Since HEK293T cells endogenously express MyD88 isoform 2 at high levels (*cf.* **Figure 1C**), we also conducted the experiment in the MyD88-deficient HEK293T-derivative cell line, I3A (33). An almost identical picture emerged, where the canonical isoform 2 induced the highest NF-κB activity (**Figure 1E**). Since both murine and human MyD88s (isoform 3) were described as dominant-negative regulators of canonical MyD88 due to lack of the ID (34, 36), we also tested whether isoforms 3 and 5 could block TLR signaling, e.g. *via* TLR5, in the HEK293T system, but this was not the case (**Figures S1C, D**). Collectively,

non-canonical MyD88 isoforms with an intact DD and ID (isoforms 1 and 4) are capable of transmitting downstream NF-κB activity and their expression may thus support the function of the canonical MyD88 (isoform 2), whereas isoforms 3 and 5 are inactive.

Primary B Cells Express Multiple *MYD88* Splice Isoforms

All analyses on *MYD88* splicing have so far focused on (mostly transformed) myeloid and epithelial cells but as aforementioned MyD88 also plays an oncogenic role in B cells *via* NF-κB signaling (11). To assess the expression levels of these isoforms in primary B cells and be able to identify them by molecular weight *via* SDS-PAGE, we also generated expression constructs without a tag as a 'molecular ladder'. Whole cell lysates from HEK293T transfected with these untagged isoforms 1 to 5 and from unmodified and MyD88 knockout reporter THP-1 cells (see Methods) were then compared alongside lysates of primary B cells from two different healthy donors. We could detect the expression of four different MyD88 isoforms, identifying isoforms 2, 3 and (probably) 4 as matching the molecular weight of the untagged expression constructs and strongly reduced or absent in the edited THP-1 cells (**Figure 1F**). In long exposures a band migrating at the height of isoform 5 was also visible in 1 donor but not THP-1 cells. Collectively, the canonical isoform 2 shows the highest protein expression levels in primary B cells and isoform 5 the lowest (**Figure 1F**).

Transformed B Cells Also Express Multiple *MYD88* Splice Isoforms

As expression patterns between primary and transformed cells may differ, we next characterized the expression of the five isoforms in several ABC and GCB DLBCL cell lines using isoform-specific primers to distinguish isoforms 1/2 from other isoforms (**Figures S2A, B**, Methods and **Table S1**). This confirmed the expression of isoforms 3, 4 and 5 at mRNA level in these cell lines (**Figure 2A**). Using lysates of these ABC and GCB cell lines and an antibody directed against the DD, multiple MyD88-specific bands were also detectable (**Figure 2B**). Taking into account the predicted molecular weights of the alternative isoforms (*cf.* **Table 1** and **Figure 1F**) and their corresponding mRNA levels in BJAB cells *vs* primary B cells (*cf.* **Figure 2A**), certain labeled bands in **Figure 2B** are likely to correspond to isoform 3, 4 and 5. This same pattern of bands was observed using a combination of 2 additional anti-MyD88 antibodies (**Figure S2C**). To enrich the alternative isoforms from whole cell lysates, we pulled down MyD88 using an antibody, which is directed against the TIR domain (exon 4) and thus should detect isoforms 1, 2 and 3. Subsequent immunoblot of the elution showed bands corresponding to isoform 2 and surprisingly isoform 4, possible due to DD-mediated heterodimer formation (6) with isoform 2 (**Figure 2C**). Any detected alternative isoforms were less prominent than isoform 2 (**Figure 2B, C**) in the DLBCL lysates. This suggests that B cells express multiple MyD88 splice isoforms both on mRNA and protein level but isoform 2 is also dominant in transformed B cells.

Primary B Cell Malignancies Show a Preference for Isoform 2

As these transformed cell lines may not reflect primary tumors, we next characterized the RNA expression of the five isoforms in primary B cell lymphoma samples and untransformed naïve B cells. Sashimi plots of RNAseq data from a total of 186 different lymphoma cases (Burkitt lymphoma, DLBCL, follicular lymphoma, follicular lymphoma-DLBCL), untransformed germinal center B cells (GCB, n=5) and naïve peripheral blood B cells (n=5, acquired by the German ICGC MMMLSeq consortium, see Methods) showed expression of all five isoforms at mRNA level (**Figures 2D** and **S2D**). Consistent with earlier mRNA and protein analysis, the canonical isoform 2 was significantly more abundant in transformed *vs* untransformed B cells, whereas other isoforms were either comparable between these groups (isoform 3) or significantly lower (isoform 1, isoform 4 and isoform 5) (**Figure 2E**). Thus, transformed primary B cell tumor samples also showed a preference for the canonical isoform 2 – but not isoform 3 (MyD88s) or other non-canonical isoforms. This was surprising as an 'NF-κB signature' has been attributed to these types of entities (14–16) and in myeloid cells NF-κB signaling was proposed to induce MyD88s (isoform 3) as aforementioned. Collectively, this suggests that, contrary to expectations, lymphoma samples show a higher ratio of canonical MyD88 (isoform 2) to MyD88s (isoform 3) than naive B cells. The analysis of sub-clusters (dependent on driver mutations) of DLBCL samples suggested that those driven by direct activators of NF-κB signaling (e.g. an 'MyD88-like' sub-cluster, see Methods) had a lower ratio of alternative splicing *vs* canonical, and specifically isoform 3, than those driven by indirect NF-κB activation (e.g. BCL2-, BCL6- and TP53-like DLBCL, see **Figures 2F** and **S2E**). In line with this, samples with NF-κB-promoting *MYD88* gain-of-function mutations, such as L265P, had a lower isoform 3 *vs* isoform 2 ratio, i.e. expressed significantly more isoform 2 *vs* isoform 3 transcripts (**Figure 2G**). At least on mRNA level, primary B cell tumors thus did not show evidence for an isoform 3-mediated negative feedback look despite an 'NF-κB signature' described for these entities.

TLR Stimulation Induces Isoform 3 Only Transiently in Stimulated B Cells

Based on what has been published regarding the induction of MyD88s *via* NF-κB signaling in myeloid cells (17, 36), we next tested whether defined NF-κB activating stimuli, e.g. LPS for TLR4 and CpG for TLR9, would lead to an upregulation of isoform 3 in freshly purified (**Figure S3A**) primary B cells. Indeed, TLR9 stimulation enhanced mRNA levels of isoform 3 and 4 at 6 h (mean fold change = approx. two-fold), but at later time points it decreased again to unstimulated levels. TLR4 stimulation induced a marginal but significant reduction of isoform 3 at 18 h (**Figure 3A**). Overall, TLR stimulation changed the relative ratios of *MYD88* splice isoforms very little and the variability between donors is high. As control, we isolated, differentiated and stimulated hMoMacs from the same donors and observed an increase upon 6 h TLR4 stimulation, in

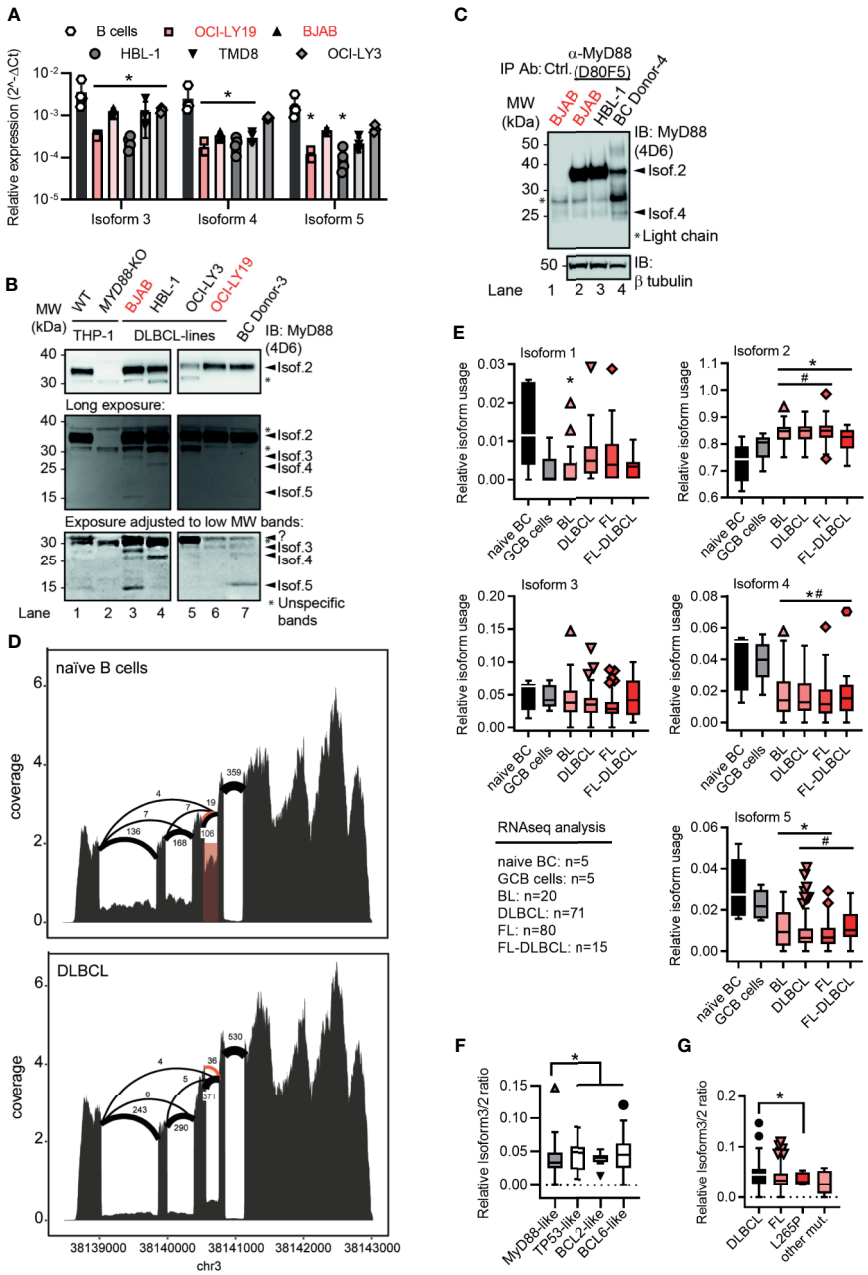

FIGURE 2 | Lymphoma cell lines and primary tumor samples show a preference for the canonical *MYD88* isoform. **(A)** RT-qPCR analysis of isoforms 3-5 in primary B cells or lymphoma cell lines (n=3-4; red GCB, black ABC). **(B)** Immunoblot from THP-1 myeloid cells, B lymphoma cell lines and primary B cells (n=3). **(C)** Immunoblot from pulldown of MyD88 isoforms using the antibody D80F5 against the TIR domain (exon 4). **(D)** Sashimi plots with mean read numbers supporting the splice junctions from naïve B cells (n=5) and DLBCL samples (n=83). The red shaded box shows intron retention and orange arcs represent an alternative donor splice site from isoforms 6 and 7. **(E)** RNAseq analysis of relative isoform usage from untransformed B cells or lymphoma samples (n=as indicated). Isoform 2 expressed as 1-(sum of all others). Other isoforms used: number of unique splice junctions divided by number of reads at the respective splice site (see Methods). **(F, G)** Ratios of isoform 3 (*MYD88s*) to isoform 2 in different DLBCL sub-clusters **(F)** and in dependence of *MYD88* mutations **(G)**. **(F)** MyD88-like n=24, BCL2-like n=9, BCL6-like n=16 and TP53-like n=19. **(G)** MyD88 L265P mutated samples (n=5) and other mutants (n=6) compared to respective MyD88 wildtype lymphomas. **(A, E–G)** represent combined data (mean+SD, or Tukey box and whiskers) from 'n' biological replicates (each dot represents one replicate). In B one representative of 'n' technical replicates is shown. * or # = p<0.05 according to two-way ANOVA **(A)**, Mann-Whitney (**E**, comparison to naïve B cells (*) or to GCB cells (#)), or Wilcoxon **(F, G)**.

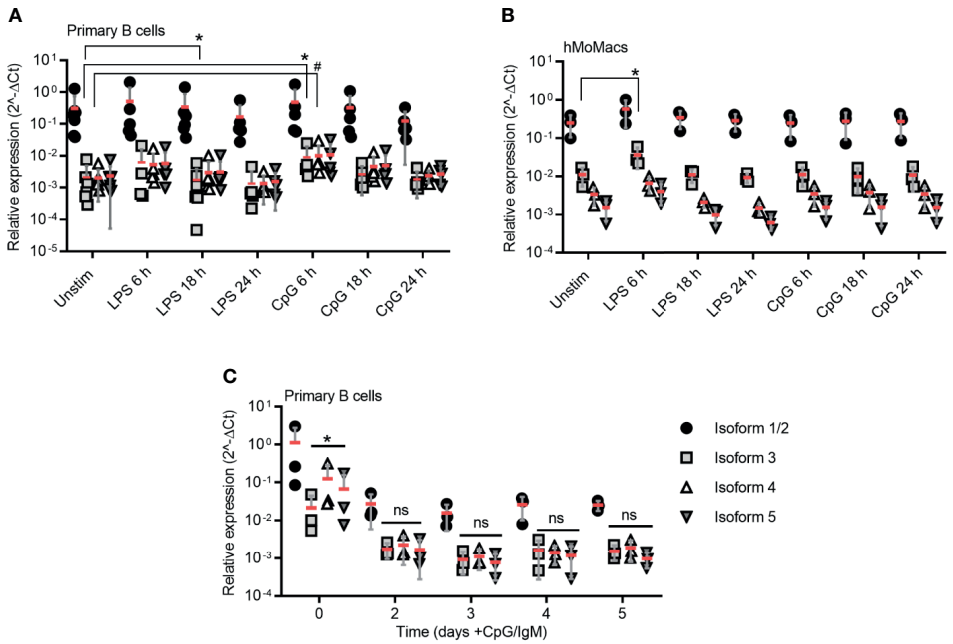

FIGURE 3 | TLR stimulation induces isoform 3 only transiently in primary B cells. **(A–B)** RT-qPCR analysis of isoforms 1/2 to 5 in primary B cells (**A**, n=5-7) and hMoMacs (**B**, n=3) stimulated with LPS or CpG as indicated (n=3). **(C)** RT-qPCR analysis of isoforms 1/2 to 5 in primary B cells stimulated with CpG and IgM to induce proliferation (n=3). A-C represent combined data (mean+SD from 'n' biological replicates (each dot represents one replicate). ns, non-significant; * or # = p<0.05 according to Kruskal-Wallis test **(A)**, ordinary one-way ANOVA **(B)** or two-way ANOVA **(C)**.

line with earlier studies (**Figure 3B**), although it has to be borne in mind, that these earlier studies mainly tested in murine macrophages or human epithelial cells (17, 26, 36). Conversely, when B cells were stimulated until proliferation with TLR9 CpG + IgM, surprisingly, *MYD88* transcription was reduced altogether and did not lead to higher relative induction of the MyD88s (isoform 3, **Figure 3C**), despite the fact that TLR stimulation was effective at driving cellular proliferation as assessed by CFSE proliferation assays (**Figure S3B**). Therefore, we conclude that proliferating B cells, like lymphoma samples, show and maintain a preference for canonical MyD88 signaling. Furthermore, in B cells sustained NF-κB signaling does not induce or coincide with a shift towards inhibitory isoforms as reported for myeloid cells regarding MyD88s (isoform 3). Rather, the canonical, signaling-competent isoform 2 dominates

Novel MyD88 Isoforms With TIR Truncation in B Cells Are Supportive of NF-κB Signaling

In the process of RNAseq analysis we noticed additional alternative splicing events, namely either usage of another donor splice site within the exon 3 (leading to isoforms 6 and 7) or the retention of the exon 3-4 intron (here termed isoform 8), see **Figures 2D**, **4A, B**, **Figures S4A, B** and **Table 1**. The novel splice site within exon 3 (20 nt upstream of a canonical donor) showed a Human Splicing Finder (HSF) score of 81. Typically, a score above 65 is considered a strong splice site (37), indicating these additional splicing events are highly plausible. This alternative donor site leads to a premature STOP codon and thus results in additional isoforms with a truncated TIR domain (**Figures 4A, B** and **S4A, B**), which have not been reported so far. When expression constructs corresponding to isoforms 6-8 were transfected into HEK293T cells, proteins of the expected size (29 kDa for isoform 6, 24 kDa isoform 7 and 26 kDa for isoform 8; plus 6 kDa from the StrepHA-tag) were detectable (**Figure 4C** and **Table 1**). The isoform 8 construct was generated from an hypothetical sequence, which was confirmed by sequencing BJAB amplification product upon PCR using specific primers (**Table S1** and **Figure S4B**). To gain an insight into their ability to signal to NF-κB, we performed NF-κB dual luciferase assays in normal HEK293T and I3A cells as before. Evidently, isoforms 6 and 8 were able to induce downstream NF-κB activation in HEK293T cells, whereas isoform 7 did not (**Figures 4D, E**).

Isoform 6-8 transcripts were also detectable in the lymphoma samples (**Figures 4F–H**) and, as with the other non-canonical isoforms, they were significantly less abundant in lymphoma cells *vs* naive B cells. In the 289 RNA-seq samples of the ICGC Chronic Lymphocytic Leukemia (CLL) dataset, 7 isoforms could be readily detected and quantified, with the canonical isoform showing the highest relative abundance, followed by isoform 6, while isoform 5 showed the lowest abundance (**Figure 4I**). Furthermore, there were noticeable reads mapping to the exon 3-4 intron (**Figure S4C**) confirming isoform 8 in CLL. Additionally, we could also detect isoform 8 in primary B cells (**Figure S4D** and

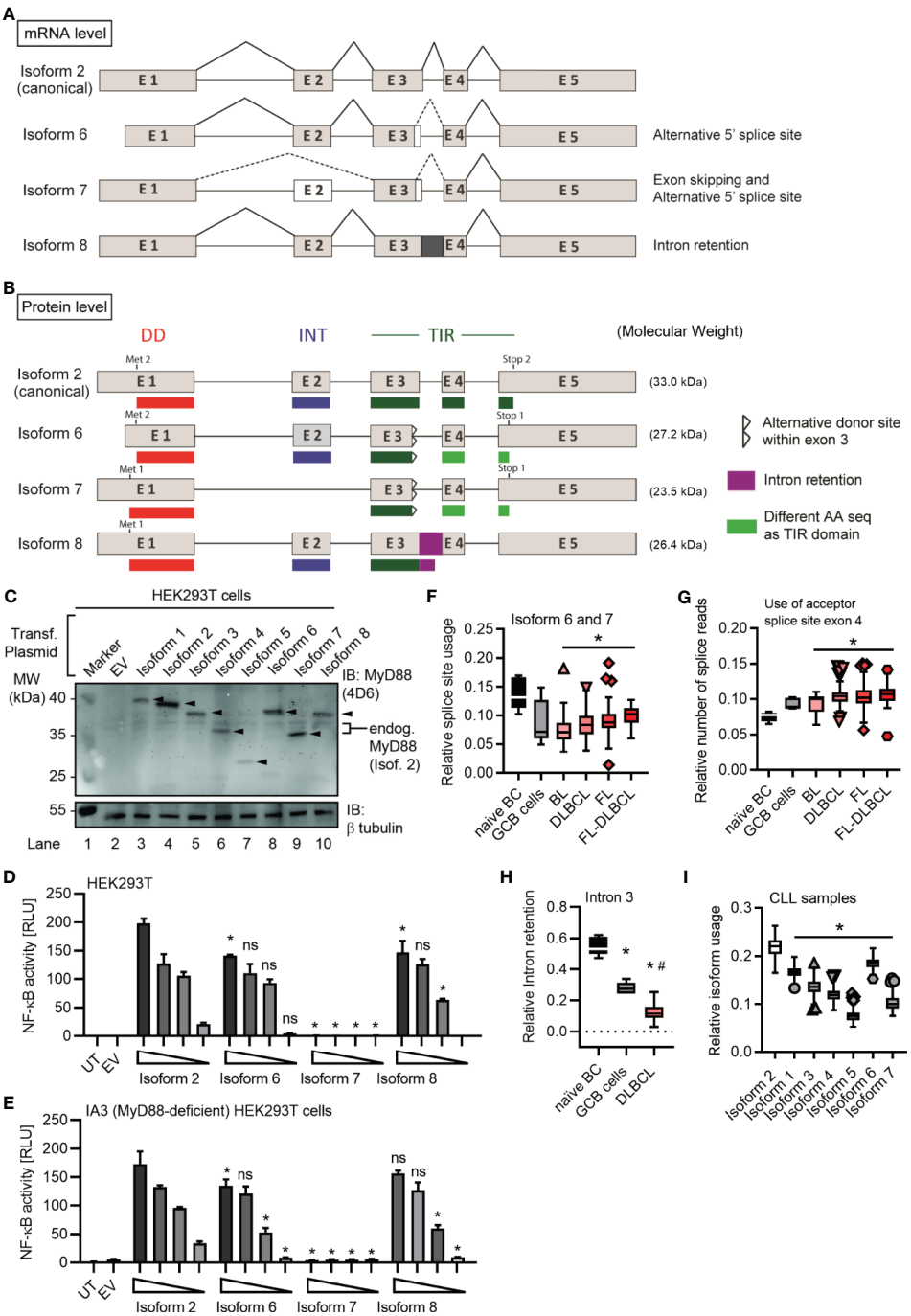

FIGURE 4 | *MYD88* can give rise to three additional MyD88 isoforms. **(A, B)** Schematic representation of novel *MYD88* isoforms on mRNA and protein level according to references in **Table 1** and hypothetical sequence for isoform 8. **(C–E)** HEK293T cells were transfected with plasmids for different *MYD88* isoforms and lysates analyzed for expression or pathway activation by immunoblot (**C**, n=2) or NF-κB dual luciferase assay (**D, E** n=3), respectively. **(E)** as in D but using MyD88-deficient I3A cells (n=3). **(F–H)** RNAseq analysis from untransformed B cells or lymphoma samples (n=as indicated in **Figure 2E**). Intron retention presented as relative number of splice reads using the acceptor splice site of exon 4 **(G)** or coverage of intron 3 compared to mean of flanking exons 3 and 4 **(H)**. **(I)** RNAseq analysis from CLL samples (n=289). In **C–E** one representative of 'n' technical replicates is shown, for **D, E**, as mean + SD from three repeats. **F–I** represent combined data (Tukey box and whiskers) from 'n' biological replicates (each dot represents one replicate). ns, non-significant; * or # = p<0.05 according to two-way ANOVA comparing to isoform 2 **(D, E)** or Wilcoxon Mann-Whitney **(F-I)** in comparison to naïve B cells (*, **F–H**) and to GCB cells (#, **F–H**) or isoform 2 **(I)**.

hMoMacs (**Figure S4E**) by RT-qPCR. Interestingly, TLR4 stimulation in hMoMacs significantly enhanced the mRNA levels of isoform 8, another signaling competent form (cf. **Figures 4D,E**). All eight *MYD88* splice isoforms were also detectable in non-immune cells, as verified in a publicly available RNAseq dataset (31) for ovarian cancer (**Figure S5**). On the whole, there are 3 additional splice isoforms of MyD88 with truncated TIR domains out of which two, unexpectedly, can support signaling upon overexpression, similar to the canonical MyD88 isoform. This extended analysis highlights an even higher diversity of splice variants emanating from the *MYD88* oncogene than previously thought. Furthermore, splicing in B cell lymphomas appears to strongly favor the canonical *MYD88* isoform without diverting splicing events to alternative or signaling-incompetent splice isoforms. Importantly, we find no evidence for a significant induction of MyD88s (isoform 3) as a restrictor of TLR pathway activity.

DISCUSSION

Alternative splicing has emerged as a frequent phenomenon employed for fine-tuning or regulating signaling pathways and plays a pivotal role in the adaptive immune system (38, 39). However, decisive regulators of innate immune pathways have also been subject to alternative splicing: Since its discovery in 2002, the induction of MyD88s *via* NF-κB signaling loop has been viewed as a classical example of an inflammation-restricting negative feedback loop in innate immunity (17, 27). Hence, all the numerous subsequent studies on MyD88 splicing have exclusively focused on this isoform (23, 24, 40–42) and have been largely limited to myeloid cells, primarily in the murine system.

We here provide a comprehensive characterization of all currently reported human *MYD88* splice isoforms. This includes the novel isoforms 6-8, which are the only variants to contain partial TIR domains. During the course of this analysis, isoforms 6 and 7 were added to Genebank but had not been confirmed or studied in detail. Isoform 8 is a novel and surprisingly frequent splicing event not reported before and found abundantly in naïve B cells. Our analysis suggests that, with the exception of isoforms 3 (MyD88s), 5 and 7, isoforms (4, 6 and 8) may induce downstream NF-κB activity in overexpression assays. Whether they can nucleate or engage in the Myddosome in response to TLR signaling in the absence of a complete TIR domain remains to be studied. Potentially, isoforms 4, 6 and 8 may also be signaling incompetent. Thus, all *MYD88* splice isoforms, except isoforms 1 and 2, may lead to dysfunctional MyD88 proteins. This would make our observations made on transcript levels even more striking as then none of the alternative splicing events would be able to counteract constitutive NF-κB signaling *via* isoform 2. Consequently, the oncogenic influence of isoform 2 is likely to be even more dominant.

Furthermore, we show that *MYD88* splicing is much more multi-faceted than previously reported: Our data indicate that

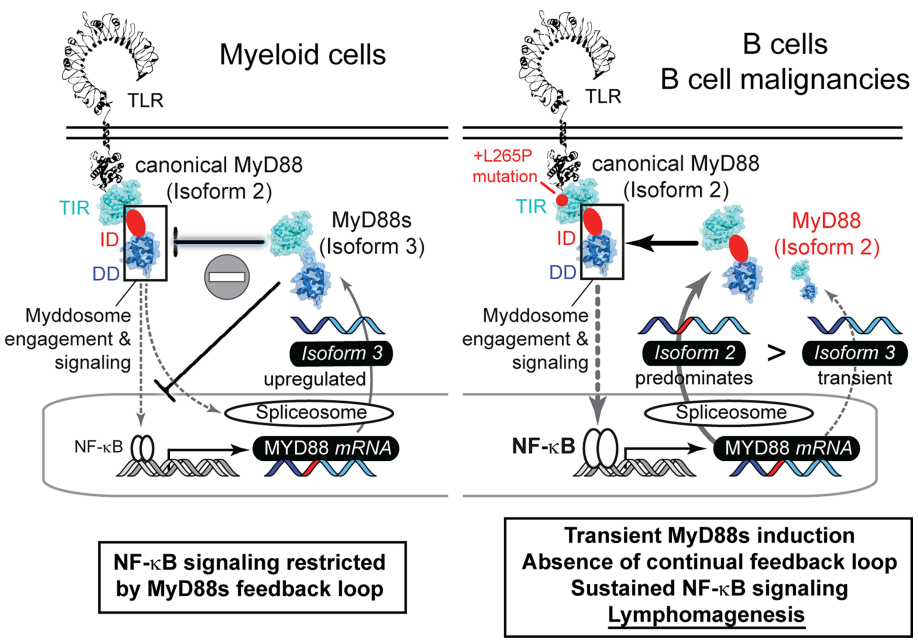

FIGURE 5 | The failure of proliferative B cells to induce non-canonical *MYD88* splice variants correlates with lymphomagenesis *via* sustained NF-κB signaling. Graphical abstract summarizing splicing patterns in myeloid cells (left, based on literature) and B cells (right, focus in this study).

whereas normal B cells use a richer repertoire of splice isoforms, the transformed status rather displays a reduced diversity and appears to lack alternative splice events. The reason for this is unknown but our data warrant a further investigation in additional cohorts and entities, e.g. Waldemström's macroglobulinemia, in future. Based on our data it appears that the preference for canonical isoform 2 and thus unrestricted NF-κB signaling may be favored in the oncogenic process. BCL2, BCL6 or TP53-driven lymphomas, which have an indirect effect on the NF-κB signature, showed lower levels of canonical *MYD88* and higher levels of isoform 1 and isoform 4, compared to MyD88-like lymphomas (**Figures 2F** and **S2E**). This fits well with the observation that the gain-of-function mutation, L265P, leads to extended NF-κB hyperactivation and is a hallmark of oncogenic B cells (7, 8). Of note, our data indicate that B cells lack a sustained negative feedback mechanism of MyD88s induction to rescue mutated cells from MyD88-driven oncogenesis: For example, TLR stimulation induced MyD88s in TLR-stimulated hMoMacs and B cells at short time points, but MyD88s was not prominently expressed or regulated under the extended presence of NF-κB stimuli in B cells and lymphoma cell lines. Thus, B cells with increased NF-κB activity, due to L265P mutation or other mechanisms, cannot get "reigned in" (controlled) *via* MyD88s expression, unlike some myeloid cells, then continued NF-κB pro-survival activity may result (**Figure 5**). Our data thus provide an explanation why oncogenic mutations have only been reported in B cell lymphoma, rather than tumors arising from myeloid cells, whose MyD88s induction loop possibly renders them more resistant to MyD88 pathway induced NF-κB activity.

Our observations that alternative splicing of genes in the MyD88 dependent pathway are important candidates in oncogenesis agree with the recent description of oncogenic *IRAK4* isoforms, albeit in myeloid malignancies (43). It is intriguing to speculate whether the aforementioned negative feedback loop, that is absent in proliferative B cells, prevents *MYD88* mutations from manifesting themselves, but does not prevent oncogenic signaling arising from the next downstream pathway member, IRAK4. Undoubtedly, with the availability of powerful sequencing techniques the analysis of alternative splice isoforms of MyD88 pathway members for discovering novel non-mutational cancer drivers is both possible and warranted. In the substantial percentage of cases without druggable driver mutations this may offer opportunities for targeting e.g., *via* antisense oligonucleotide-mediated exon skipping (44, 45). In this therapeutic sense, MyD88s or the other signaling incompetent isoforms described here may provide a blueprint for such an approach in B cell lymphomas.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. B cell lymphoma, naive B cells and Germinal center B cells RNAseq libraries are from European genome-phenom archive at EBI: https://www.ebi.ac.uk/ega/home. Chronic Lymphocytic Leukemia (CLL) RNAseq data from the ICGC-CLL Consortium (https://dcc.icgc.org/releases). Ovarian cancer RNAseq libraries from the ICGC/OVAU project (Australian Ovarian Cancer Study, https://dcc.icgc.org/projects/OV-AU).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Medical Faculty, University of Tübingen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YC, O-OW and SD performed experiments. YC, SB, SF, SN, SD, JA, and SO analyzed data. RS and SO were involved in sample collection. YC and AW conceived and AW supervised the entire study. YC and AW wrote the manuscript and all authors provided additions and comments to 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/fimmu.2021.616451/full#supplementary-material

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