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Rationale for targeting tumor cells in their microenvironment for mantle cell lymphoma treatment

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ABSTRACT
Mantle cell lymphoma (MCL) is an aggressive non-Hodgkin lymphoma associated with poor prognosis, and despite recent improvements in the therapeutic strategies for treating MCL, its management remains challenging. While improvements in next generation sequencing technology have greatly increased our understanding of the intrinsic abnormalities of MCL, the role of extrinsic signaling remains largely unknown. Recent studies have highlighted the central role of the MCL microenvironment in tumor cell survival, drug resistance and proliferation. Characterization of the diverse MCL tumoral niches and comprehension of the crosstalk between tumor cells and surrounding cells within the MCL microenvironment are needed to increase treatment efficacy. Here, we reviewed the recent findings regarding the MCL microenvironment that could be rapidly translated into new therapeutic strategies to overcome drug resistance during MCL treatment.

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Introduction
Mantle cell lymphoma (MCL) is a rare B-cell malignancy, representing 6% of non-Hodgkin lymphomas (NHLs) with an incidence of 0.8 and 0.2 per 100,000 men and women, respectively (4:1 ratio). The median age at diagnosis is 65–70 years, with an overall survival (OS) of 5–7 years. MCL has one of the poorest outcomes of all NHLs [1]. MCL cells are IgM+ CD19+ CD5+ CD23– B cells, and while they initially accumulate in the lymph nodes or the spleen, they disseminate early in extranodal tissues [2].

At the molecular level, MCL cells are characterized by the t(11;14)(q13;q32) translocation, which results in aberrant expression of the cyclin D1 and thus dysregulation of the cell cycle [3]. However, although the cyclin D1 overexpression appears as the primum movens, it is not sufficient for MCL development. Indeed, diagnosis of MCL is associated with the occurrence of secondary abnormalities, which are deletion and/or mutation of tumor suppressors and overexpression of oncogenes. Recent progress in next generation sequencing technology has highlighted an important genomic instability that results in recurrent gains of MYC and deletions of RB1, ATM, CDKN2A or TP53 [4]. Mutation or overexpression of several genes, such as ATM, CCND1, SOX11, TP53 or UBR5, also contributes to the pathogenesis of MCL and can be associated with poorer prognosis [5,6]. CDKN2A and TP53 deletions have been shown to be independent prognostic factors separate from the proliferation index [4]. Additionally, various studies have shown that the transcription factor SOX11 is overexpressed in 90% of MCL cases, with SOX11 being negatively associated with clinically indolent cases [7,8]. Taken together, these findings offer novel perspectives in understanding MCL physiopathology [9].

In addition to these intrinsic abnormalities, it has been shown that extrinsic signaling from the microenvironment has a central role in MCL expansion [10,11]. As previously observed for other NHLs, MCL chemoresistance, survival and proliferation could be impacted by surrounding cells [12]. In this review, we present novel insights regarding the MCL microenvironment and discuss how these findings could offer new therapeutic opportunities to overcome drug resistance during the treatment of MCL.

Composition and dynamics of MCL tumoral niches
Mantle cell lymphoma owes its name to its primary expansion zone, the mantle zone, in lymph nodes,
but extranodal manifestations in the bone marrow, blood or the gastrointestinal tract are often associated with the diagnosis [2]. These tumoral niches are dynamic, and they host crosstalk between tumoral cells, accessory cells and soluble factors [13] (Figure 1).

A complex chemokine network

The attraction of tumoral and accessory cells to their specific tumoral niches is based on a complex chemokine network. Chemotaxis of MCL cells is controlled by several chemokines, such as CXCL12, CXCL13 and CCL19, that bind to the chemokine receptors CXCR4, CXCR5 and CCR7, respectively, which are highly expressed in MCL cells. Interestingly, whereas CXCL12 also attracts normal B cells, CCL19 is specific for the homing of MCL cells [14]. MCL also shapes its tumor microenvironment by attracting accessory cells through chemokine secretions. The expression of CCL4 and CCL5 is increased in tumor cells compared with normal B cells, and they are known to attract monocytes [15]. CCL17 and CCL22 are also detected in MCL blood samples, suggesting they play a role in the recruitment of immune cells, such as monocytes or T cells, in the lymph nodes [16].

**Immune cells: macrophages and T-cells have the first role**

Only a few studies have addressed the immune cell composition of MCL tumor niches so far. In peripheral blood and lymph nodes, an evaluation of absolute lymphocyte counts shows that the numbers of CD4⁺ T cells and the CD4:CD8 ratio positively correlate with OS [17,18]. Absolute counts of CD3⁺, CD4⁺ and CD8⁺ T cells are lower in aggressive forms of MCL [18]. Despite the low levels of T cells, regulatory T cells (CD25⁺, CD4⁺), known to negatively impact many cancer types, have been described as having a high frequency in the microenvironments of several NHLs, including MCL [19]. Moreover, the expression of the protein program cell death 1 (PD-1) in T cells is observed in MCL lymph nodes, mainly in the germinal centers, which suggests an interaction with tumoral cells [20,21]. Indeed, PDL-1, the ligand of PD-1, is expressed in MCL cells and leads to the inhibition of the T cell anti-tumoral response *in vitro* [22]. Of note,
numerous studies have shown the importance of lymphoid-like interactions through the CD40/CD40L axis for proliferation and tumor cell growth [10,23–26] and a recent study highlighted the co-localization of primary MCL cells and autologous T-cells in a model of patient-derived xenograft [27]. These preliminary data should encourage further study of the precise T cell phenotypes in the MCL microenvironment.

Several works have shown that absolute monocyte counts negatively correlate with OS [28], and that CD163+ macrophages are present in lymph nodes, suggesting a role of myeloid cells in the MCL microenvironment. Preliminary studies highlight that myeloid cells support primary MCL cell survival and are involved in lymphangiogenesis through VEGF-C production [29,30]. However, the interactions and signaling networks between MCL cells and macrophages are still unidentified and warrant further investigations.

**The mesenchymal cells participate to the network**

Follicular dendritic cells (FDCs), cells of mesenchymal origin, have low levels of CD23, CD35 and CD54 in MCL compared to reactive lymph nodes [31]. The distribution of FDCs can differentiate between groups of MCL patients that have different outcomes: the diffuse pattern of lymph node FDCs is associated with a poorer outcome than the nodular pattern [32]. FDCs support MCL cell survival through a cell-cell contact mechanism, but the molecular signaling has not yet been identified. In bone marrow, MCL cells and mesenchymal stromal cells (MSC) participate in soluble crosstalk that is essentially driven by the CXCL12/CXCR4 axis and BAFF that promotes the survival of tumor cells [33,34]. Cell–cell interactions occur in the niche through VLA-4/ICAM-1 binding [35] and the adhesion molecule JAM-C, as described in a xenograft mouse model [36].

**MCL microenvironment promotes tumor survival and proliferation**

The pro-tumoral ecosystem that supports MCL is still poorly understood; however, whereas proliferation is often detected in lymph nodes in vivo, MCL cells rarely proliferate in the peripheral blood and bone marrow. In fact, our data suggest a differential proliferation of the same clone in function of its tissue localization in vivo [10,37]. Furthermore, Saba et al. recently confirmed the central role of the MCL microenvironment demonstrating that the B-cell receptor (BCR) and nuclear factor κB (NF-κB) pathways, in addition to the proliferation of tumoral cells, is restricted to the lymph nodes and reduced in the peripheral blood [11].

**BCR signaling is activated in MCL**

BCR signaling plays a central role in the development, proliferation and survival of normal B cells, and a high rate of early responses to BCR signaling inhibition has recently increased interest in this pathway in regards to MCL. Whereas MCL is defined as a naïve B cell malignancy, studies have shown that, in addition to carrying a biased IGHV repertoire, somatic hypermutations (at least one mutation) are detected in more than 70% of MCL samples, and that 14% are highly mutated (<97% germline), suggesting antigen encounter [38]. Of note, highly mutated cases are mostly SOX11 negative MCL cells that are associated with an indolent clinical outcome [39]. However, in contrast to several NHLs, no autoantigens have been described to be associated with MCL [40]. At the plasma membrane, the BCR is associated with a heterodimer composed of the proteins CD79A and CD79B. BCR activation results in phosphorylation of the dimer, which allows for its transduction into the cell. BCR activates a series of kinases, such as SYK, BTK and PLCγ2 that leads in the activation of several pathways: NF-κB, PI3K/AKT, and MAPK [40]. It has been shown a specific BCR signaling pattern associated with MCL characterized by strong levels of phospho-(p)-SYK, p-PLCγ2 and p-AKT [41]. MCL cell lines and primary samples have an active BCR pathway [42], but the molecular nature of in vivo BCR activation, either tonic (antigen-independent) or chronically active (antigen-dependent), remains to be defined [40]. Nevertheless, gene expression signatures show high BCR activation in lymph node resident cells compared to peripheral blood cells, which reinforces the hypothesis that the microenvironment is implicated in MCL [11].

**The CD40/CD40L axis is central**

The CD40/CD40L axis is a major component of B and T-cell communication networks and is well known to be involved in normal B cell activation as well as in the survival and proliferation of various malignant B cells [12]. In MCL, T cells are suspected to provide CD40L molecules in lymph nodes, and soluble CD40L is present in peripheral blood of MCL patients at a high level compared to healthy donors [43]. Considering this, several studies highlighted the relevance of CD40/CD40L in MCL models in vitro based on soluble CD40L [24], activators of CD40 [23], or transfected CD40L-expressed fibroblasts [10,25,26].
Importantly, whereas peripheral blood MCL cells rarely proliferate, CD40 activation activates progression of MCL cells into the cell cycle ex vivo, suggesting that this extrinsic signal plays a central role in the proliferation of MCL in vivo. We recently demonstrated that CD40 activation in the presence of MCL-specific growth factors recapitulates molecular signatures that are characteristic of MCL lymph nodes, such as cell cycle, BCR, NF-κB/NIK and survival (Bcl-2 family).

**Soluble factors play a major role**

In addition to direct contacts between accessory cells and MCL cells, autocrine and paracrine secretion of several soluble factors could have a major role within MCL niches. Interestingly, various cytokines and chemokines are present at high levels in the blood of MCL patients, such as interleukin IL-12, CXCL3 and CCL4, and some of these are correlated with poor survival (IL-8, CCL3 and CCL4) [44]. In addition, primary MCL cells express receptors for IL-10, BAFF, IL-15, APRIL, CXCL12) in several NHLs but their precise role in MCL is still unknown [12,48]. In addition to supporting survival, soluble factors are involved in MCL cell proliferation. Indeed, CD40L-dependent proliferation is potentiated by IL-4 or IL-10 [23,25]. However, although IL-4 has been used in various models in vitro, it is unlikely that it has an in vivo role in MCL because of low IL4-R expression in MCL cells and the dramatic IL-4-induced expression of CD23, which is typically unexpressed in MCL cells in vivo [49].

IL-6 and IL-10 activate STAT3, and it has been proposed that STAT3 and p-STAT3 expression witness IL-10 implications in both primary and MCL cell lines [50]. IL-6 induces the activation of PI3K/akt and the NF-κB pathways [45,51], whereas BAFF is a potent inducer of the alternative NF-κB pathway [34]. Finally, the CXCL12/CXCR4 axis also promotes MCL cell survival through MAPK activation, particularly in the context of the bone marrow microenvironment [33,35].

**How the microenvironment drives chemoresistance**

**Chemoresistance involves cellular adhesion-mediated drug resistance (CAM-DR)**

As previously described in other malignancies, molecular interactions associated with the homing of MCL cells can result in CAM-DR. Both the CXCL12/CXCR4 and the ICAM-1/VLA-4 axis facilitate the interactions and adhesion between MScs and MCL cells. These interactions result in the active phosphorylation of the ERK, AKT and NF-κB pathways that leads to CAM-DR against chemotherapeutic agents such as fludarabine and cyclophosphamide [35,52]. CAM-DR is also observed for proteasome inhibitors where bortezomib treatment enhances CXCR4 expression, which consequently favors MCL resistance [33].

**The NF-κB pathways are activated**

Classical NF-κB activation results in the phosphorylation and degradation of the IκBα subunit, followed by the translocation of the p50/65 complex in the cell nucleus. The alternative NF-κB pathway is independent of IκBα and consists of the proteolysis of the p100 protein into p52. Several mutations in NF-κB pathways (BIRC3, TRAF2, CARD11, NIK or IKBKB) have been described in more than 10% of MCL patients [53] and, as observed for BTK mutation [37], could be involved in acquired resistance to novel therapies that target BCR signaling. However, extrinsic signals could also control NF-κB activity in MCL cells. Indeed, CD40L or MSCs activate both the classical and alternative pathways [26,34]. BAFF signaling induces the alternative NF-κB pathway and activates the transcription of targeted genes, including TNFSF13B, for the formation of an autocrine loop [54]. In MCL, the NF-κB pathway has a predominant role and is associated with survival and proliferation, whereas its inhibition leads to cell cycle arrest and apoptosis [55]. In addition to tumor promotion, several studies have shown that an active NF-κB pathway is associated with the acquisition of chemoresistance against not only traditional chemotherapy drugs, but also against targeted drugs, such as ibrutinib [53]. Resistance to the Bcl-2-specific BH3 mimetic Venetoclax has also been associated with microenvironment-dependent NF-κB activation [26].

**The imbalanced expression bcl-2 family proteins favor survival**

The Bcl-2 family is composed of multidomain anti-apoptotic members (Mcl-1, Bcl-2, Bcl-xL, Bcl-W and A1),
multidomain pro-apoptotic members (Bak and Bax) and BH3-only pro-apoptotic members (Bim, Bid, Bad, Bik, Puma, and Noxa). MCL is characterized by overexpression of the anti-apoptotic Bcl-2, and the apoptotic cascade is almost systematically altered [56]. Various evidence shows that Bim downregulation is a central event in MCL physiopathology. Indeed, the homozygous deletion of Bim leads to the development of MCL in a t(11;14) mouse model [57], and five out of the seven widely used MCL cell lines (Z138, Mino, Jeko, UPN1 and SP53) do not express Bim. However, only rare cases of homozygous deletion or mutation of Bim have been reported in MCL primary samples. These discrepancies could be explained by the integration of the central role of the MCL microenvironment. Indeed, Bim is downregulated by many extrinsic signaling factors such as CD40 activation [10], MSC interaction [10] and FDC co-culture [58]. However, molecular signaling that is involved in the microenvironment-dependent regulation of Bim regarding MCL is still unclear.

In addition to the dramatic downregulation of Bim, CD40 signaling induces the NF-κB-dependent upregulation of Bcl-xL, leading to a pro-survival signal. This Bcl-2 family unbalance leads to the microenvironment-dependent loss of mitochondrial priming and, consequently, confers resistance to chemotherapeutic agents such as bendamustine or aracytine. In addition, whereas peripheral blood MCL cells are highly sensitive to the Bcl-2-specific BH3 mimetic Venetoclax, cells that are in protective niches appear to be resistant [10,26].

Taken together, these data highlight that the lymphoma ecosystem must be integrated into future mechanism-based therapeutic strategies.

**Novel therapeutic strategies integrating the key role of the microenvironment**

For over a decade, major therapeutic advances have increased the OS of MCL patients. In particular the use of rituximab for maintenance [59,60], the use of intensive therapies such as autologous stem cell transplantation and the use of high doses of aracytine [61], represent the most recent progress in MCL therapy. Standard chemotherapy has probably reached its limit as an MCL treatment. The emergence of new targeted therapy, such as BTK inhibition and Bcl-2 targeted therapy, is changing the paradigm of treatment in relapsed/refractory MCL and may soon challenge front-line treatment. Among innovative strategies, specifically targeting the MCL microenvironment could be promising. Several drugs, such as lenalidomide, bortezomib and, more recently, ibrutinib, already directly affect the microenvironment or impair its crosstalk with MCL cells, but new agents in development, such as checkpoint inhibitors and BH3 mimetics, could also improve MCL treatment.

**Targeting the microenvironment: IMIDs and checkpoint inhibitors**

As a single agent, the immunomodulatory drug (IMID) lenalidomide has moderate activity against MCL cells and is approved for the treatment of relapsed/refractory MCL [62]. Various studies have also shown good response associated with rituximab (57% of overall response rate [ORR] and 36% of complete response [CR]) [63], and dexamethasone (52% ORR and 12% CR) [64]. Lenalidomide activates anti-tumoral cells, such as T and NK cells in vitro, and increases Tγδ expansion and cytotoxicity in coculture assays [65]. In a xenograft mouse model, lenalidomide inhibits lymphangiogenesis by downregulating Prox1, podoplanin and VEGFR-3 and decreasing the number of VEGF-C+ tumor-associated macrophages [29]. However, no significant modifications in the level of neo-angiogenic factors have been detected in vivo in MCL patients treated with lenalidomide [64].

In addition to the IMIDs, other targeted immunoregulatory strategies are under development. Immune checkpoint inhibitors are among the most promising options for treatment of melanoma and Hodgkin lymphoma. Preliminary data suggest that blocking the PD1/PDL1 axis could be of interest based on encouraging responses in other NHLs. In follicular lymphoma, an anti-tumoral response to immune reactivation upon the implementation of a PD-1 blockade is observed [66].

**NF-κB pathway and CD40-CD40L axis**

Bortezomib is a reversible inhibitor of the ubiquitin-proteasome complex and an approved molecule for treating relapsed/refractory MCL. Bortezomib has proven its efficiency in MCL treatment as a single agent (ORR: 33% CR: 8%) [67], and its association with rituximab and dexamethasone has an ORR of 81%, with 44% of CR [68]. At the molecular level, multiple mechanisms of action of proteasome inhibitors have been proposed, such as downregulation of the NF-κB pathway through IkB degradation inhibition in MCL cells [55]. As previously described, the NF-κB pathway is central in the extrinsic signaling observed in MCL cells, which could participate in the clinical
efficacy of proteasome inhibitors for treatment of the disease [55]. Indeed, we observed that bortezomib can counteract microenvironment-dependent drug resistance by inhibiting NF-κB-dependent Bcl-xL expression [10].

Unfortunately, bortezomib efficacy is limited by major side effects, and more selective therapy targeting the NF-κB pathway could be of interest. We recently showed that the type II anti-CD20 monoclonal antibody Obinutuzumab directly impairs NF-κB target-genes, such as BCL2L1, which consequently counteracts microenvironment-dependent drug resistance in primary MCL cells [10]. Moreover, as previously noted, the CD40/CD40L axis plays a central role in MCL NF-κB activation by the microenvironment. Targeting the CD40/CD40L axis with antagonistic antibodies could be of interest in MCL treatment.

The BCR pathway inhibition promotes MCL egress
Ibrutinib binds covalently to the kinase BTK and irreversibly inhibits BCR signaling. Ibrutinib has recently been approved to treat relapsed MCL patients and has shown efficacy as a single agent in MCL treatment with an ORR of 68% and a CR of 21% [69]. Currently, numerous clinical trials associating ibrutinib to chemotherapy or rational targeted therapy are ongoing. Ibrutinib interferes with the homing of MCL cells, leading to lymphocytosis in vivo, by downregulating CXCR4 expression and migration factors CCL22, CCL4 and CXCL13 in MCL cells [16]. Bone marrow-resident cells seem to be particularly affected by ibrutinib-dependent homing inhibition [70]. BTK inhibition also directly affects MCL survival and proliferation by reducing the autocrine secretion of IL1β, TNFa and CCL5 [47]. Little is known about the impact of ibrutinib on accessory cells, but initial studies in chronic lymphocytic leukemia show that ibrutinib reduces anti-tumor NK activity and phagocytosis by macrophages that are both mediated by anti-CD20. These phenomena may be involved in MCL relapse [71,72].

Selective targeting of Bcl-2, Bcl-xL and Mcl-1
BH3 mimetics selectively target anti-apoptotic proteins that are essential for B-cell malignancy survival and drug resistance. The first study using the Bcl-2-specific BH3 mimic Venetoclax in patients with relapsed or refractory NHL confirmed tolerance and efficacy, with an ORR of 75% and a CR of 21% [73]. We previously described that the MCL microenvironment modulates the expression of members of the Bcl-2 family to promote tumor survival, especially through induction of the anti-apoptotic Bcl-xL and, to a lesser extent, Mcl-1, and through the inhibition of the pro-apoptotic protein Bim [10]. By integrating extrinsic signaling, we observed that the BCL2/(BCL2L1 + MCL1) mRNA ratio is a strong predictor of Venetoclax sensitivity [26]. Accordingly, whereas peripheral blood MCL cells are Bcl-2-dependent and highly sensitive to Venetoclax, the microenvironment-dependent imbalance of the Bcl-2 family induces drug resistance, which could lead to relapse. In addition to Bcl-2-specific BH3 mimetics, Bcl-xL and Mcl-1-specific BH3 mimetics are in development and will be critical to targeting cells in their protective niches. Interestingly, preliminary data on these novel BH3 mimetics show encouraging results in B-cell lymphoma.

Therapeutic perspectives: example of the OAsIs trial
We have recently demonstrated that lymphocytosis following ibrutinib treatment sensitizes MCL cells that egress into peripheral blood to the Bcl-2-specific BH3 mimic Venetoclax [26]. In addition, the type II anti-CD20 Obinutuzumab counteracts Bcl-xL upregulation through inhibition of the microenvironment-dependent NF-κB pathway, leading to increased mitochondrial priming and Venetoclax sensitization [10]. Based on these preclinical observations, we have designed a multicenter phase I/II clinical trial for MCL treatment consisting of treating with the sequential combination of ibrutinib and Obinutuzumab followed by Venetoclax (OAsIs trial NTC#02558816). This mechanism-based clinical trial integrates the lymphoma ecosystem and will rapidly provide information on the efficacy of such a rational strategy.

Conclusions
Increased understanding of MCL biology leads us to reconsider therapeutic strategies for MCL patients. The emergence of novel targeted therapies offers an opportunity to build innovative therapeutic approaches and will soon challenge the role of conventional chemotherapy. However, the cost of such therapeutic strategies could be a challenge, particularly for treating rare diseases and treatment must be based on a strong biological rationale that especially integrates both intrinsic and extrinsic signals.

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