Review

Dysregulated CARD11 signaling in the development of diffuse large B cell lymphoma

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ABSTRACT

CARD11 is a crucial scaffold protein that controls antigen-induced activation of lymphocytes. Upon antigen receptor signaling, CARD11 engages several signaling pathways, leading to the activation of NF-κB, mTOR, and JNK. CARD11 mutations are frequently found in patients with non-Hodgkin lymphoma and their ability to induce aberrant lymphocyte proliferation may be enhanced by mutations in regulators of CARD11 signal transduction. Here we describe how dysregulated CARD11 activity can promote lymphomagenesis through branched signaling pathways whose components and intermediates provide targets for novel diagnostic and therapeutic approaches.

Statement of novelty: This review discusses how gain-of-function CARD11 mutations promote lymphomagenesis by engaging branching signaling pathways and how these different pathways provide multiple targets for therapies.

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common lymphoid malignancy among adults and represents 30%–40% of non-Hodgkin lymphoma cases (Fisher and Fisher 2004). DLBCL is a fast-growing, aggressive type of non-Hodgkin lymphoma that has a median survival of <1 year if untreated (Rovira et al. 2015). Through gene expression profiling, DLBCL has been classified into activated B cell (ABC) and germinal center B cell (GCB) subtypes. The GCB DLBCL subtype expresses genes that define normal germinal center B cells, while the ABC DLBCL subtype exhibits a transcriptional profile similar to mature plasma cells (Alizadeh et al. 2000). The standard of care for ABC DLBCL is a combination of the anti-CD20 antibody rituximab and a 4-drug chemotherapy regimen of cyclophosphamide, doxorubicin, vincristine, and prednisone collectively referred to as R-CHOP; unfortunately, this treatment fails in more than 50% of ABC DLBCL patients (Coiffier and Sarkozy 2016). A thorough understanding of mutations that influence DLBCL disease progression will be necessary to innovate diagnostic tools and targeted therapies and improve outcomes for these patients.

One common signaling cascade that is dysregulated in several types of lymphoid malignancies is the nuclear factor kappa B (NF-κB) pathway. Nearly all cases of ABC DLBCL are characterized by constitutive activation of NF-κB, which has been shown to drive lymphomagenesis (Davis et al. 2001). NF-κB regulates the differentiation, cell cycle progression, proliferation, and survival of lymphocytes. Therefore, it is not surprising that certain mutations in signaling proteins upstream of NF-κB have been shown to cause aberrant lymphocyte activation and are associated with DLBCL cases. An obligate component of antigen receptor

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signaling upstream of NF-κB activation is the scaffold protein CARD11. Approximately 10% of human ABC DLBCL biopsies exhibit gain-of-function (GOF) mutations in CARD11 that lead to enhanced NF-κB activation (Lenz et al. 2008). In addition to regulating NF-κB, CARD11 also engages branching signaling pathways leading to mammalian target of rapamycin (mTOR), Forkhead box protein O1 (FOX01), and c-Jun N-terminal kinases (JNK) activation through mechanisms that are not fully understood (Blonska et al. 2007; Oeckinghaus et al. 2007; Chen 2012; Wray-Dutra et al. 2018; Wei et al. 2019). Investigating the complex integration of multiple signaling pathways that may become dysregulated by mutant CARD11 is essential for understanding the role of CARD11 in the stages of lymphocyte oncogenesis. This review will summarize the molecular mechanisms by which mutations in both CARD11 and regulators of CARD11 drive lymphomagenesis and will provide supporting evidence that intermediates within the CARD11 signaling pathways serve as important therapeutic targets for treating DLBCL (Figure 1).

CARD11 signaling to NF-κB

Several studies have established the role of CARD11 in antigen-induced lymphocyte activation (Bedsaul et al. 2018). In the absence of antigen receptor signaling, CARD11 remains in an inactive conformation due to an inhibitory domain (ID) that contains 4 small Repressive Elements (REs) that prevent cofactor interaction (Jattani et al. 2016*a*, 2016*b*). Antigen receptor triggering leads to the phosphorylation of CARD11 serine residues 564, 567, 577, and 657 within the ID, in part by PKCβ in B cells and PKCθ in T cells, both of which are thought to phosphorylate S564 and S657 (Matsumoto et al. 2005; Sommer et al. 2005). Phosphorylation of CARD11 by other kinases also plays a role in promoting maximal CARD11 activity, including the phosphorylation of S567 by IKKβ (Shinohara et al. 2007). Though the mechanism remains unknown, phosphorylation of these serine residues in the ID is thought to neutralize the inhibitory function of the REs and allow CARD11 to undergo a conformational change to an open and active state. Once converted to an active scaffold, CARD11 is able to recruit several cofactors that bind to sites located in the CARD, LATCH, and Coiled-coil domains, such as Bcl10, MALT1, TRAF6, and HOIP (Ruland et al. 2001; Che et al. 2004; Sun et al. 2004; Yang et al. 2016). This multiprotein complex then activates the IKK complex, leading to the phosphorylation of the IκBα inhibitory protein and its subsequent ubiquitinylation and proteasomal degradation (Figure 1). NF-κB then translocates into the nucleus and regulates the transcription of pro-proliferative, pro-inflammatory, and anti-apoptotic genes that are important for lymphocyte function. Following activation, the signaling cofactors dissociate and CARD11 returns to its inactive state (Bedsaul et al. 2018).

Mutations that disrupt the autoinhibition of CARD11 have been linked to immunodeficiency and lymphoproliferative diseases. CARD, LATCH, and Coiled-coil domain mutations have been found in DLBCL biopsies, including C49Y, G123S, and G123D (Lenz et al. 2008; Compagno et al. 2009). Mutations in these domains disrupt autoinhibition by 4 REs (Jattani et al. 2016*b*) and increase signaling to NF-κB by 80- to 160-fold. This enhanced activity results in constitutive Bcl10 and HOIP association with CARD11, leading to the generation of polyubiquitinylated Bcl10 ($LinUb_n$-Bcl10), a key signaling intermediate that determines the quantitative output of CARD11 signaling (Lamason et al. 2010; Chan et al. 2013; Yang et al. 2016). Several studies have documented a critical role for hyperactive CARD11 signaling in promoting lymphomagenesis.

RNA interference and CRISPR/Cas9 screens have shown that most ABC DLBCL cell lines depend on CARD11-Bcl10-MALT1 (CBM) complex signaling (Ngo et al. 2006; Reddy et al. 2017; Phelan et al. 2018). Furthermore, DLBCL-derived CARD11 mutations introduced into activated B cells ex vivo and transplanted into *RAG1*−/− recipient mice were shown to induce aberrant B cell proliferation, antibody secretion, and plasmacytic differentiation (Jeelall et al. 2012). In addition, mice expressing the strongly hyperactive CARD11 L225LI mutation under the control of the *Rosa26* promoter in the B cell lineage succumbed to aggressive B cell lymphoproliferation a few days after birth, demonstrating that heightened CARD11 signaling can be sufficient to cause a disease phenotype resembling ABC DLBCL (Knies et al. 2015). NF-κB signaling was found to be essential for CARD11 L225LI B cell survival because treating these cells with the IKK inhibitor Bay11-7082 prevented their survival and proliferation in vitro. Interestingly, other mouse models expressing GOF CARD11 alleles do not readily develop lymphoma, suggesting that the accumulation of additional mutations is necessary to drive

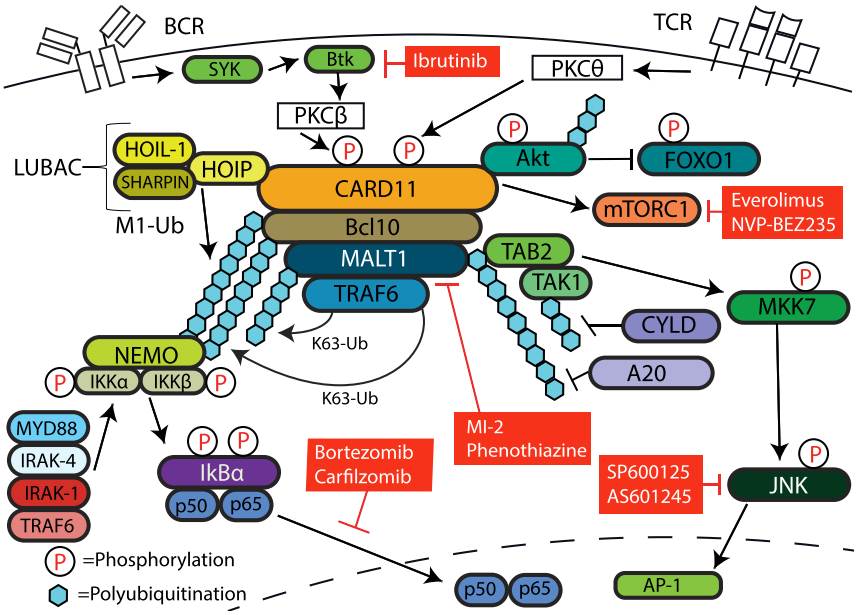

Figure 1: B cell receptor and T cell receptor signaling to CARD11 activates a network of signaling pathways that offer therapeutic targets. Therapeutics are indicated by the red boxes.

malignancy of B cells that express these GOF CARD11 mutations (Wray-Dutra et al. 2018; Wei et al. 2019). The molecular mechanisms that cause some GOF CARD11 mutations to promote lymphoma while others do not are still not well understood.

Certain GOF mutations found in DLBCL biopsy samples such as C49Y and G123S are also present in patients with B cell expansion with NF-κB and T cell anergy (BENTA) disease (Snow et al. 2012; Buchbinder et al. 2015). Occurring predominately in children, BENTA patients present with B cell expansion, splenomegaly, and susceptibility to recurrent ear, sinus, and infections of Epstein-Barr virus, molluscum contagiosum virus, or BK virus. BENTA patients have T cell numbers within normal pediatric ranges, but their T cells are hyporesponsive to ex vivo stimulation. Patients have reduced numbers of class-switched and memory B cells and their naive B cells have reduced capacity to differentiate into long-lived plasma cells (PCs) in vitro (Arjunaraja et al. 2017). Two BENTA patients have developed B cell tumors in adulthood, suggesting that the increased survival and expansion of BENTA B cells could allow for the acquisition of more mutations, and therefore predispose individuals to developing lymphoma (Lu et al. 2018). However, more studies are needed to identify and verify the other cooperating mutations that may be driving B cell lymphoma in BENTA patients.

GOF CARD11 mutations are not restricted to ABC DLBCL and BENTA disease and have also been identified in other DLBCL subtypes as well as in other malignancies including Acute T cell Leukemia/Lymphoma, Sézary syndrome, and Angioimmunoblastic T cell lymphoma (Compagno et al. 2009; da Silva Almeida et al. 2015; Kataoka et al. 2015; Wang et al. 2015; Vallois et al. 2016; Bedsaul et al. 2018). For example, the CARD11 variants F176C and F902C found in Angioimmunoblastic T cell lymphoma, and S615F and E262K found in Sézary syndrome, were confirmed to exhibit hyperactive CARD11 signaling to NF-κB (da Silva Almeida et al. 2015; Vallois et al. 2016). Determining the role of various CARD11 mutations in promoting these distinct lymphoid malignancies is an area of active investigation that may provide new insights to improve diagnostics and treatment options for CARD11-mediated diseases.

mTOR and Akt-FOXO1 signaling

In addition to signaling to NF-κB, CARD11 also engages the mTOR pathway, which is known to regulate genes involved in cell growth, survival, and proliferation

(Figure 1). CARD11 is thought to activate mTOR complex 1 (mTORC1) through direct interaction with ASCT2, by inducing ASCT2 gene expression, or both. ASCT2 then promotes glutamine uptake into the cell. Both CARD11 and MALT1 are required for optimal mTORC1 activation by antigen receptor signaling (Hamilton et al. 2014; Nakaya et al. 2014). Recently, it has been suggested that increased CARD11 signaling to mTORC1 could promote ABC DLBCL (Wray-Dutra et al. 2018). To elucidate the role of aberrant CARD11-mediated mTORC1 activation in ABC DLBCL, mice were engineered to express the L251P GOF CARD11 mutation from the *Rosa26* locus downstream of a lox-STOP-lox cassette and were then mated to Mb1-Cre mice to restrict Cre recombinase activity to B cells beginning at the pro-B cell stage (Mb1-aCard11). Germinal center (GC) B cells from the Mb1-a Card11 transgenic mice exhibited increased phosphorylation of S6 and 4E-BP1 as measured by flow cytometry, indicating increased mTOR signaling. Following sheep red blood cell (SRBC) immunization, GC B cells expressing CARD11 L251P also exhibited increased cell cycling and class switching, indicating enhanced terminal differentiation into short-lived PCs (Arjunaraja et al. 2017). Restricting aCARD11 expression to GC B cells using Cγ1-Cre also resulted in a smaller GC compartment and earlier PC production. Furthermore, the GC B cells also exhibited elevated levels of activation-induced-deaminase (AID) and decreased expression of the transcription factor FOXO1, which may help explain the aberrant phenotypes associated with GOF CARD11 in B cells (Wray-Dutra et al. 2018).

AID is the enzyme responsible for isotype switching and producing the diversity observed in the B cell receptor (BCR) repertoire through the process of somatic hypermutation. Overactive AID is associated with malignancies that have been attributed to hypermutations occurring in oncogenes as well as unintended genomic translocations (Maul and Gearhart 2010). FOXO1 is known to regulate levels of AID and the loss of FOXO1 has been shown to skew GC B cells toward the dark-zone phenotype (Dominguez-Sola et al. 2015; Sander et al. 2015). The dark zone is the compartment of the GC that predominately contains B cells undergoing proliferation and somatic hypermutation, while in the light zone B cells encounter and respond to selection signals (Victora et al. 2012). The results of Wray-Dutra et al. (2018) raised the possibility that some GOF CARD11 mutants promote ABC DLBCL lymphomagenesis through the enhanced differentiation of GC-derived B cells into PCs in concert with the accumulation of AID-mediated genetic aberrations that arise during the GC response. However, it appears that not all GOF CARD11 mutants regulate FOXO1 and B cell differentiation in the same way (Wei et al. 2019).

In B cells, phosphatidylinositol 3-kinase (PI3K) and mTORC2 can phosphorylate and activate the serine/threonine kinase Akt, which then phosphorylates FOXO1, suppressing its transcriptional activity and causing it to be excluded from the nucleus and degraded (Yusuf et al. 2004; Burgering 2008). FOXO1 is known to regulate cell cycle arrest through enhancing expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$, down-regulating the D-type cyclins D1 and D2, and inactivating the S-phase repressor pRb (Medema et al. 2000; Schmidt et al. 2002). The suppression and degradation of FOXO1 allows for cell cycle progression, thereby increasing cell proliferation. It has been suggested that CARD11 signaling negatively regulates Akt activation through mechanisms that may involve Akt interaction with CARD11 via the Coiled-coil domain and K63-linked polyubiquitinylation of Akt, which affects Akt membrane recruitment and subsequent activation (Yang et al. 2009). Interestingly, Wei et al. (2019) determined that GOF CARD11 variants bind Akt with different affinities, leading to distinct outcomes of Akt activation and B cell differentiation.

To study how GOF CARD11 mutants differentially regulate the Akt/FOXO1 signaling pathway, Wei et al. (2019) used CRISPR/Cas9 genomic editing to generate knockin mice expressing mutations associated with BENTA disease, ABC DLBCL, and GCB DLBCL. Compared to wild-type CARD11, the GCB DLBCL variants K215M and L232LI were shown to enhance Akt interaction with CARD11, which suppressed Akt activation and enhanced FOXO1 protein and target gene expression. In contrast, the G123S variant found in both ABC DLBCL and BENTA patients exhibited reduced interaction with Akt, which led to enhanced Akt activation and reduced FOXO1 protein and target gene expression. These differences in Akt association and FOXO1-dependent gene expression correlated with changes in B cell differentiation. For example, the CARD11 K215M mutation further promoted GC B cell development in mixed bone marrow chimera experiments and K215M-expressing GC B cells displayed

increased levels of FOXO1 protein. In contrast, the CARD11 E134G mutation found in BENTA patients led to a markedly reduced GC compartment and GC B cells expressing this mutation exhibited lower levels of FOXO1 protein (Wei et al. 2019). The differential effects of GOF CARD11 mutations on Akt signaling to FOXO1, and the downstream impact on B cell differentiation, suggest that this property could influence the tendency of a particular CARD11 variant to promote one subtype of DLBCL or another in the context of lymphomagenesis. This notion warrants further investigation.

JNK signaling

CARD11 is also required for antigen receptor signaling to JNK, a kinase that controls cellular responses to stress stimuli and regulates apoptosis (Figure 1). CARD11 signaling specifically engages JNK2 in lymphocytes through a process that involves the CARD11 cofactors Bcl10, MALT1, and TAK1. Upon signal-induced Bcl10 oligomerization, the K63-linked polyubiquitinylation of MALT1, and the subsequent recruitment of TAB2 to ubiquitinylated MALT1, the kinases TAK1 and MKK7 phosphorylate and thereby activate JNK2 (Blonska et al. 2007; Oeckinghaus et al. 2007; Chen 2012) (Figure 1).

Knies et al. (2015) demonstrated the importance of JNK signaling in ABC DLBCL. The researchers found that the human ABC DLBCL cell lines HBL-1, OCI-Ly3, and OCI-Ly10, which exhibit constitutive JNK activation, were sensitive to SP600125, an ATP-competitive inhibitor of JNK2. Strikingly, they also found that 55% of human ABC DLBCL biopsies stained positive for phosphorylated JNK2, while all tested GCB DLBCL biopsies stained negative (Knies et al. 2015). Knies et al. (2015) also examined the role of JNK signaling in a mouse model in which the human DLBCL-derived mutation CARD11 L225LI was expressed from the *Rosa26* locus in a CD19-Cre-dependent manner. CARD11 L225LI-expressing B cells displayed high levels of c-Jun, constitutive JNK phosphorylation and activation, and increased concentrations of the phosphorylated forms of the AP-1 transcription factor subunits c-Jun and activating transcription factor 2 (ATF2). Autonomously proliferating CARD11 L225LI-expressing B cells isolated from these mice were susceptible to killing by SP600125 treatment in culture, similar to their susceptibility to the NF-κB pathway inhibitor Bay11-7082, indicating both pathways are important in sustaining proliferation by GOF CARD11.

AP-1 transcription factors are composed of heterodimeric complexes of Jun and ATF subfamilies, and it is not fully understood how the individual AP-1 family members regulate oncogenic B cell survival (Eferl and Wagner 2003). Juilland et al. (2016) showed that high expression of c-Jun, JunB, and ATF3 in ABC DLBCL cells is dependent on CARD11, MALT1, and the Toll-like receptor (TLR) signaling adaptor MyD88. Furthermore, blocking AP-1 function with a dominant-negative A-Fos construct or by silencing of ATF2 or ATF3 led to decreased viability in the HBL-1 ABC DLBCL cell line. Juilland et al. (2016) also observed that high nuclear protein expression of ATF3 appears to be a specific feature of non-GC/ABC DLBCL patient biopsies, further highlighting the importance of ATF3-containing AP-1 complexes downstream of chronic CARD11 signaling in ABC DLBCL. Taken together, these results indicate that the JNK signaling pathway is a potential therapeutic target that could be especially effective in DLBCL cases found to contain hyperactive CARD11 signaling.

Key role of E3 ligases and deubiquitinases

An important mechanism to attenuate CARD11 signaling is the removal of key polyubiquitinylated signaling intermediates. A20 is a ubiquitin editing enzyme that negatively regulates NF-κB signaling through a variety of mechanisms. For example, A20 can disrupt and cause steric hindrance of ubiquitin-mediated protein interactions and act as a deubiquitinase that cleaves K63-linked polyubiquitin chains on MALT1, thereby repressing NF-κB activation (Figure 1) (Düwel et al. 2009; Shembade et al. 2010; Srinivasula and Ashwell 2011). A20 is frequently mutated in DLBCL, with about 33% of ABC DLBCL patients having biallelic inactivation of A20 as a result of point mutations or epigenetic silencing (Compagno et al. 2009; Kato et al. 2009). Two different studies have shown that when A20 is introduced into the lymphoma cell lines SUDHL2, RC-K8, and KM-H2, which have biallelic *TNFAIP3* (A20) mutations causing inactivation and constitutive NF-κB signaling, these cell lines exhibit decreased NF-κB signaling, arrested cell growth, and eventually undergo apoptosis (Compagno et al. 2009; Kato et al. 2009).

Another negative regulator of NF-κB signaling is the cylindromatosis tumor suppressor protein CYLD, which suppresses IKK complex activation (Figure 1). CYLD cleaves K63-linked polyubiquitin from positive regulators of NF-κB signaling, such as IKKγ/NEMO, TRAF2, TRAF6, TRAF7, RIP1, and TAK1 (Brummelkamp et al. 2003; Kovalenko et al. 2003; Trompouki et al. 2003; Yoshida et al. 2005). CYLD also functions as a negative regulator of JNK and AP-1 by deubiquitinylating TAK1 (Reiley et al. 2007). Consistent with the notion that CYLD has the potential to function as a tumor suppressor in lymphomas that have constitutive CARD11 signaling, Xu (2019) found that ABC DLBCL cell lines HBL-1 and OCI-Ly10 and patients with non-GCB DLBCL exhibit constitutive phosphorylation of CYLD, a modification that represses its function. Both CYLD and A20 are cleaved by MALT1 to maximize JNK and NF-κB activation, respectively (Coornaert et al. 2008; Staal et al. 2011). MALT1 protease inhibitors may therefore be particularly effective at reducing constitutive CARD11 signaling by preventing MALT1 from silencing negative feedback mechanisms.

The E3 ubiquitin ligase RNF181 is another negative regulator of normal and oncogenic CARD11 signaling. Pedersen et al. (2016) showed that RNF181 limits the steady-state level of Bcl10 through K48-linked ubiquitinylation and degradation and in doing so, limits the proliferation of DLBCL cells that are dependent on active CARD11 signaling for growth and survival. Increasing the function or expression levels of the proteins that mediate K48-linked Bcl10 ubiquitinylation could provide a novel method to inhibit GOF CARD11 signaling.

Because the CARD11 signaling pathway depends on ubiquitinylation for downstream signaling, mutations in key E3 ubiquitin ligases that regulate CARD11 signaling could further increase NF-κB activation. The recruitment of Bcl10 and HOIP, the catalytic subunit of the Linear Ubiquitin Chain Assembly Complex (LUBAC), to CARD11 allows HOIP to conjugate linear ubiquitin chains to Bcl10 (Yang et al. 2016) (Figure 1). This $LinUb_n$-Bcl10 then binds to the IKK complex, promoting its activation. Mutations in the *RNF31* gene, which encodes HOIP, were found in 8% of ABC DLBCL samples (Y. Yang et al. 2014). CARD11 variants found in ABC DLBCL samples increased the association of CARD11 with both HOIP and Bcl10 in the absence of antigen receptor signaling (Chan et al. 2013; Yang et al. 2016). Because the level of NF-κB activation of each oncogenic CARD11 variant correlated with increased Bcl10 association and steady-state levels of $Lin(Ub)_n$-Bcl10, the ability of a CARD11 mutant to generate $Lin(Ub)_n$-Bcl10 likely determines its potential to promote lymphomagenesis. Therefore, $Lin(Ub)_n$-Bcl10 generation by CARD11 variants may serve as an effective tool for screening the signaling potency of future CARD11 mutations that are associated with disease.

Bridging BCR and TLR signaling

In addition to mutant CARD11 and the BCR signaling pathway, Toll-like receptors (TLRs) can also activate NF-κB in ABC DLBCL. MyD88, an adaptor protein for TLR signaling, is critical for NF-κB activation in response to pro-inflammatory stimuli such as IL-1, IL-18, and LPS (Adachi et al. 1998; Kawai et al. 1999). ABC DLBCL tumors have been shown to have co-occurring mutations in CD79B/A, A20, CARD11, and MYD88 (Ngo et al. 2011). Phelan et al. (2018) studied how hyperactive MyD88 can specifically interact with the CARD11 signaling pathway by performing BioID experiments using the L265P GOF MyD88 mutant. CARD11 and MALT1 were both biotinylated in ABC DLBCL cells expressing a L265P MyD88-BioID construct, providing evidence for close proximity of MyD88 to the CBM complex. Furthermore, CARD11/Bcl10 cytoplasmic puncta visualized by fluorescent proximity ligation assays were reduced by knockdown of TLR9 or MyD88 in double mutant cell lines. MALT1/MyD88 and Bcl10/MyD88 cytoplasmic puncta were reduced by knocking down CD79A, TLR9, and CARD11, which suggests the BCR and TLR9 may cooperate to control the assembly of a MyD88 and CBM supercomplex in ABC DLBCL cells. Additionally, knockdown of both MyD88 and CARD11 in HBL-1 cells via shRNA infection led to greater cell death compared to knockdown of either gene alone, suggesting that MyD88 and CARD11 signaling pathways cooperate to promote cell survival (Ngo et al. 2011). Silencing of MyD88 by shRNA in ABC DLBCL cell lines has also been shown to reduce expression of c-Jun, JunB, and ATF3, suggesting that hyperactive MyD88 has a role in mediating JNK signaling in ABC DLBCL (Juilland et al. 2016). If the crosstalk between MyD88 and CARD11 signaling pathways observed in ABC DLBCL is dependent on the co-occurrence of specific mutations in pathway components,

the targeting of this crosstalk may be a fruitful approach for precision treatment of lymphoma.

Therapies targeting CARD11 signaling

The prevalence of CARD11 mutations in lymphoma cells acquiring secondary resistance underscores the need to develop new therapeutic strategies that account for the specific mutations in each patient's lymphoid cancer. Some therapies that have shown promise in treating ABC DLBCL are specifically ineffective for patients harboring CARD11 mutations. A common target for treating B cell malignances is the non-receptor tyrosine kinase BTK, which acts downstream of the BCR to activate PKCβ (Figure 1). The BTK inhibitor ibrutinib has shown promise as a new therapy for treating ABC DLBCL, with Phase II trials showing a 40% objective response rate in patients (Aalipour and Advani 2014). However, ibrutinib was ineffective when tumors expressed *TNFAIP3* or GOF CARD11 mutations, as these mutations activate the pathway downstream of BTK (Aalipour and Advani 2014; Wilson et al. 2015).

A new strategy to treat DLBCL is to target the signaling cofactors of CARD11. As a central component of the CBM complex, MALT1 is an attractive target and inhibition of MALT1 shows promise to attenuate constitutive CARD11 signaling. The MALT1 inhibitor zVRPR-fmk was shown to inhibit the growth of ABC DLBCL cell lines in which CARD11 mutations drive constitutive MALT1 activation, but not to inhibit growth of GCB DLBCL cell lines in vitro (Ferch et al. 2009; Hailfinger et al. 2009). Other small molecule inhibitors of MALT1 have also shown good potential. Phenothiazines have elicited selective toxic effects for MALT1-dependent ABC DLBCL cells in vitro and in vivo (Nagel et al. 2012), while the MALT1 inhibitor MI-2 inhibited growth of ABC DLBCL cell lines in vitro and xenotransplanted ABC DLBCL tumors (Fontan et al. 2012). In addition, ABC DLBCL cells that harbor mutations in CD79 or CARD11 were treated with a combination of the BTK inhibitor ibrutinib and the MALT1 inhibitor S-mepazine and were found to exhibit markedly reduced MALT1 protease activity and NF-κB pro-survival factors that ultimately resulted in cell death (Nagel et al. 2015).

Another therapeutic strategy is to target signaling events downstream of the CBM complex such as the phosphorylation, ubiquitinylation, or degradation of IκBα, which sequesters NF-κB in the cytoplasm in the absence of signaling (Beg et al. 1992). For example, bortezomib blocks the proteolytic activity of the proteasome, thereby preventing signal-induced IκBα degradation (McConkey and Zhu 2008). When DLBCL patients received bortezomib in combination with chemotherapy, significantly higher overall survival was observed in patients with ABC DLBCL compared to patients with GCB DLBCL (Dunleavy et al. 2009). Furthermore, the second-generation proteasome inhibitor carfilzomib is an irreversible inhibitor, in contrast to the reversible binding of bortezomib, and is more selective for chymotryptic activity of the 20S proteasome than bortezomib (Demo et al. 2007). Carfilzomib has been shown to induce cell death in DLBCL lymphoma cell lines, including in rituximab-resistant lymphoma cell lines, and in primary B cell lymphoma patient samples (Gu et al. 2013). Since lymphoma cells with GOF CARD11 mutations can acquire resistance to common treatment options, inhibitors directly targeting CARD11 and signaling intermediates to NF-κB have the potential to be particularly effective at treating cancers with these specific CARD11 mutations.

Drugs targeting the other signaling pathways regulated by CARD11 are also currently being investigated. Targeting mTOR signaling proteins such as mTORC1 will likely improve outcomes for some patients, as it has been shown that DLBCL patients with an active mTORC1 signature have unfavorable responses to R-CHOP treatment compared to DLBCL patients who exhibit an inactive mTORC1 signature (Sebestyén et al. 2012). Inhibitors of mTOR signaling are under investigation for their effectiveness in treating DLBCL. The first generation mTOR inhibitors are called rapalogs because they are chemically derived from rapamycin. Rapalogs are allosteric inhibitors that block the interaction between mTOR and its intracellular receptor, FBPK12. Providing rapalogs as a monotherapy for cancer has had limited success, with modest response rates in major solid tumors (Don and Zheng 2010) that could be due to incomplete mTOR inhibition or their inability to prevent a negative feedback loop that activates Akt. Rapalogs have had success when combined with drugs that target other known oncogenic drivers. For example, the mTORC1 inhibitor everolimus and the anti-CD20 antibody rituximab were combined in a large Phase II study in relapsed/refractory DLBCL and showed an objective

response rate of 38% (Merli et al. 2015). Second generation mTOR inhibitors have been shown to overcome pro-survival feedback loops by inhibiting both mTORC1 and mTORC2 through targeting the ATP binding site in the mTOR kinase domain. The second generation mTOR inhibitor NVP-BEZ235 in combination with the immunomodulatory agent lenalidomide has been shown to effectively reduce cell proliferation in the non-GCB DLBCL derived cell lines OCI-Ly10, OCI-Ly3, and SUDHL2 and suppress tumor growth in an OCI-Ly10 xenograft mouse model (Jin et al. 2016). In addition, the combination of the BTK inhibitor ibrutinib and the ATP-competitive mTOR inhibitor AZD2014 was shown to strongly induce apoptosis in ABC-type DLBCL cell lines and suppress tumor growth in an OCI-Ly10 xenograft mouse model (Ezell et al. 2014). Clinical trials are still underway for a variety of cancer types to determine the efficacy and tolerability of second generation mTOR inhibitors, including NVP-BEZ235, PF-04691502, and GDC-0980 (Yang et al. 2019).

As alluded to previously, JNK is another therapeutic target for the treatment of lymphomas that express GOF CARD11 mutations. Two ATP-competitive JNK inhibitors, SP600125 and AS601245, have shown promise in preclinical studies to treat malignancies, such as T cell acute lymphoblastic leukemia (Cui et al. 2009). Both inhibitors are widely used for in vitro studies and results suggest that they could be used to treat a variety of other cancers, including colon and stomach cancers (Wu et al. 2020). However, there is a need to develop new JNK inhibitors that selectively inhibit specific isoforms of JNK to improve their efficacy on the distinct cancer types.

Conclusion

GOF CARD11 mutations are associated with an increasing number of lymphoid malignancies and B cell proliferative diseases, such as BENTA. DLBCL tumors can utilize chronically active forms of CARD11 or disrupt the negative regulation of CARD11 signaling to convert the transient and self-limiting antigen-dependent response that normally restricts lymphocyte growth into a sustained proliferative signal. Because CARD11 acts as a signaling hub for a variety of pathways, including NF-κB, mTOR, and JNK, there are ongoing efforts to elucidate all the branching effects of CARD11 signaling to provide insight into the nature of lymphoma development. CARD11 signal transduction becomes amplified when mutations occur that dysregulate the polyubiquitinylation of downstream signaling intermediates or when hyperactive TLR signaling cooperates with GOF CARD11 to drive oncogenesis. As discussed previously, certain drugs targeting the cofactors of CARD11 signaling are already showing promise as therapeutic strategies. The potential for pharmacological interference of CARD11 signaling remains to be fully explored, including the development of inhibitors that prevent the formation of the CBM complex by blocking the interactions between Bcl10 and CARD11 or MALT1 (C. Yang et al. 2014). New strategies to limit aberrant CARD11 activation without crippling the immune homeostasis role of the CBM complex will be needed to mitigate various lymphomas and lymphoproliferative diseases involving dysregulated CARD11. Examination of these future therapies in different lymphoma subsets and as part of novel combination therapies could reveal promising options for precision medicine in lymphoma treatment.

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