Review Article

The biology of the tumor microenvironment in DLBCL: Targeting the "don't eat me" signal

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Diffuse large B-cell lymphoma (DLBCL) is the most common type of malignant lymphoma with biologically and clinically heterogeneous features. Recently, the tumor microenvironment of this disease has been recognized as an important biological aspect of tumor development and therapeutic targets. Recurrent genetic alterations play significant roles in immune recognition of lymphoma cells. In particular, novel genetic alterations promoting phagocytosis were identified, suggesting a potential therapeutic strategy targeting the "don't eat me" signal.

Keywords: microenvironment, phagocytosis, TMEM30A, Diffuse large B-cell lymphoma

INTRODUCTION

The molecular genetic characteristics of mature B-cell lymphoma were recently elucidated, leading the way to personalized medicine based on the identified gene mutations and molecular genetic classification.[1-3] Diffuse large B-cell lymphoma (DLBCL) is the most common type of malignant lymphoma, accounting for approximately 40% of all malignant lymphomas with biologically and clinically heterogeneous features.[4] Biologically, DLBCL is subdivided into an activated B-cell-like type (ABC-DLBCL) and a germinal center B-cell-like type (GCB-DLBCL) depending on the cell of origin (COO) of the tumor.[5-9] The COO is classified by its gene expression profile, and a recent large-scale gene mutation analysis proposed new genetic classifications and identified drug target genes.[10,11] In addition, the importance of the tumor microenvironment (TME) of DLBCL has increased with the development and employment of novel immunotherapeutic strategies, such as CAR-T therapy, in the current standard of care for this disease.[12] A recent comprehensive study identified several driver genetic alterations impacting the checkpoint of immune recognition.[13,14] In particular, the discovery of novel genetic alterations affecting the macrophage engulfment ("eat me" and "don't eat me" signal) suggests a potential therapeutic target focusing on the cross-talk between macrophages and lymphoma cells.[15]

In this review, we describe the recent advances in the understanding of the biology of TME in aggressive B-cell lymphomas. We highlight the clinical and biological significance of *TMEM30A* genetic alterations, and discuss the potential development of predictive and prognostic biomarkers for next-generation immune-checkpoint inhibitors targeting phagocytosis.

GENETIC LANDSCAPE OF DLBCL

Recent advances in genetic technologies using next-generation sequencing revealed several recurrent genetic abnormalities in DLBCL.[1-3] Large-scale genetic analyses with clinical information have been reported by two independent groups. These studies established new molecular classifications based on multiple genetic abnormalities correlated with the prognosis of DLBCL.[10,11] Chapuy *et al.* performed a multi-omics analysis of more than 300 DLBCL cases that demonstrated that DLBCL can be divided into five groups (C1, C2, C3, C4, C5) based on the combination of recurrent genetic abnormalities. This enabled the risk stratification of DLBCL patients and promoted targeted therapy. For example, cases classified as C5 frequently have *CD79B* and *MYD88*-L265P mutations, poor outcomes are observed in cases of ABC-DLBCL, and sensitivity to BTK inhibitors or lenalidomide is noted. Furthermore, C1 and C5 were significantly enriched in ABC-DLBCL, and C3 and C4 were enriched in GCB-DLBCL, further dividing COO into two groups with different prognoses (e.g., C5 and C3 as the unfavorable group, and C1 and C4 as the favorable group).[10] Schmitz *et al.* also reported that approximately half of DLBCL cases can be divided into four classifications (MCD,

BN2, N1, and EZB) based on the pattern of genetic abnormality.[16] Importantly, these two groups can be consistently genetically classified by clinical outcomes (e.g., C5 and MCD are characterized by CD79B and MYD88 mutations, ABC-DLBCL enrichment, and poor outcome), suggesting highly reproducible genetic subtypes that can serve as a foothold for personalized medicine for DLBCL. However, the correlation between the genetic subtypes and TME composition remains unclear, which may limit the clinical implications of subtyping for future immune-therapeutic strategies.

BIOLOGY OF TME OF DLBCL

DLBCL is less dependent on its microenvironment, consistent with the complete disorganization of normal lymphoid structure. However, increasing evidence suggests that the immune system is essential for disease development and outcome of DLBCL, similar to other B-cell lymphomas and solid cancers. The features of the TME differ between different lymphoma types.[13,17] In DLBCL, disrupted cross-talk between lymphoma cells and the microenvironment plays a role in the ability of lymphoma cells to escape immune surveillance of the host. The mechanisms of immune escape include i) hiding from the immune system by losing or reducing recognition molecules (altering immune recognition), ii) suppressing antitumor immune function, and iii) creating a lymphoma-supportive microenvironment.[18] Altering immune recognition, in particular, is deeply involved in tumor development and progression in DLBCL, and its molecular basis has been actively investigated.

Attenuated expression of MHC systems plays a key role in the immune escape of DLBCL.[19,20] MHC class I (MHC-I) proteins, which are present on most nucleated cells, mediate the presentation of self, non-self, and neo-peptides to cytotoxic CD8+T cells.[21,22] Frequent deficiency of MHC-I expression on the surface of DLBCL cells was observed in

DLBCL based on genetic mechanisms such as inactivation of beta-2-microglobulin (B2M).[23] In contrast, the COO of B-cell lymphomas is a professional antigen-presenting cell. Thus, MHC class II (MHC-II) is normally expressed, and selection in the light zone (LZ) of the germinal center (GC) involves antigen presentation via MHC-II to T follicular helper (TFH) cells and follicular dendritic cells (FDCs).[24,25] Thus, antigen presentation must be concealed for these cells to escape death in GC-driven B-cell lymphomas. Accordingly, MHC-II expression is often lost in GC-derived neoplasms. In the COO-specific context, loss of MHC-II expression occurs more often in ABC-DLBCL than in GCB-DLBCL.[26] As GC B-cells transition to plasma cells, the expression of transactivators of MHC-II is silenced, resulting in the subsequent loss of MHC-II expression. Of note, a recent study demonstrated that loss of MHC-II expression also defines tumors originating from the dark zone (DZ) of GC, which is associated with an inferior prognosis and immune "cold" microenvironment.[27] Moreover, the impact of MHC-II deficiency on the immune microenvironment and outcome was much stronger in GCB-DLBCL than in ABC-DLBCL, reflecting a substantial degree of dependence on microenvironmental cells for survival and proliferation signals in the DLBCL subtype.[27] Clinically, loss of MHC class II expression in DLBCL tumor cells correlates with poor patient survival and lower numbers of TILs,[28-30] mainly due to reduced immune reactivity against tumors by immune chemotherapies.

Mutational landscape studies highlighted the recurrent mutations involved in immune recognition in DLBCL (Figure 1). Inactivating mutations and deletions in the B2M gene impair MHC-I assembly and cell surface expression. These events occur in 30% of DLBCL cases. Copy number loss of HLA-I loci at chromosome 6p21 is also a recurrent genetic event associated with reduced MHC-I expression in lymphoma cells.[31] The genetic mechanism of the loss of MHC-II proteins in DLBCL is more complicated. In
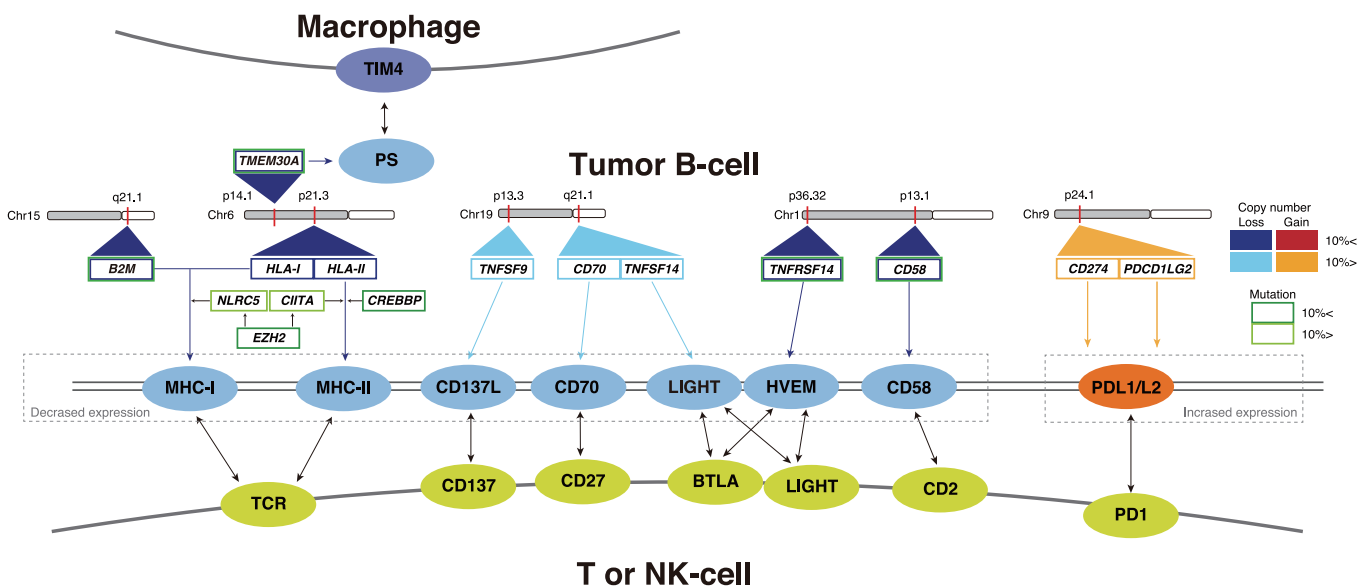
Fig. 1. Recurrent genetic alterations impacting the tumor microenvironment of DLBCL.

addition to deleted HLA-II loci, they inactivate *CIITA*, an essential activator of the MHC class II gene, by inactivating somatic mutations, whereas *CIITA* translocations are recurrent genetic events in cHL and PMBCL.[2,32,33] Of note, recent analyses revealed that MHC-II is downregulated by epigenetic aberrations. For example, *CREBBP* mutations downregulate MHC-II expression, which results in reduced T-cell infiltration in follicular lymphoma (FL) and GCB-DLBCL.[34,35] CREBBP binds and acetylates the regulatory sequences of several genes involved in antigen presentation/processing, including the CIITA transactivator and multiple MHC class II loci, thus functioning in tumor immune escape. Moreover, HDAC3-specific inhibitors can rescue the expression of MHC-II in *CREBBP*-mutant lymphoma cells and restore the ability of TILs to kill DLBCL cells in an MHC class II-dependent manner.[35] *EZH2* Y641 mutation is also associated with marked silencing of both MHC-I and MHC-II genes, and *EZH2*-mutant lymphomas in both mice and humans exhibit reduced expression of these genes, and a reduction in lymphoma-infiltrating CD4+ and CD8+ T cells. EZH2 inhibitors also restored MHC-I and MHC-II expression in *in vitro* models.[27] Collectively, these studies strongly suggest the potential of epigenetic reprogramming to prime the host immune system, providing an attractive rationale for the combination treatment strategy of epigenetic modifiers with immune-checkpoint inhibitors for a subset of DLBCL.

Conversely and consistent with the low expression of programmed death-ligand 1(PD-L1) in DLBCL, gain-of-function genetic changes in the PD1/PD-L1 axis are rare in DLBCL patients (~10%).[1,31,36] In particular, the C1 genetic subtype harbors gains, amplifications, and translocations of the PD-L1/PD-L2 locus associated with increased expression, but its frequency is low (20% of this subtype).[10] This suggests that the PD1/PD-L1 axis does not play a major role in the immune architecture of tumors, which explains the lower activity of immune-checkpoint inhibitors in DLBCL patients than in those with other cancers.[37]

DISCOVERY OF NOVEL GENETIC ALTERATIONS OF *TMEM30A* IN DLBCL

In addition to the aforementioned recurrent genetic alterations that induce immune escape, our previous mutation landscape study uncovered recurrent mutations of *TMEM30A* with a novel biological mechanism impacting the macrophage checkpoint.[14] *TMEM30A* mutations have several biologically and clinically distinct features. First, although the TMEM30A mutation is a gene mutation found in DLBCL with a frequency of approximately 5%–10%, 80% of TMEM30A mutations have loss-of-function mutations (nonsense, frameshift, and splice-site mutations), which is the highest proportion of loss-of-function mutations among the 57 driver genes examined in this study. Furthermore, copy number analysis revealed that *TMEM30A* is located in a gene region (6q14.1) that is prone to copy number attenuation. Indeed, more than 80% of cases with loss-of-function *TMEM30A* mutation have copy number attenuation resulting

in the deficiency of both alleles, reflecting tumor suppressor features based on a double-hit theory. Furthermore, gene expression analysis confirmed that the *TMEM30A* mutation also reduced its own gene expression.

Another major feature of the *TMEM30A* mutation is that it appears specifically in aggressive B-cell lymphoma (BCL). Search of a public database confirmed the presence of the mutation only in aggressive BCL, including DLBCL, primary central nervous system lymphoma, and transformed FL, although almost no mutations were identified in other hematological malignancies or solid cancers. Of note, in six cases with transformed FL, the *TMEM30A* mutation appeared only when DLBCL was diagnosed at the time of recurrence in all cases, whereas biopsy material obtained at FL diagnosis did not have the *TMEM30A* mutation. This supports the specific appearance of the *TMEM30A* mutation in aggressive BCL.

The clinical importance of the *TMEM30A* mutation has also been reported. The prognostic significance of all gene mutations (mutations, copy number abnormalities, and fusion genes) in a comprehensive analysis revealed that the *TMEM30A* mutation is a strong favorable prognostic factor in R-CHOP therapy, especially in cases with bi-allelic alterations of *TMEM30A*. The prognostic effects were independent of the clinical prognostic factors and COO. Importantly, favorable prognostic effects of the *TMEM30A* mutation were confirmed in the reanalysis of another study dataset.[10,11] The *TMEM30A* mutation is a constituent gene mutation of the C1 and BN2 groups, which have a good prognosis in the genetic classification proposed by previous studies, suggesting the reproducibility of the prognostic significance of the *TMEM30A* mutation. TMEM30A loss-of-function drives lymphomagenesis by increasing BCR-dependent signaling. Thus, the *TMEM30A* mutation is a tumor suppressor gene that appears specifically in DLBCL and is a prognostic factor in DLBCL patients who received R-CHOP therapy.

Concerning the biological insight underlying the clinical and genetic significance of the *TMEM30A* mutation, previous studies demonstrated that TMEM30A is one of the main players regulating the "eat me" signal that promotes phagocytosis of macrophages.[38-40] Based on these studies, we examined the relationship between phosphatidylserine (PS) exposure and phagocytosis using primary samples, cell lines, and animal models. The study confirmed that TMEM30A knockout DLBCL promotes PS exposure and phagocytosis. Furthermore, in an experiment using an inhibitor of signal regulatory protein alpha (SIRPα)—another "don't eat me" signal that suppresses phagocytosis—the tumor suppressive effects increased and the survival time was significantly prolonged in knockout mice compared with normal TMEM30A mice. Thus, TMEM30A has the potential to predict the therapeutic response to macrophage checkpoint inhibitors. Moreover, the increased phagocytosis caused by *TMEM30A* mutation may be related to the biological background of the good prognosis of *TMEM30A* mutation-positive DLBCL (Figure 2).
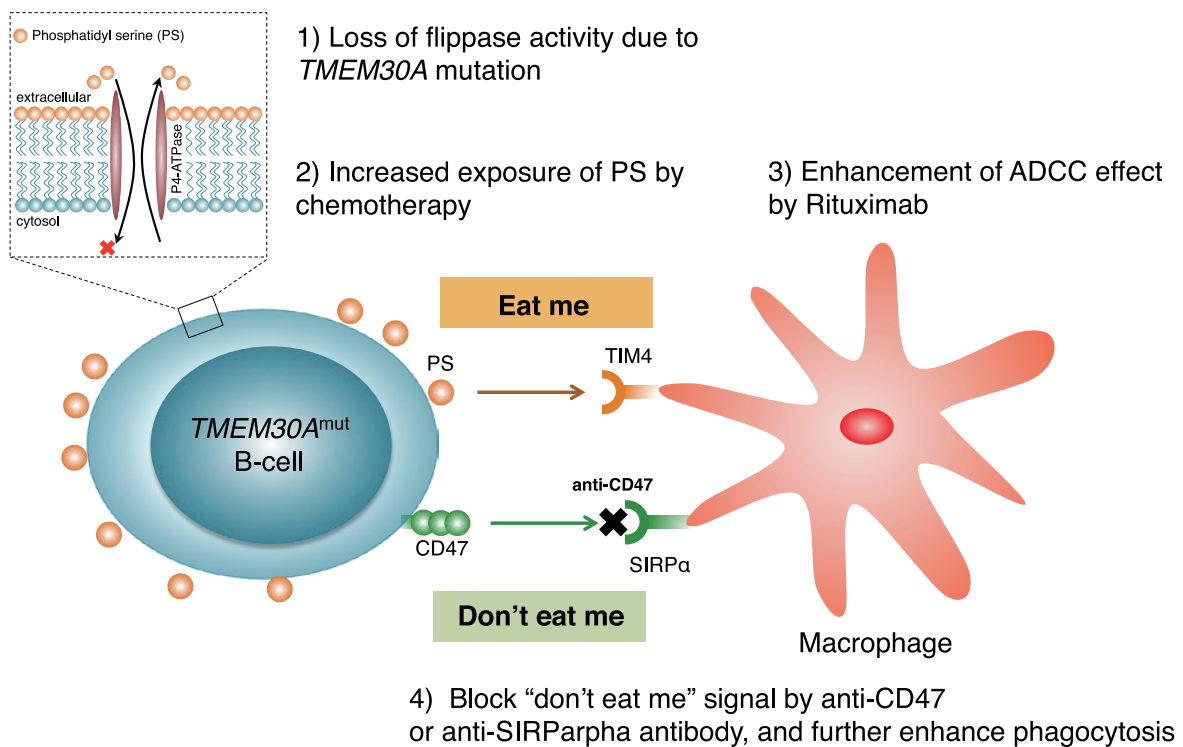
1) Loss of flippase activity due to *TMEM30A* mutation

2) Increased exposure of PS by chemotherapy

3) Enhancement of ADCC effect by Rituximab

4) Block "don't eat me" signal by anti-CD47 or anti-SIRParpha antibody, and further enhance phagocytosis

Fig. 2. Biological significance of TMEM30A in BCL.

CLINICAL IMPACT OF "EAT ME" AND "DON'T EAT ME" SIGNALS IN BCL

Inhibition of macrophage-mediated phagocytosis has emerged as an essential mechanism for tumor immune evasion and is an attractive therapeutic target as a next-generation immune-checkpoint inhibitor. During apoptosis, phagocytosis is mainly induced by exposure to PS on the cell membrane surface.[41-43] PS is predominantly confined to the inner leaflet of the plasma membrane in cells, but it is externalized on the cell surface during apoptosis. This externalized PS is required for the effective phagocytosis of apoptotic cells by macrophages. In 2014, Segawa *et al.* discovered that CDC50A (TMEM30A) plays an essential role in flipping PS inside the plasma membrane to avoid engulfment of living cells. Importantly, they knocked out TMEM30A in a mouse model and demonstrated that the deficiency of TMEM30A promoted PS exposure on the cell membrane surface, resulting in the elimination of cancer cells by macrophage engulfment.[38]

The "don't eat me" signal based on the phagocytosis checkpoint axis, CD47- SIRPα, was identified in the 1990s.[44] CD47 was first identified as a "marker of self" in red blood cells, and was highly upregulated in malignant hematopoietic and non-hematopoietic cells.[45] CD47 inhibits cellular phagocytosis through its interaction with SIRPα expressed on phagocytic cells, which include macrophages and dendritic cells. Due to the inhibitory signal of phagocytosis, CD47 overexpression confers an unfavorable prognostic effect in several cancer types.[46,47] Chao *et al.* reported that CD47 expression correlates with an aggressive phenotype in non-Hodgkin

lymphoma and an overall poor clinical prognosis following immune chemotherapies in DLBCL.[48] The indicated biological and clinical significance of "don't eat me" signal prompted the idea of drug-related inhibition of the CD47 signal to increase macrophage phagocytic activity (Table 1). Indeed, several CD47 inhibitors impaired tumor growth, inhibited metastatic spread, and inhibited tumor regression in a preclinical model.[49,50] Moreover, this activity reportedly increased when combined with cancer targeting antibodies that provide exogenous prophagocytic signals. For example, synergistic suppression of tumor growth was observed when CD47-targeting agents were combined with monoclonal antibodies such as rituximab.[51] These antibodies have active Fc domains that bind to Fc-gamma receptors on macrophages, resulting in the stimulation of phagocytosis. This mechanism provides rationale for exploring combinations of antitumor antibodies with CD47 targeting agents in clinical trials.

Among candidate CD47 blockades, Hu5F9-G4 (5F9) is a humanized IgG4 monoclonal antibody undergoing clinical trials.[50] 5F9 was engineered with a human IgG4 isotype to minimize potential off-target effects on normal tissues. 5F9 binds to CD47 on tumor tissues and its antitumor effects are primarily dependent on the inhibition of CD47 signaling, although for optimal activity, an IgG4 Fc domain is required. Indeed, a recent clinical trial demonstrated marked anti-lymphoma effects with CD47 blockade in relapsed/refractory DLBCL. Advani *et al.* first reported the significant efficacy of 5F9 in combination with rituximab, without severe toxicities, in 15 patients with relapsed and refractory DLBCL, and seven FL patients exhibiting resistance to immune chemotherapies. Overall, 11 patients responded to the agent,

Table 1. Development of novel agents targeting "don't eat me" signal

Name	Target	Clinical	Disease
Hu5F9-G4	CD47	Phase I/II	lymphomas
TI-061	CD47	Phase I/II	solid cancers
TTI-622	SIRPα	Phase I	lymphomas
TTI-621	SIRPα	Phase I	solid and hematologic malignancies
SRF231	CD47	Phase I	solid and hematologic malignancies
SHR-1603	CD47	Phase I	solid and hematologic malignancies
OSE-172	SIRPα	Phase I	solid cancers
NI-1701	CD47	Phase I	hematologic malignancies
IBI188	CD47	Phase I	solid and hematologic malignancies

including five complete responses of DLBCL. In addition, 91% of patients remained in remission at a median follow-up of 6.2 months in the DLBCL group.[52] An ongoing phase II trial enrolling more patients is seeking to validate these findings.

CONCLUSION

DLBCL is a disease with a complicated pathogenesis that is based on genetic alterations of tumor cells, composition of the TME, and the escape from attack by tumor-associated immune cells. As a mechanism of immune escape, several genetic alterations that affect immune recognition and elimination have been identified. These recent biological insights regarding immune evasion by lymphomas have enabled the development of multiple promising immunotherapeutic strategies. Among them, CD47 blockades are less toxic and induce stable responses in patients with relapsed or refractory DLBCL. TMEM30A is a potentially novel biomarker for next-generation checkpoint inhibitors.

CONFLICT OF INTEREST

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