IMMUNOTHERAPY AND THE REGULATORY IMMUNE SYSTEM IN BLOOD CANCERS: FROM MECHANISMS TO CLINICAL APPLICATIONS

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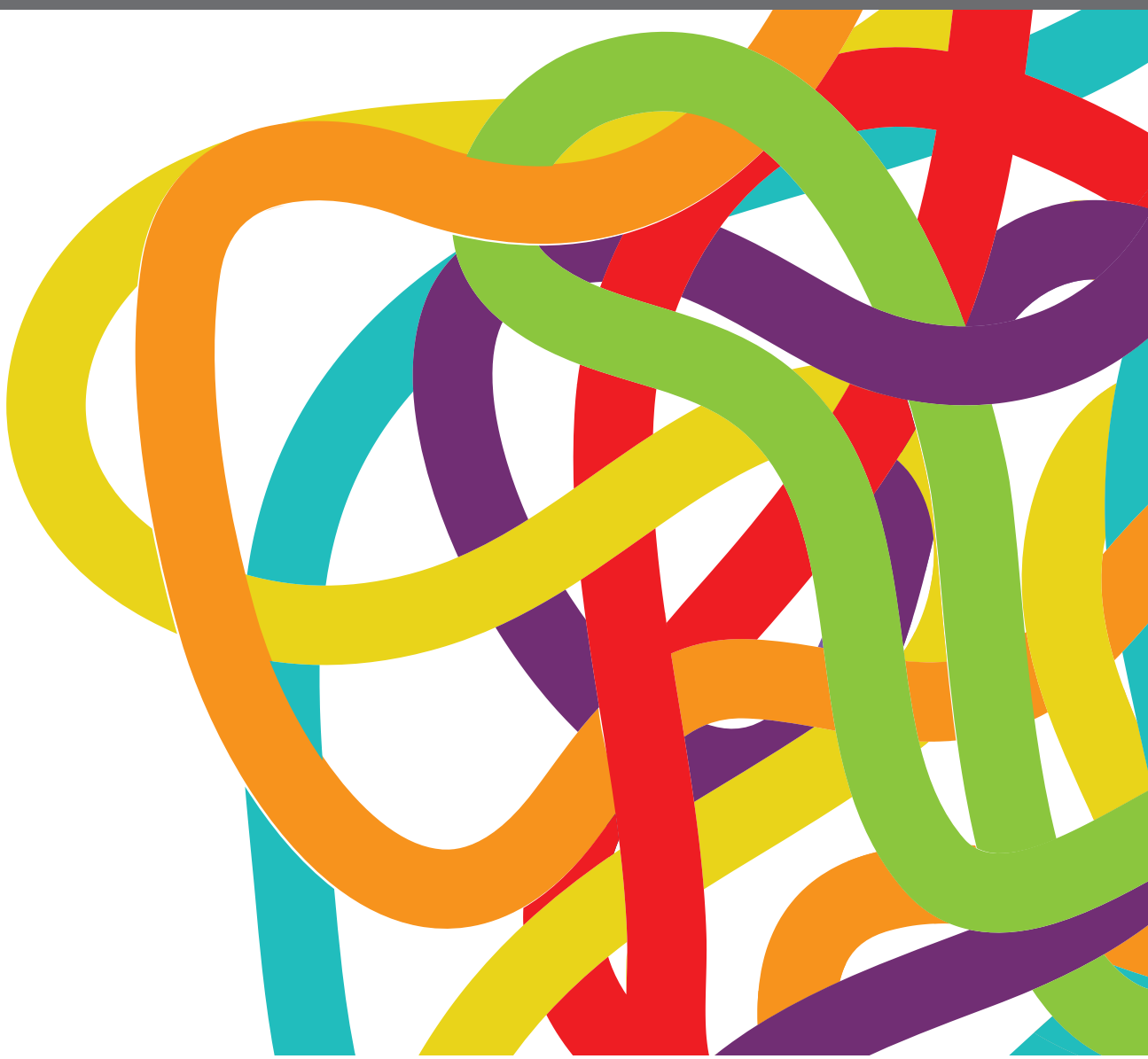

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IMMUNOTHERAPY AND THE REGULATORY IMMUNE SYSTEM IN BLOOD CANCERS: FROM MECHANISMS TO CLINICAL APPLICATIONS

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REVIEW
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Cancer Immunotherapy in Diffuse Large B-Cell Lymphoma

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Remarkable progress has been made in the field of cancer immunotherapy in the past few years. Immunotherapy has become a standard treatment option for patients with various cancers, including melanoma, lymphoma, and carcinomas of the lungs, kidneys, bladder, and head and neck. Promising immunotherapy approaches, such as chimeric antigen receptor (CAR) T cell therapy and therapeutic blockade of immune checkpoints, in particular cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 pathway (PD-1/PD-L1), have boosted the development of new therapeutic regimens for patients with cancer. Immunotherapeutic strategies for diffuse large B-cell lymphoma (DLBCL) include monoclonal anti-CD20 antibody (rituximab), monoclonal anti-PD-1 antibodies (nivolumab and pembrolizumab), monoclonal anti-PD-L1 antibodies (avelumab, durvalumab, and atezolizumab) and chimeric antigen receptor (CAR) T cell therapy. In this review, we outline the latest highlights and progress in using immunotherapy to treat patients with DLBCL, with a focus on the therapeutic blockade of PD-1/PD-L1 and CAR T cell therapy in DLBCL. We also discuss current clinical trials of PD-1/PD-L1 and CAR T cell therapy and review the challenges and opportunities of using immunotherapy for the treatment of DLBCL.

Keywords: DLBCL, NHL, immunotheray, PD-1, PD-L1, CTLA-4, Chimeric antigen receptor (CAR) T cells therapy, immune checkpoint

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma. Approximately 60% of DLBCL patients are cured using standard chemotherapy that includes monoclonal anti-CD20 antibody (rituximab), cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). However, 30–40% of DLBCL patients will develop relapse or have refractory disease that cannot be cured with the standard R-CHOP therapy, indicating the need for more effective therapies for this patient subset. For patients with high-risk DLBCL who often fail R-CHOP therapy, especially patients with high-grade B-cell lymphoma with *MYC* and *BCL2* or *BCL6* translocation, dose-adjusted rituximab, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin (DA.R-EPOCH) regimen is a commonly used high intensity regimen.

The development of rituximab was an early step in the application of immunotherapy for the treatment of lymphoma, as it was the first monoclonal antibody (mAb) approved by the US Food and Drug Administration (FDA) for the treatment of patients with advanced stage or relapsed low-grade non-Hodgkin lymphoma, in 1997 (1). See comment in PubMed Commons below Rituximab is a chimeric (mouse and human) monoclonal antibody directed against the B-cell antigen CD20. Rituximab acts via a number of mechanisms including direct antibody

dependent cellular cytotoxicity, apoptosis induction, and complement mediated cell death (2). Other monoclonal antibodies that target B-cell antigens, such as CD19 and CD22, also have been developed. CD19 is a specific B cell marker widely expressed during all phases of B cell development until terminal differentiation into plasma cells, with a potential efficacy on a large panel of B cell malignancies. Although initial attempts to target CD19 were unsuccessful, accumulated studies demonstrated targeting CD19 has a therapeutic potential for patients with B cell malignancies (3, 4).

More recently, a number of innovative immunotherapy approaches have shown promising results in patients with relapsed or refractory DLBCL, leading to numerous ongoing clinical trials. CTLA-4 is a negative regulator of T-cell activation, which inhibits anti-tumor immune responses. Blockade of CTLA-4 using the monoclonal antibody ipilimumab improves anti-tumor activity. Ipilimumab was the first immune checkpoint inhibitor approved by the US FDA for the treatment of patients with malignant melanoma. However, the role of the CTLA-4 pathway in DLBCL remains to be elucidated. A phase I clinical trial of ipilimumab in 18 patients with relapsed/refractory B-cell NHL included 3 patients with DLBCL (NCT00089076). Two of these patients had clinical responses and 1 achieved a complete response that lasted more than 31 months. In this study, investigators reported that ipilimumab was well tolerated at the doses used, and that ipilimumab has anti-tumor activity resulting in durable responses in a minority of DLBCL patients (5).

Two highly promising strategies designed to harness the immune system to treat patients with DLBCL are therapeutic blockade of the PD-1/PD-L1 pathway and chimeric antigen receptor (CAR) T cell therapy. These approaches are triggering a paradigm shift in cancer immunotherapy.

PD-1/PD-L1 SIGNALING PATHWAY

PD-1/PD-L1 pathway blockade with nivolumab, pembrolizumab, atezolizumab, avelumab, and durvalumab has demonstrated activity in multiple solid tumor malignancies (6–17). Monoclonal anti-PD-1 antibody (nivolumab) was granted designation as a breakthrough therapy for the treatment of patients with relapsed or refractory classical Hodgkin lymphoma on May 17, 2016. The FDA recently granted accelerated approval to another monoclonal anti-PD-1 antibody (pembrolizumab) for the treatment of adult and pediatric patients with refractory primary mediastinal large B-cell lymphoma, or who have relapsed after two or more prior lines of therapy (June 13, 2018). More clinical trials of PD-1 and PD-L1 monoclonal antibodies are currently ongoing (**Figure 1**). Despite the potential activity of PD-1–blocking antibodies in DLBCL, a subset of patients experiences progressive disease after an initial, often short response (18, 19). Additional research is therefore needed to better understand the reasons for host resistance and to prevent immune-related adverse events.

Mechanisms of PD-1/PD-L1 Signal Pathway Blockade

The immune system protects the body against illness and infection by bacteria, viruses, fungi, or parasites. Simultaneously, the immune system has the capacity to recognize tumors, inhibit tumor development, and eliminate malignant cells. Cancer cells, however, can evolve and therefore escape from immune surveillance and attack. The mechanisms of cancer immune escape mainly include: reducing the expression of tumor antigens; increasing co-inhibitor expression (e.g., PD-L1, CTLA-4) (20) (**Figure 2**); secreting suppressive cytokines (e.g., TGF-β and IL-10); and lastly orchestrating an immunosuppressive microenvironment (21, 22).

PD-1 (CD279), a member of the CD28 and CTLA-4 immunoglobulin superfamily, interacts with two B7 family ligands: PD-L1 (CD274 and also known as B7-H1) and PD-L2 (CD273 and also known as B7-DC). PD-1 is expressed on the surface of activated T cells, B cells, natural killer cells, and macrophages as well as by a large proportion of tumor infiltrating lymphocytes (TILs) (15). PD-1 exerts an important immune checkpoint function in the regulation of T-cell mediated immune responses. PD-1 delivers inhibitory signals that regulate T-cell activation, exhaustion, and tolerance through binding to its ligands PD-L1 and PD-L2. PD-L1 and PD-L2 have distinct patterns of expression (23). PD-L1 is expressed primarily by antigen-presenting cells (APC), as well as by a variety of non-hematopoietic cells and tumor cells. PD-L1 expression is induced by pro-inflammatory cytokines, including type I and type II interferons, tumor necrosis factor α (TNF-α) and vascular endothelial growth factor (VEGF) (24, 25). PD-L2 is expressed primarily by dendritic cells and macrophages, and is induced by IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (26).

In addition to PD-1, PD-L1 also interacts with CD80 expressed on T cells and inhibits T cell responses, whereas PD-L2 also binds to a novel partner repulsive guidance molecule b (RGMb), and plays an important role in pulmonary tolerance (27). Further investigation is needed to explore how these novel pathways are involved in anti-tumor immune responses.

Negative regulation of the PD-1 pathway may be accomplished via multiple mechanisms. The engagement of PD-1 with PD-L1/PD-L2 may suppress T cell activation by competing directly with CD28 for CD80/CD86 binding, resulting in impaired T cell activation and decreased IL-2 production (28). PD-1 binding to PD-L1/PD-L2 results in tyrosine phosphorylation of the PD-1 cytoplasmic regions ITIM and ITSM, which bind the phosphatases SHP-1 and SHP-2, leading to decreased T cell activation and cytokine production (29). PD-1 signaling also inhibits CD28-mediated activation of phosphatidylinositol 3-kinase (PI3K), leading to decreased activation of Akt and reduced expression of transcription factors associated with cell effector functions including GATA3, T-bet, and Eomes (30). Signaling through PD-1 decreases tyrosine phosphorylation of the TCR ζ chain and ZAP-70 (31). PD-1 signaling inhibits the expression of transcription factors associated with effector cell functions, including GATA-3, T-bet, and Eomes (32).

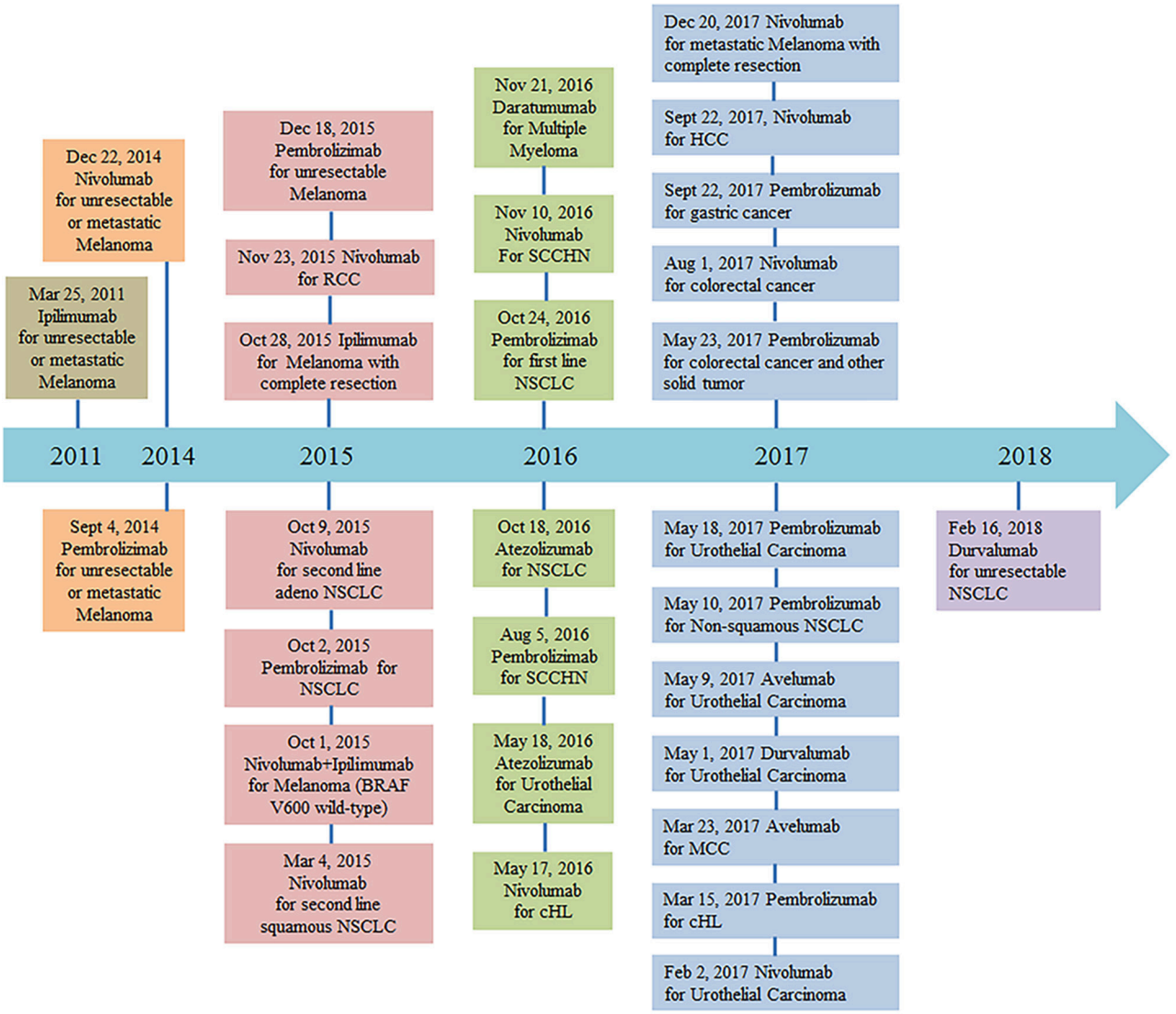

FIGURE 1 | FDA approval timeline of immune checkpoint inhibitors for the treatment of malignancies (https://www.fda.gov/drugs, retrieved Mar 7, 2018). Abbreviations: NSCLC, non–small cell lung cancer; RCC, renal cell carcinoma; cHL, classical Hodgkin Lymphoma; SCCHN, squamous cell carcinoma of the head and neck; MCC, merkel cell carcinoma; HCC, hepatocellular carcinoma.

Clinical Immunotherapy of PD-1/PD-L1 Inhibitors in DLBCL

As already mentioned, 30–40% of DLBCL patients fail standard therapy and have relapsed or refractory disease (33). PD-1 and PD-L1 expression are not usually a striking feature of patients with cancer (34–36), although several studies have reported over-expression of PD-L1 in specific lymphoma subsets (37, 38). Immune blockade of the PD-1/PD-L1 interaction by monoclonal antibodies can restore the antitumor activity of cytotoxic T cells. Immunotherapy using PD-1/PD-L1 inhibitors has become a clinically validated treatment and has produced durable objective responses and improved overall survival (OS) in patients with solid and hematologic neoplasms. Several monoclonal antibodies targeting the PD-1/PD-L1 pathway are currently in early clinical development including two anti-PD-1 antibodies (nivolumab and pembrolizumab) (**Table 1**), and three anti-PD-L1 antibodies (avelumab, durvalumab, and atezolizumab) (**Table 2**).

Nivolumab is a human IgG4 anti-PD-1 monoclonal antibody. Multiple phase I/II studies of nivolumab are evaluating (or planning to evaluate) its efficacy in combination with agents such as ipilimumab (NCT03305445), rituximab and chemotherapy (NCT03259529), varlilumab (anti-CD27) (NCT03038672), the IDO1 inhibitor epacadostat (NCT02327078), and lenalidomide (NCT03015896) in participants with DLBCL.

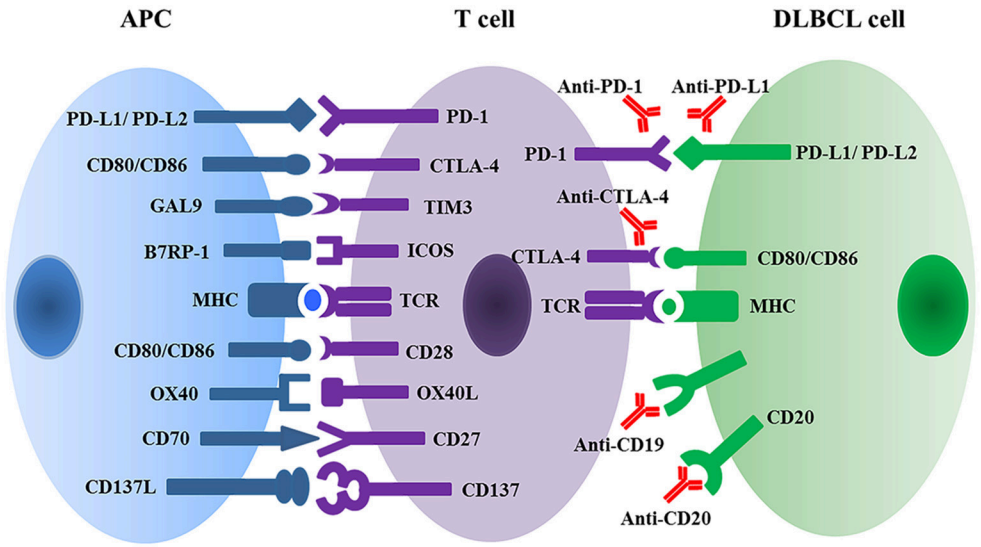

FIGURE 2 | Multiple immune checkpoint and ligand-receptor interactions between T cell and APC or DLBCL malignant cells regulate T cell activation and anti-tumor activity. APC, antigen presenting cell; DLBCL, diffuse large B cell lymphoma; PD1, programmed cell death protein 1; PD-L, programmed cell death ligand; GAL9, galectin 9; TIM3, T cell membrane protein 3; B7RP1, B7-related protein 1; ICOS, inducible T cell co-stimulator; MHC, major histocompatibility complex; TCR, T cell receptor.

Although early results in phase I studies were promising, only one phase II study has been reported for the use of PD-1/PD-L1 inhibitors in DLBCL patients, "A single-arm, open-label, phase 2 study of nivolumab (BMS-936558) in subjects with relapsed or refractory DLBCL after failure of autologous stem cell transplant (ASCT) or after failure of at least two prior multi-agent chemotherapy regimens in subjects who are not candidates for ASCT". In this study, 161 participants were enrolled and 121 participants entered the treatment period. Participants were enrolled, but not treated due to adverse events ($n = 2$), withdrawal of consent ($n = 2$), death ($n = 2$), or they no longer met study criteria ($n = 34$). Finally, 102 participants completed the treatment period. Nivolumab 3 mg/kg administered as an IV infusion on treatment day 1 of each 14 day cycle until disease progression or discontinuation due to toxicity, withdrawal of study consent, or the study ends. Nivolumab therapy resulted in an overall response rate (ORR) of 10.3% in the ASCT-failed group (complete response [CR], 3.4%; partial response [PR], 6.9%) and 2.9% in the ASCT ineligible group (CR, 0%; PR, 2.9%). The median duration of response was 11.4 months in the ASCT-failed group and 8.3 months in the ASCT-ineligible group (NCT02038933).

A phase I trial of nivolumab monotherapy recruited patients with heavily pretreated relapsed or refractory lymphoid malignancies including 11 patients with DLBCL. Four (36%) patients responded (2 CR and 2 PR). The median follow-up duration for patients with DLBCL was 22.7 weeks; 1 of 4 patients with DLBCL has had an ongoing response, and 2 patients continue to be followed (18).

Pembrolizumab is another humanized IgG4 anti-PD-1 monoclonal antibody. Various phase I/II studies of the PD-1 antibody pembrolizumab are still ongoing, either as a single agent or in combination with antibodies, small molecular inhibitors, immunotherapeutic vaccine, dendritic cell therapy, and CAR T cell treatment in participants with DLBCL.

Atezolizumab is a human IgG1 monoclonal antibody that targets PD-L1. Seven phase I/II studies of atezolizumab are ongoing to evaluate its efficacy in combination with other agents such as CAR T cells, antibodies, small molecular inhibitors, and chemotherapy in participants with DLBCL.

Durvalumab is a human IgG1 monoclonal antibody that targets PD-L1. One phase II clinical trial of durvalumab as a single agent is ongoing to assess the progression-free survival (PFS) two years after ASCT in high-risk DLBCL patients. Five phase I/II studies are underway to evaluate the efficacy of durvalumab in combination with antibodies, small molecular inhibitors, and chemotherapy as well as CAR T cell therapy in participants with DLBCL. Another phase II clinical trial of durvalumab in combination with monoclonal antibodies directed against CD20, OX40 and CTLA4 designed to determine the optimal dose of MEDI6469 (anti-OX40) that is safe and tolerable in participants with DLBCL was terminated early at the sponsor's discretion due technical problems (NCT02205333).

Another human IgG1 anti-PD-L1 monoclonal antibody is avelumab. An early phase I study of avelumab as a single agent is ongoing to evaluate the feasibility of adding induction and maintenance avelumab to standard R-CHOP therapy in patients with stage II, III, and IV DLBCL (NCT03244176). An ongoing phase III study is evaluating the efficacy of avelumab in combination with a variety of agents for relapsed or refractory DLBCL patients; these agents include utomilumab (anti-4-1BB/CD137), rituximab, azacitidine,

TABLE 1 | Ongoing PD-1 inhibitors trials in DLBCL.

PD-1 inhibitor	Trial name	Status	Phase	Intervention/treatment	Immunological target
Nivolumab	NCT03305445	Not yet recruiting	I/II	Nivolumab, Ipilimumab	PD-1, CTLA-4
	NCT03259529	Recruiting	I/II	Nivolumab, Rituximab, Bendamustine hydrochloride, Gemcitabine	PD-1, CD20
	NCT02038933	Active, not recruiting	II	Nivolumab	PD-1
	NCT03311958	Not yet recruiting	I	Nivolumab	PD-1
	NCT03038672	Not yet recruiting	II	Nivolumab, Varlilumab	PD-1, CD27
	NCT02327078	Recruiting	I/II	Nivolumab, Epacadostat	PD-1
	NCT03015896	Recruiting	I/II	Nivolumab, Lenalidomide	PD-1
Pembrolizumab	NCT03340766	Not yet recruiting	I	Pembrolizumab, Blinatumomab	PD-1, CD19,CD3
	NCT03349450	Not yet recruiting	II	Pembrolizumab, DPX-Survivac, Cyclophosphamide	PD-1
	NCT02362997	Recruiting	II	Pembrolizumab	PD-1
	NCT03401853	Not yet recruiting	II	Pembrolizumab, Rituximab	PD-1, CD20
	NCT03255018	Recruiting	II	Pembrolizumab	PD-1
	NCT03150329	Recruiting	I	Pembrolizumab, Vorinostat	PD-1
	NCT02541565	Recruiting	I	Pembrolizumab, Rituximab, Cyclophosphamide, Doxorubicin Hydrochloride, Prednisone, Vincristine Sulfate	PD-1, CD20
	NCT02650999	Recruiting	I/II	Pembrolizumab	PD-1
	NCT03287817	Recruiting	I/II	Pembrolizumab, AUTO3	PD-1, CD19/22
	NCT03309878	Not yet recruiting	I/II	Pembrolizumab, Mogamulizumab	PD-1, CCR4
	NCT02178722	Recruiting	I/II	Pembrolizumab, INCB024360	PD-1
	NCT02950220	Recruiting	I	Pembrolizumab, Ibrutinib	PD-1
	NCT01953692	Active, not recruiting	I	Pembrolizumab, Lenalidomide	PD-1
	NCT03035331	Recruiting	I/II	Pembrolizumab, Dendritic Cell Therapy,	PD-1
	NCT02446457	Active, not recruiting	II	Pembrolizumab, Rituximab, Lenalidomide	PD-1, CD20
	NCT02362035	Active, not recruiting	I/II	Pembrolizumab, Acalabrutinib	PD-1

bendamustine, gemcitabine, and oxaliplatin, (NCT02951156). A recent phase I trial is studying the side effects and optimal dosing of avelumab, utomilumab, rituximab, ibrutinib, and combination chemotherapy for treating patients with DLBCL or relapsed/refractory mantle cell lymphoma, but is not yet recruiting (NCT03440567).

Pidilizumab (MDV9300, Medivation, Inc) was originally considered a monoclonal antibody binding to PD-1. This agent yielded encouraging results in phase II clinical trials for DLBCL. However, recent evidence suggests that PD-1 is not the target of pidilizumab. The FDA has lifted its partial clinical hold on the investigational new drug (IND) application for pidilizumab (MDV9300) in hematological malignancies and has confirmed that the phase II clinical trial in patients with relapsed or refractory DLBCL, as well as other studies that cross reference the IND, may now proceed. The partial clinical hold was not related to any safety concerns. The investigator brochure, protocols, and informed consent documents related to the phase II trial have satisfactorily been revised to reflect that the manufacturer's understands that PD-1 is not the target of pidilizumab. No patients had yet been enrolled in the trial which commenced in late 2015. Patients who were receiving pidilizumab through investigator-sponsored trials have continued to receive treatment and the investigators have been informed to update their protocols and informed consent documents to state that pidilizumab is not an anti-PD-1 antibody, but an anti-Delta-like ligand 1 antibody (39, 40).

Immune checkpoint blockade has promising potential in DLBCL therapy. A subgroup of patients with advanced cancers may respond to single-agent immune checkpoint blockade, however, most patients do not respond to monotherapy (41). In order to enhance the antitumor efficacy, a combination of multiple therapeutic approaches is urgently needed. Many clinical trials are ongoing to evaluate the synergistic efficacy of immune checkpoint inhibitors in combination with other

TABLE 2 | Ongoing PD-L1 inhibitors trials in DLBCL.

PD-L1 inhibitor	Trial name	Status	Phase	Intervention/treatment	Immunological target
Atezolizumab	NCT02926833	Recruiting	I/II	Atezolizumab, Axicabtagene Ciloleucel	PD-L1
	NCT03422523	Not yet recruiting	II	Atezolizumab, Rituximab, Gemcitabine, Oxaliplatin	PD-L1, CD20
	NCT02596971	Active, not recruiting	I	Atezolizumab, Obinutuzumab, Rituximab, Bendamustine, Cyclophosphamide, Doxorubicin, Prednisone, Vincristine	PD-L1, CD20
	NCT03321643	Not yet recruiting	I	Atezolizumab, Rituximab, Gemcitabine, Oxaliplatin	PD-L1, CD20
	NCT02729896	Recruiting	I	Atezolizumab, Obinuzumab, Rituximab, PolatuzumabVedotin	PD-L1, CD20, CD79b
	NCT02220842	Recruiting	I	Atezolizumab, Obinutuzumab, Tazemetostat	PD-L1, CD20
	NCT03276468	Not yet recruiting	II	Atezolizumab, Obinutuzumab, Venetoclax	PD-L1, CD20
Durvalumab	NCT02549651	Recruiting	I	Durvalumab, Tremelimumab, AZD9150	PD-L1, CTLA-4
	NCT03212807	Not yet recruiting	II	Durvalumab, Lenalidomide	PD-L1
	NCT03241017	Not yet recruiting	II	Durvalumab	PD-L1
	NCT03003520	Recruiting	II	Durvalumab, Rituximab, Doxorubicin, Vincristine, Cyclophosphamide, Prednisone, Lenalidomide	PD-L1, CD20
	NCT02401048	Active, not recruiting	I/II	Durvalumab Ibrutinib	PD-L1
	NCT02706405	Recruiting	I	Durvalumab Autologous Anti-CD19CAR-4-1BB-CD3zeta-EGFRt-expressing CD4+/CD8+ Central Memory T-lymphocytes JCAR014, Cyclophosphamide, Fludarabine Phosphate	PD-L1
	NCT02205333	Terminated	I/II	Durvalumab, MEDI6469, Rituximab, Tremelimumab	PD-L1, OX40, CD20, CTLA-4
Avelumab	NCT03244176	Recruiting	I	Avelumab	PD-L1
	NCT02951156	Recruiting	III	Avelumab, Utomilumab, Rituximab, Azacitidine, Bendamustine, Gemcitabine, Oxaliplatin,	PD-L1, 4-1BB, CD20
	NCT03440567	Not yet recruiting	I	Avelumab, Utomilumab, Rituximab, Ibrutinib, Carboplatin, Etoposide Phosphate, Ifosfamide	PD-L1, 4-1BB, CD20

agents, which mainly includes co-inhibitory blockade (anti-CTLA-4), co-stimulatory agonists (anti-OX40, anti-4-1BB), rituximab (anti-CD20) and conventional chemotherapy. Both PD-1 and CTLA-4 are expressed on T cells, but they play different regulatory functions via different signaling pathways in suppressing T cell activation and proliferation. The combined therapy of anti-PD-1 and anti-CTLA-4 has demonstrated synergistic efficacy and improve antitumor activities. In contrast, both OX40 and 4-1BB are members of the tumor necrosis factor (TNF) family of co-stimulatory receptors, expressed on the surface of $CD4^+$ and $CD8^+$ T cells. Agonist antibodies anti-OX40 and anti-4-1BB promote T cell activation, growth, and survival and enhance antitumor functions. Conventional chemotherapy in combination with immune checkpoint blockade has shown synergistic efficacy by releasing multiple tumor neoantigens or modifying the tumor microenvironment (**Tables 1**, **2**).

Challenges and Opportunities for Blocking the PD-1/PD-L1 Pathway

Targeting the PD-1/PD-L1 pathway in patients with DLBCL is a promising treatment strategy. However, there are adverse events associated with PD-1/PD-L1 inhibitors that reflect the actions of the PD-1 pathway in the regulation of immune responses. PD-1 pathway blockade can cause immune-related adverse events that may affect almost all tissues. Toxicities related to immune checkpoint inhibitors typically include dermatologic manifestations, diarrhea, colitis, hepatotoxicity, endocrinopathies, and pneumonitis (42–44). Based on the experience of immune-checkpoint inhibitors in patients with solid tumors, the occurrence of grade 3–4 immune-related adverse events is approximately 20% with ipilimumab, compared with 5–10% with nivolumab or pembrolizumab (45). Generally, PD-1 pathway blockade is associated with fewer and less severe toxicities compared with CTLA-4 blockade. Toxicities can be managed with immune-modulating agents including corticosteroids and infliximab. Early studies suggest that combination therapy with CTLA-4 and PD-1 inhibitors may increase efficacy, but at the cost of increased toxicity (46). However, the combination of anti-CTLA-4 and anti-PD-1 antibodies demonstrated a similar safety and efficacy profile compared to a previous report for anti-PD-1 monotherapy in Hodgkin lymphoma, non-Hodgkin lymphoma (NHL), and multiple myeloma (19).

In patients with NHL, severe immune-related adverse events have been rare to date. A phase I trial of ipilimumab in patients with relapsed/refractory B-cell lymphoma is designed to evaluate safety, immunologic activity, and potential clinical efficacy. Diarrhea has been reported frequently among patients receiving ipilimumab, in 56%, with 28% of these patients developing grade 3–4 adverse events (5). Among patients with relapsed NHL receiving nivolumab within a phase Ib trial, 4% developed grade 3–5 pneumonitis (18). Another adverse event is fatigue, reported to occur in 13–56% of patients, mostly grade 1–2 (5). In clinical practice, adverse events associated with nivolumabhave been well tolerated and this agent has exhibited antitumor activity in extensively pretreated patients with relapsed or refractory B- and T-cell lymphomas (5, 18).

Biomarker data might be useful in guiding dose and regimen selection in early clinical development. However, a correlation between the expression of PD-L1 by DLBCL cells and response to PD-1 inhibitors has not been confirmed and remains controversial (39). Evaluation of PD-L1 expression by tumor-cells as a predictive marker has been inconclusive. This observation might be due to complex dynamics of expression depending on the tumor microenvironment and the lack of standardized immunohistochemical assessment of PD-L1 expression (47).

CAR T CELL THERAPY

CAR T-cells are autologous, polyclonal T lymphocytes genetically engineered to express a tumor-targeting receptor, directing the T cells to bind to a specific tumor-associated antigen. CAR T cells are composed of an extracellular single chain variable fragment (scFv) and intracellular signaling domains that allow T cells to effect functions independent of major histocompatibility complex (MHC) antigens. Depending on differences in the intracellular signaling domains and cytokine secretion, CAR T cells have been classified as first-, second-, third- and fourth-generation. First-generation CAR T cells consisted of an extracellular scFv and a single intracellular signaling domain CD3ζ. The limited activity of this generation was probably attributable to their inability to adequately activate T cells, especially in cases where tumor cells did not express T cell co-stimulatory molecules (48). Subsequently, second (and third and fourth)-generation CAR T cells included co-stimulatory domains, such as CD28 or CD137 (4-1BB), to improve expansion and persistence of T cells (49, 50). Kochenderfer first reported the anti-tumor efficacy of an anti-CD19 CAR T cell containing the CD28 costimulatory domain in aggressive lymphoma (51). In order to enhance the activation of CAR T cells, third-generation CAR T cells were designed by combining two signaling domains among CD28, CD27, 4-1BB, ICOS, and OX40 (52–56). Including two co-stimulatory domains into CAR T cells can improve the tumor cell-killing efficacy. However, because of the activation of multiple intracellular signaling caused by the co-stimulatory domains of third-generation CAR T cells, abundant cytokines might be released which may result in a life-threatening cytokine storm (57). In order to enhance their tumor cell-killing efficacy and impact local suppressive cells, fourth-generation CAR T cells were engineered with an inducible expression component, such as cytokine IL-12, and also are known as T cells redirected for universal cytokine-mediated killing (TRUCKs). TRUCKs not only increase the activation of CAR T cells, they also induce cytokines and attract innate immune cells to eliminate antigen-negative cancer cells (58). In addition, for safety considerations, an inducible caspase 9 self-withdrawal genetic design allows for rapid elimination of infused CAR T cells once the anti-tumor mission is accomplished (59, 60).

Clinical Trials of CAR T Cells as Therapy in DLBCL

CAR T cell therapies have been most efficacious in patients with B-cell acute lymphoblastic leukemia; less data are available for patients with DLBCL. According to the American Cancer Society, ∼72,000 children and adults in the US will be diagnosed with non-Hodgkin lymphoma in 2017; 60% of these cases are aggressive neoplasms with the most common type being DLBCL. The typical survival duration of patients with DLBCL who have disease progression after chemotherapy or ASCT is 9 months. The cumulative promising data indicate that immunotherapy using CAR T cells offers hope for achieving long-term survival in patients with relapse/refractory DLBCL or follicular lymphoma (FL).

Investigators from Kite Pharma developed a clinical trial of CD19-CAR T cells (NCT02348216) that was approved on October 2017, becoming the first CAR T therapy approved by the FDA for the treatment of adults with relapsed or refractory DLBCL after two or more lines of systemic therapy. CD19-targeting CAR T cell therapy showed that 42% of patients with refractory DLBCL remained in remission at 15 months following treatment with axi-cel (marketed as Yescarta). Axi-cel CAR T cell therapy is the second gene therapy approved by the FDA and the first for adult patients with DLBCL after failing at least two other kinds of treatment; the types of large B-cell lymphoma in this study include DLBCL not otherwise specified (NOS), primary mediastinal large B-cell lymphoma, DLBCL arising from follicular lymphoma, and cases that fit into the new World Health organization category of high grade B-cell lymphoma (e.g., DLBCL with double hit genetics). This study, named ZUMA-1, also reported measurable responses in 82% of patients and complete responses in 54%. Over half (56%) of patients were alive at 15 months following therapy, with some remaining cancer-free for 2 years post-treatment. Among the 111 patients who were enrolled, axi-cel was successfully manufactured for 110 and administered to 101. The median age of these patients was 58 years (range, 23–76 years). Most (85%) patients in the study group had stage III or IV disease; 77% had disease that was resistant to second line or subsequent therapies, 21% had disease relapse after transplantation, 69% had received at least three previous therapies, and 26% had a history of primary refractory disease. Among the 101 patients who received axi-cel, the ORR was 82%, with a 54% CR. With a median follow-up of 15.4 months, 42% of patients continued to have a response, with 40% in CR. The overall rate of survival at 18 months was

52%. The most common adverse events of grade 3 or higher during treatment were neutropenia (78% of the patients), anemia (43%), and thrombocytopenia (38%). Grade 3 or higher cytokine release syndrome (CRS) and neurologic events occurred in 13 and 28% of patients, respectively. Three patients died during treatment. In this multicenter study, patients with refractory DLBCL who received CAR T-cell therapy with axi-cel had high levels of durable response, with a safety profile that included myelosuppression, CRS, and neurologic events (61).

Kochenderfer and colleagues at the National Cancer Institute were the first to report a partial response (PR) lasting 32 weeks after infusing autologous T cells directed against CD19 in a patient with FL (62). This group later published seven patients with DLBCL: four patients achieved a CR, two achieved a PR, and one had stable disease (SD) (57). Recently, Kochenderfer et al. reported results for 22 patients with advanced-stage lymphoma in a clinical trial of CAR-19 T cells preceded by low-dose chemotherapy, including 19 patients with DLBCL, two patients with FL, and one patient with mantle cell lymphoma. Patients received a single dose of CAR-19 T cells 2 days after a low-dose chemotherapy conditioning regimen of cyclophosphamide plus fludarabine. This study showed that CAR-19 T cells are an effective therapy for lymphoma patients and with lower doses of chemotherapy than they previously used; the ORR was 73%, with 55% achieving CR and 18% achieving PR. Eleven of 12 patients remain in CR and grade 3 or 4 neurologic toxicities in about half of the patients resolved completely (51).

Investigators from the University of Pennsylvania Medical Center have collaborated with Novartis to develop a second-generation CD19-CAR T cell named CTL019. This CAR consists of a murine anti-CD19 scFv, a CD8 hinge, a transmembrane domain, 4-1BB (co-stimulatory molecule), and CD3ζ. This group has conducted a phase IIa clinical trial of CTL019 cells in patients with relapsed or refractory CD19+ non-Hodgkin lymphomas (NCT02030834); 29 patients (19 DLBCL; 8 FL; 2 MCL) enrolled and 20 patients received CTL019 per protocol dose (12 DLBCL; 7 FL; 1 MCL). Pre-infusion chemotherapy regimens were EPOCH ($n = 2$); cyclophosphamide ($n = 9$); radiation + cyclophosphamide ($n = 2$); bendamustine ($n = 6$); cyclophosphamide-fludarabine ($n = 1$). Cytokine release syndrome occurred in 15 patients (13 grade 2; 2 grade 3). Neurologic toxicity occurred in 3 patients: transient delirium (1 grade 2, 1 grade 3) and 1 possibly related, grade 5 encephalopathy. For 18 patients evaluable for response at 3 months (12 DLBCL; 6 FL), the ORR was 67% (DLBCL 50%; FL 100%). At a median follow up 6 months, progression-free survival for evaluable patients was 59% (DLBCL 37%; FL 100%). This report shows that CTL019 cells induce durable responses in patients with relapsed/refractory DLBCL and FL with acceptable toxicity (63).

Recently, interim results from a global, pivotal multi-center phase II JULIET trial (NCT02445248) of CTL019 (tisagenlecleucel) showed durable complete responses in adults with relapsed/refractory DLBCL. The ORR at 3 months was 45% (23 of 51 patients evaluated), with 37% achieving CR and 8% achieving PR. The patients with CR remained stable from 3 months through data cutoff among the study cohort (64).

Investigators from the Fred Hutchinson Cancer Research Center, Memorial Sloan Kettering Cancer Center, and Seattle Children's Research Institute have collaborated with Juno Therapeutics to conduct several clinical trials of CD19-CAR T cell products: JCAR014, JCAR015, JCAR017, JCAR021, and others. Among them, updated results from the ongoing TRANSCEND study of JCAR017, which contains the 4-1BB costimulatory domain, in patients with relapsed or refractory aggressive non-Hodgkin lymphoma were presented during 2017 American Society of Hematology meeting. The core group ($n = 49$) included patients with DLBCL (NOS and transformed from follicular lymphoma) who were ECOG performance status 0–1. These patients represented a highly refractory population based on factors associated with a poor prognosis, including older patient age, double, or triple hit genetics (*MYC* and *BCL2* and/or *BCL6* rearrangement), and the DLBCL being refractory to chemotherapy. Dose level 1 (DL1 = 50 million cells) showed a 3 month ORR of 52% (11/21 patients) and a 3 month CR rate of 33% (7/21). Dose level 2 (DL2 = 100 million cells), the dose in the pivotal cohort of the TRANSCEND study, showed a 3 month overall response rate (ORR) of 80% (12/15) and a 3 month complete response (CR) rate of 73% (11/15) in the core group. These data support a dose response relationship. Across both doses in the core group, the best overall response was 84% (41/49 patients) and the best overall CR rate was 61% (30/49). There was no increase in CRS or neurotoxicity (NT) rates associated with the higher dose or between the full and core groups. Across doses in the full group, 1 of 69 (1%) patients experienced severe CRS and 10 (14%) patients experienced severe NT. Twenty-one of 69 (30%) patients had any grade CRS and 14 (20%) patients had any grade NT. 64% (44/69) of patients had no evidence of CRS or NT. The most common treatment-emergent adverse events other than CRS and NT that occurred at ≥25% in the full group included neutropenia (41%), fatigue (30%), thrombocytopenia (30%), and anemia (26%) (65–67).

Challenges and Opportunities for CAR T Cell Therapy

CAR T cells have shown promising efficacy in patients with DLBCL, including those with relapsed or refractory DLBCL. However, this therapy can be associated with unexpected toxicities that can be life-threatening, including CRS, NT, and "on-target off-tumor" recognition. The challenges are to reduce toxicity, prolong disease-free survival, and to determine which factors can predict relapse of DLBCL after successful CAR T cell therapy.

Cytokine release syndrome is a systemic inflammatory response to the activation and proliferation of CAR T cells. The clinical features of CRS include high fever, fatigue, nausea, malaise, hypotension, cardiac dysfunction, renal impairment, hepatic failure, capillary leak, and disseminated intravascular coagulation (68). CRS is associated with a dramatic elevation of inflammatory cytokines in the serum including C reactive protein (CRP), interferon-γ, ferritin, granulocyte macrophage colony-stimulating factor, IL-10, and IL-6 following CAR T-cell infusion (69–72). CRS occurs most frequently within the first 2 weeks after CAR T cell infusion. Clinical management

schemes of CRS include administration of steroids and the IL-6 receptor blocking antibody, tocilizumab (68, 73). However, steroids blunt the anti-tumor function of CAR T cells and the long-term impact of tocilizumab on CAR T cell function remains unclear. It remains a challenge to control CRS without inhibiting the anti-tumor efficacy of CAR T cell therapy.

Neurologic adverse events have been observed in many patients receiving CD19-CAR T cell therapy. Reversible symptoms of NT, including confusion, delirium, expressive aphasia, encephalopathy, and seizures, have been reported in several studies (51, 69, 74–77). In some patients, CD19-CAR T cells have been found in cerebrospinal fluid (74, 76). Whether neurological toxicities are solely restricted to CD19-specific CAR T cells or are associated generally with CAR T cell therapy remains unclear and the potential causes of NT remain to be elucidated. The postulated pathophysiological mechanisms include cytokine diffusion and/or translocation of activated CAR T cells across the blood brain barrier.

On-target off-tumor recognition side effects caused by depletion of healthy CD19-positive B-cells by CAR T cells are also an issue. B cell aplasia is a common adverse event in CAR T cells trials targeting B cell malignancies (75, 77, 78). Off-tumor recognition side effects in CAR T cell treated patients also can occur as a result of cross-reactivity of the engineered antigen binding domain with a non-related surface protein.

Selective depletion of CAR T cells can be approached by the use of "self-withdrawal CARs" in which is inserted an inducible caspase 9 (ICasp9) (79, 80). Current T-cell engineering approaches redirect patient T cells to tumors by transducing them with antigen-specific T-cell receptors (TCRs) or CARs that target a single antigen. However, healthy tissues that express the targeted antigen may undergo CAR T cell-mediated damage. A novel strategy that combines antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells (81). In trials using CD19-targeting T-cells, CD19-negative clones have expanded and caused progressive disease (82). The approach of increasing the specificity of CARs is to combine more CAR T cells to recognize multiple targets. This treatment strategy may help broaden the applicability and avoid some of the side effects of targeted T-cell therapies. In addition, a novel agent that blocks IL-35 may support CAR T cell therapy by reducing the inhibitory effect of regulatory T cells that may be of value in the future (83). Furthermore, several small molecule inhibitors, such as ibrutinib (Bruton tyrosine kinase inhibitor) (84), ABT-199 (Bcl-2 inhibitor) (85), and JQ-1 (bromodomain inhibitor) (86), has shown impressive potential for treating DLBCL patients. CAR T immunotherapy in combination with a small molecule inhibitor is likely to provide greater benefit for the treatment of patients with DLBCL.

CONCLUSIONS

Cancer immunotherapy that harnesses the host immune system in novel ways to kill tumor cells is emerging. Immunotherapy offers promising opportunities with the potential to induce sustained remissions, and is expected to become a "game changer" for the treatment of patients with cancer. Novel immunotherapy regimens, PD-1/PD-L1, and CTLA-4 checkpoint inhibitors, and CAR T cells have shown promising potential in the treatment of patients with DLBCL.

Early clinical trials using PD-1/PD-L1 checkpoint inhibitors including two anti-PD-1 antibodies (nivolumab and pembrolizumab), and three anti-PD-L1 antibodies (avelumab, durvalumab, and atezolizumab), have shown great promise. CAR T cell therapy also has shown remarkable activity in patients with refractory DLBCL. Yescarta, a CAR T cell immunotherapy, has been approved by the FDA for use in adults with large B-cell lymphoma after at least two other kinds of treatment have failed. Numerous ongoing clinical trials will undoubtedly offer the hope of achieving long-term survival in patients with relapsed or refractory disease.

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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Microenvironment Cell Contribution to Lymphoma Immunity

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Lymphoma microenvironment is a complex system composed of stromal cells, blood vessels, immune cells as well as extracellular matrix, cytokines, exosomes, and chemokines. In this review, we describe the function, localization, and interactions between various cellular components. We also summarize their contribution to lymphoma immunity in the era of immunotherapy. Publications were identified from searching Pubmed. Primary literature was carefully evaluated for replicability before incorporating into the review. We describe the roles of mesenchymal stem/stromal cells (MSCs), lymphoma-associated macrophages (LAMs), dendritic cells, cytotoxic T cells, PD-1 expressing CD4+ tumor infiltrating lymphocytes (TILs), T-cells expressing markers of exhaustion such as TIM-3 and LAG-3, regulatory T cells, and natural killer cells. While it is not in itself a cell, we also include a brief overview of the lymphoma exosome and how it contributes to anti-tumor effect as well as immune dysfunction. Understanding the cellular players that comprise the lymphoma microenvironment is critical to developing novel therapeutics that can help block the signals for immune escape and promote tumor surveillance. It may also be the key to understanding mechanisms of resistance to immune checkpoint blockade and immune-related adverse events due to certain types of immunotherapy.

Keywords: lymphoma, microenvironment, T cell subsets, stromal cells, lymphoma exosomes

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BACKGROUND

The cellular context in which lymphoma cells thrive has only recently become an important focus of inquiry. The roles of what used to be considered passive bystanders are quickly becoming elucidated in order to parse out potential targets for immunotherapy. Although our understanding of cytogenetic abnormalities and molecular pathways in lymphoma are in advance of solid organ tumors, the same cannot be said of the tumor microenvironment. In this section, we summarize some of the major components of the lymphoma microenvironment and their contribution to lymphoma immunity.

The primary goal of this review is to address the interplay between lymphoma cells and the cells of the lymphoma microenvironment and to understand how this communication leads to mechanisms of immune evasion and tumor proliferation. Our manuscript will also present some of the controversies in the field and present the limitations in our understanding of the roles and responsibilities of the microenvironment cell in lymphoma pathogenesis.

INTRODUCTION

Lymphomas are a diverse group of clonal neoplasms arising from B and T lymphocytes, and natural killer (NK) cells and are characterized by infiltration of lymphoid structures.

Most of these neoplasms correspond to the normal stages of B-cell or T-cell differentiation and hence can be classified accordingly (1). Advances in structural and functional genomics have highlighted the underlying genetic aberrations and oncogenic regulatory pathways leading to a better understanding of the molecular pathogenesis of lymphomas (2). In contrast, the integral role played by microenvironment in lymphomagenesis and progression has only been recently highlighted and needs to be explored in greater depth.

LYMPHOMA MICROENVIRONMENT

In addition to somatic mutations and inflammation, the role of tumor microenvironment (TME) in acquisition of key characteristics of cancer pathogenesis and progression, like sustained tumor proliferative signaling, resisting cell death, evasion of growth suppressors, and immune escape mechanism is becoming important in the study of lymphoma pathogenesis (3). The lymphoma microenvironment is increasingly being recognized as a dynamic and interactive supporting network of immune cells, stromal cells, cytokines, blood vessels, and extracellular matrix components, including sclerosis, whose composition is guided by the neoplastic cells and which in turn, influence tumor initiation, progression, and drug resistance (4). The key factors influencing the composition of microenvironment include lymphoma subtypes and signaling interactions between the lymphoma cells and microenvironment cells. The various components of a typical lymphoma microenvironment are outlined in **Table 1**.

A deeper knowledge of interactions between lymphoma cells and its non-malignant microenvironment would be critical in understanding the differences between the pathogenesis and prognosis of various lymphoma subtypes and potential new therapeutic targets.

TABLE 1 | Components of lymphoma microenvironment.

A.	IMMUNE CELLLS 1. Cytotoxic T cells (CTLs) 2. Follicular B helper T cells (T_{FH}) 3. Regulatory T cells (Tregs) 4. Natural Killer cells (NK) 5. Bystander B cells
B.	STROMAL CELLS 1. Mesenchymal stromal cells (MSC) 2. Lymphoma associated macrophages (LAMs) 3. Myeloid-derived suppressor cells (MDSCs) 4. Dendritic cells
C.	ANGIOGENESIS
D.	EXTRACELLULAR COMPONENTS 1. Extracellular matrix (ECM) 2. Cytokines/Chemokines 3. Lymphoma exosome

MESENCHYMAL STROMAL CELLS (MSCs)

MSCs have both anti-inflammatory as well as immunosuppressive properties. The latter characteristic can aid tumor cells to escape immune surveillance. Investigators have found that co-injection of MSCs with neoplastic (A20) B cells promotes B cell lymphoma growth in the lacrimal glands of immunocompetent mice and were associated with marked increased in CD4+ forkhead box P3 (FoxP3) + T cells and myeloid-derived suppressor cells (5). In murine model of lacrimal gland B-cell lymphomas, those lymphoma cells that were coinjected with MSCs were found to have increased CD4+ Foxp3+ regulatory T cells as well as CD11b+ Ly6C+Ly6G– MDSCs. These coinjected tumors demonstrated less apoptosis and had up-regulated immune-associated molecules such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, transforming growth factor beta (TGF-β), and arginase. Hence, it appears that MSCs help create an immunosuppressive milieu in the context of lacrimal gland B-cell lymphomas (5). Likewise, other investigators have found that MSCs promote tumor growth in mice with p53 mutations that develop spontaneous lymphomas (6).

MSCs have also been postulated to differentiate into the fibroblastic reticular cells and follicular dendritic cells necessary for the infiltration of follicular lymphoma in the bone marrow (7). Investigators have demonstrated that marrow MSCs from patients with follicular lymphoma, which has a relatively high rate of marrow involvement, overexpress chemokine (C-C motif) ligand 2 (CCL2) and aid in sustaining the growth of malignant B cells. These findings suggest an integral role of stromal cells in the infiltration and persistence of lymphoma in medullary sites (7).

LYMPHOMA-ASSOCIATED MACROPHAGES (LAMs)

LAMs are the macrophage/circulating monocyte lineage cells found in close association with lymphoma. Their roles appear to differ based on tumor type. Elevated numbers of LAMs have been correlated with poor prognosis in certain tumors. In individual studies of advanced stage classic Hodgkin lymphoma (CHL) as well as in meta-analyses, a high-density of LAMs is a strong predictor of adverse outcomes in adult patients (8, 9).

LAMs appear to demonstrate dual predictive roles in follicular lymphoma. High levels of CD68+ or CD163+ LAMs are associated with poor outcome in follicular lymphoma treated with conventional chemotherapy prior to the rituximab era, whereas this effect was diminished or even inverted when rituximab is used in combination (10). In murine models, anti-CD20 monoclonal antibody (mAb) mediated depletion of B cells relied upon the macrophage expression of Fc-gamma receptors (FcγR) (11).

Therapeutically, it has also been shown that relatively novel immunomodulatory drugs such as pomalidomide convert the polarization status of macrophages from M2 to M1 in mouse

models of central nervous system (CNS) lymphoma (12). This appears to be achieved by reducing signal transducer and activator of transcription (STAT) 6 signaling while enhancing STAT1 signaling and thereby pomalidomide increases the phagocytic activity of macrophages. This finding argued for the therapeutic activity of pomalidomide against CNS lymphomas.

In CHL, an increased number of CD68 positive LAMs have been significantly associated with a shorter progression-free survival, increased likelihood of relapse after stem cell transplantation and an overall shortened disease-specific survival, making them a potential risk stratification biomarker (13). Such studies were carried out in patients treated with standard chemotherapy so it is unknown whether they would be consistent in patients receiving novel therapies that may alter the microenvironment.

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

MDSCs are myeloid lineage cells that appear to suppress immune surveillance, particularly in the bone marrow. They can accumulate in the context of a wide variety of pathologic conditions, including cancer, and inflammation (14). MDSCs have been shown to form mature osteoclasts in response to nuclear factor KB ligand (RANKL), increasing bone resorption. They are thought to influence the ability of tumors to spread into the marrow niche (15). Tumors can encourage the accrual of MDSCs by secreting factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), and interferon-γ (IFN-γ) (16). Elevated levels of MDSCs have been demonstrated in lymphoma, leukemia and multiple myeloma (17).

When normal peripheral blood mononuclear cells were incubated with monocytes from patients with B-cell non-Hodgkin lymphoma (NHL), a reduction in T-cell proliferation as well as decreased Th1-response was seen via measurement of IFN-γ production. Using anti-CD14 immunomagnetic beads to decrease the monocyte population resulted in restored T-cell proliferation. These findings could not be attributed to any significant difference in percentage of monocytes in the peripheral blood of patients vs. healthy controls. Furthermore, the CD14 positive monocytes in patients with NHL showed reduced HLA-DR expression, which is associated with decreased immune function and possibly more aggressive lymphoma (18).

In CHL patients, a group of investigators showed that at initial diagnosis, all subsets of MDSCs were higher in the lymphoma patients compared to healthy controls. While the patients underwent therapy, MDSC subsets declined. The patients who achieved complete response had lower CD34+ MDSCs, monocytic MDSC, and polymorphonuclear MDSCs in their peripheral blood compared to the non-responders. In particular, the undifferentiated CD34+ MDSCs were proposed as a possible biomarker for outcome (19).

More recently, patients with diffuse large B-cell lymphoma (DLBCL) were found to have higher circulating CD14+ HLA-DR^{lo} monocytic MDSCs, which was in concordance with two other studies. The level of these MDSCs correlated with a worse clinical prognosis and was associated with regulatory T cells (Tregs) proliferation (20). Such findings suggest that MDSCs may be a rational target for novel therapies in patients with aggressive lymphomas.

Studies of MDSCs in peripheral T-cell lymphomas and NK/T-cell lymphomas are few and understandably limited in the number of primary human tumors tested. One of the larger studies was conducted in 32 extranodal NK/T cell lymphoma patients. Similar to that found in other lymphoma subtypes, patients with the tumor had higher levels of CD33+ CD11b+ HLA-DR– MDSCs. These MDSCs had increased expression of IL-17, arginase-1 and cytokine-inducible nitric oxide synthase (iNOS) and suppressed T cell proliferation. The higher levels of MDSCs were associated with shorter progression-free survival and overall survival (21). In cutaneous T-cell lymphomas, programmed death-ligand 1 (PD-L1) was expressed by MDSCs as well as by tumor cells themselves and was associated with inhibition of T-cell proliferation and promotion of regulatory FoxP3+ T cells (22).

DENDRITIC CELLS

Dendritic cells are some of the most powerful antigen-presenting cells in the body, aiding in the activation of cytotoxic T cells as well as naïve helper T cells. It has been shown that direct follicular dendritic cell contact with the neoplastic cells of mantle cell lymphomas and other NHL can protect them from apoptosis. This was mediated by upregulation of microRNA-181a (miR-181a), which reduced the levels of proapoptotic Bcl-2-like protein 11 (Bim). Inhibition of miR-181a led to restoration of Bim, releasing the dendritic cell suppression of apoptosis in lymphoma cell lines and primary lymphoma cells (23).

In the setting of follicular lymphoma, tumors with gene expression signatures that included genes highly expressed by dendritic cells and monocytes were associated with poor outcomes. In contrast, those tumors with gene expression signatures containing genes encoding T cell markers and macrophages were associated with prolonged survival (15). However, follow-up studies did not show compatible findings when immunohistochemical assays substituted gene expression analysis (24, 25).

In vitro studies were initially promising when DCs were pulsed with either tumor antigen or whole tumor lysate to stimulate immune responses from T cells. While *in vivo* translation into hematologic malignancies have not demonstrated durable responses, these studies were performed in patients with advanced disease (26). Hence, it is possible that combination with other immunotherapy in less advanced disease may be promising.

CHEMOKINES AND CYTOKINES

The microenvironment of CHL is a good model to study the role of chemokines and chemokine receptors in the interaction

between microenvironment cells and the Hodgkin Reed-Sternberg (H-RS) cells toward the formation and sustenance of lymphoma microenvironment. The tumor microenvironment of CHL (constituting 99% of the tumor) is composed of B cells, T cells, eosinophils, plasma cells, neutrophils, macrophages, dendritic cells, and fibroblasts, and is largely derived from the dysregulated chemokine secretion by the H-RS cells and TME cells (27). The key cytokines playing an active role in the process, include IL-7, IL-10, TGF-β, chemokine ligand 5 (CCL 5), chemokine ligand 1 (CCL1), and Galectin-1 (28, 29).

The T cells surrounding Reed-Sternberg cells express CCL5, which acts as a chemo-attractant for monocytes, eosinophils, basophils and mast cells as well as CD4 positive T cells (30, 31). C-C chemokine receptor type 3 (CCR3) + Th2 cells and eosinophils are attracted by the CCL1(eotaxin) produced by fibroblasts surrounding RS cells (32, 33). Earlier on, chemokine receptors like C-C chemokine receptor type 5 (CCR5) were thought to be only expressed by the non-neoplastic bystander cells. However, subsequent studies have shown constitutive expression of CCL5 receptor (CCR5) on H-RS cells by immunohistochemistry, flow cytometry, and western blot (34). CCL5, along with other chemokines released by either H-RS cell, Hodgkin cell stimulated fibroblasts or T cells are central to the recruitment of CD4+ T lymphocytes and eosinophils into the classic HL microenvironment. Chronic inflammation at the site of tumor, driven by chemokines and cytokines, has also been found to promote tumor progression (35).

CYTOTOXIC T CELLS (CTLs)

Increased numbers of infiltrating CD8 positive T cells, many expressing cytotoxic markers like TIA-1, as measured by both immunohistochemistry and flow cytometric analysis have been associated with better outcomes in B-cell lymphomas (36, 37). Elevated numbers of cytotoxic lymphocytes positive for programmed cell death-1 (PD-1) was also found to be associated with favorable prognosis in the setting of follicular lymphoma (38).

The cytotoxic activity of T cells is enhanced by the targeting of the PD-1 pathway, which can lead to tumor cell lysis. Tumor specific activated T cells as well as regulatory T cells express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which binds to CD80/CD86 on antigen presenting cells and leads to T cell anergy by competing with CD28 as a costimulatory molecule. Immune checkpoint blockade can augment antitumor immunity (39).

During chronic antigen stimulation, a protein called lymphocyte activation gene-3 (LAG-3) is upregulated on T cells, suppressing CD4+ T cell expansion in response to antigen as well as CD8+ T cell function (40). Specifically, LAG-3 has been shown to maintain tolerance to tumor antigens via its effects on CD8+ T cells. In murine models, LAG-3 blockade increases proliferation and effector function of antigen-specific CD8+ T cells within organs and tumors that express their cognate antigen (41). These models suggest that LAG-3 can be a target for increasing the effectiveness of cytotoxic T-cell immunity against tumor.

REGULATORY T CELLS (Tregs)

Tregs include subsets of immune suppressive cells that regulate self-tolerance and immune homeostasis. Thymic derived Tregs are involved in preventing autoimmunity while peripheral Tregs maintain tolerance in mucosal sites. Both these naturally occurring $CD25^{+}CD4^{+}$ Treg populations express FoxP3, which is a more specific marker for regulatory T cells than CD25, CD45RB, or CTLA-4 (41–43). Tregs suppress the activity of bystander T cells, natural killer cells and B cells via CTLA-4, IL-10, and TGF-β1 (44).

FoxP3+ Tregs, particularly in inflamed tissues, have been shown to express T cell immunoglobulin and mucin-domain containing-3 (TIM-3), which enhances their regulatory function. Blockade of TIM-3 signaling appears to demonstrate therapeutic benefit in preclinical tumor models (45). TIM-3 works as a co-inhibitory receptor that is also expressed on IFN-γ producing T cells as well as macrophages and dendritic cells, where it leads to inhibition of normal Th1 responses (46).

Studies in mice have shown that Tregs are present in the peripheral blood of animals and that these circulating cells can regulate humoral immune responses *in vivo*. Furthermore, it was shown that the PD-1 pathway can inhibit blood Treg function. Hence, there is reason to believe that the PD-1: PD-L1 pathway can limit the differentiation and normal function of Tregs, suggesting that manipulation of this pathway can support protective immunity (47).

On the basis of their role in lymphomagenesis, Wang et al divided Tregs into 4 groups: suppressor Tregs (suppress CD8+ CTLs), malignant FoxP3+ Tregs, direct tumor-killing Tregs, and incompetent Tregs. The association between number of Tregs and lymphoma prognosis would vary depending on the type of Tregs present. For instance, in angioimmunoblastic T-cell lymphoma, where more of incompetent Tregs or direct tumor-killing Tregs are present, the anti-tumor cytotoxicity is preserved and hence, better prognosis is associated with increase in Tregs (48).

In certain NHL where Tregs are overrepresented in biopsy specimens compared to normal lymphoid tissue; these cells appeared to be recruited by malignant B cells (49). However, the story is not straightforward. In a study of 280 CHL patients, higher numbers of intratumoral Tregs was associated with better failure free survival and also somewhat better overall survival. Similarly, in follicular lymphoma and germinal center subtype diffuse large B-cell lymphomas, there was a positive correlation between disease specific survival and numbers of intratumoral FoxP3 positive cells (50, 51). From these studies, it has been surmised that the increased Tregs contribute to immune surveillance in lymphomas by reducing overall inflammation and lymphoma cell proliferation.

FOLLICULAR B HELPER T CELLS (T_{FH})

T_{FH} cells are abundant in follicular lymphomas. In the normal germinal center, T_{FH} cells appear to be involved in CD40-mediated interactions in the germinal center. In follicular lymphoma, these cells appear to provide IL-4 stimulation to the B cells and in conjunction with CD40 interactions, aid in the proliferation of neoplastic cells through STAT5 signaling (52). Recent work suggests that circulating CD4+ C-X-C chemokine receptor type 5 (CXCR5)+ T cells serve as the memory compartment of T_{FH} cells (53). CXCR5 is the receptor for chemokine ligand 13 (CXCL13), produced by follicular dendritic cells, that promotes the entry of B cells into germinal center. Hence, the upregulated expression of CXCR5 facilitates contact between the B cells and T cells (54).

In patients with low-grade B-cell lymphomas like follicular lymphoma or marginal zone lymphomas, subsets of circulating T_{FH} cells differ from healthy controls, with reduced C-C chemokine receptor type 6 (CCR6) and increased PD-1 (55). Increased levels of PD-1 receptor have also been found in T cells from chronic lymphocytic leukemia (CLL) patients and were not explained by patient age (56). These are correlated with the overexpression of PD-L1 and PD-L2 by the CLL cells. While both CD4+ and CD8+ T cells are increased, overall there are relatively more CD8+ T cells in patients with CLL. The presence of tumor cells appears to be associated with T cells showing an exhausted phenotype. Specifically, they often express CD160, CD244, PR domain zinc finger protein 1 (BLIMP-1), in addition to PD-1 (57). T_{FH} cells have also been shown to provide support for the follicular lymphoma B cells through IL-4 and CD40 ligand production. However, the exact role of T_{FH} cells in the context of lymphoma is not fully understood. Part of the difficulty rests in the fact that they can elicit various cytokine-mediated functions simultaneously and can, in turn, be influenced by their microenvironment (58).

NATURAL KILLER (NK) CELLS

NK cells are CD16+ CD56+ cytotoxic lymphocytes of the innate immune system, which induce apoptosis even in the absence of antibodies and major histocompatibility complex. NK cells can recognize tumor antigens via killer-cell immunoglobulin-like receptors (KIRs). KIRs can have inhibitory or activating functions and depends on the intracytoplasmic region of the receptor (59). Studies have shown defective NK cell cytolytic function in CLL (60). In a large 11-year human study, low cytotoxic activity of NK cells was associated with increased cancer risk (61).

Working through dendritic cell maturation, NK cells can prune the adaptive immune response. A subset of NK cells produces IFN-γ, TNF-α, IL-10, and certain chemokines that aid in the differentiation of T cells and dendritic cells (62). In mouse models, IFN-γ and perforin protein knockouts will develop B-cell lymphomas that show suggestion of immunosurveillance defect (63). Once a tumor microenvironment is developed, TGF-β is induced and TIM-3 expression on NK cells is upregulated. The increased TIM-3 expression has been associated with lower NK-cell cytotoxicity and poor outcomes in a variety of neoplasms (64).

Studies have demonstrated an acquired quantitative as well as qualitative deficiency of NK cells in CHL microenvironment, contributing to immune evasion mechanism for lymphoma progression (65). A study quantifying immune cells in CHL found NK cell density to be five times less compared to NHL or normal tissues (66). Recent studies have shown significant reduction in NKG2D expression as well as weak cytotoxic activity in NK cells in untreated CHL patients (67). Reactivation of silenced NK cells in CHL is a potential therapeutic target and is being currently pursued. Immune checkpoint inhibitors, like Nivolumab, are being used to recover cytotoxic activity of NK cells in CHL by PD-1 inhibition. Drugs targeting heat shock protein-90 have been found to be effective in preclinical studies (68). In a recent phase 1 study, the bispecific (CD30/CD16a), tetravalent antibody, AFM13 has proven significantly effective in NK cell activation (69).

BYSTANDER B CELLS

Bystander CD 20+ B cells are more numerous in lymphocyte predominant Hodgkin Lymphoma (LP-HL) compared with CHL, where their role in tumor progression is debatable (70). B cell production of IL-10 may aid in antitumor immunosuppression by T cell inhibition (71), whereas competition with tumor cells (H-RS) for T-cell derived survival signals may halt tumor cell growth.

LYMPHOMA EXOSOME

Exosomes are microparticles that can be secreted by cells and usually range in size from 30 to 100 nm (72). Upon discovery in 1983, they were thought to be cellular waste, but are now known as carriers of signaling molecules in various contexts, ranging from malignant to autoimmune (73) and infectious states (74). They are composed of a bilayer lipid membrane and the internal contents associated with reverse invagination from the plasma membrane and can include mRNAs, microRNAs, proteins, lipids, and signaling molecules (75).

Studies have begun to elucidate the role of exosomes in the interaction between circulating tumor cells and the microenvironment. CLL-derived exosomes were shown to induce stromal cells to take on a cancer-associated fibroblast (CAF) phenotype *in vitro*. The CAFs, in turn, support a niche that promotes CLL cell adhesion, survival and growth *in vivo* (76).

Recent studies demonstrate the possibility of studying circulating lymphoma exosomes. A group from Spain demonstrated the prognostic value of tumor associated mRNA in exosomes of patients with B-cell NHL by utilizing liquid biopsies (77). In this study, BCL-6 and C-MYC positivity in the pretreatment samples predicted worse progression free survival compared to patients without.

In another recent study, exosomes produced by lymphoma B cells carrying mutated *MYD88* were reported to reprogram

the marrow microenvironment such that mast cells and macrophages were induced to promote endogenous proinflammatory signaling pathways. Hence, it is believed that exosomes play a key role in the communication of tumor cells to non-malignant cells in the bone marrow, possibly creating a tumor-friendly environment (78).

EXTRACELLULAR MATRIX (ECM)

The extracellular matrix is a network of physically and biochemically distinct macromolecules, like proteins, glycoproteins, and proteoglycans, which constitute the basement membrane and interstitial matrix and are central to the maintenance of structural integrity and regulation of cell behavior in organs (79). In solid organ tumors, dysregulated ECM has been shown to expedite cancer progression directly by affecting cancer cells causing cellular transformation, cancer stem cell expansion and disruption of tissue polarity leading to tumor invasion and metastasis (80) or indirectly by affecting stromal cells (81) and facilitating creation of tumorigenic microenvironment by promotion of angiogenesis and inflammation (82).

ANGIOGENESIS

Lymphoma tumor microenvironment also includes a rich scaffold of vessels that supply nutrients to the proliferating cells. Much of the prior clinical studies have focused on vascular endothelial growth factor (VEGF) inhibition (83, 84) in preventing tumor angiogenesis. However, the addition of bevacizumab does not currently appear to improve efficacy above that found in R-CHOP chemotherapy alone in the setting of aggressive B cell lymphomas (85).

Platelet-derived growth factor (PDGF) type BB recruits PDGF receptor-expressing pericytes to neovessels, thus promoting vascular maturation and stabilization (86). It appears that PDGF can also be involved in the expression of other stromal angiogenic factors like basic fibroblast growth factor and VEGF (87).

Inhibition of platelet derived growth factor receptor B (PDGFRB) with imatinib mesylate or sunitinib malate has shown some efficacy in carcinoma models (88–90) but has not yet been thoroughly evaluated in the context of lymphomas. One study showed impaired growth of lymphoma in both human xenograft and mouse allograft models with the use of imatinib, a tyrosine kinase inhibitor of PDGFRB. These investigators show decreased microvascular density and *in vivo*, imatinib induced apoptosis of tumor associated PDGFRB positive pericytes and loss of perivascular integrity (91).

The tumor endothelium has also been shown to prevent T cell homing, and hence, can serve as a barrier against immunotherapy. Lessons can be learned and possibly refined from studies carried out in solid organ tumors, such as ovarian cancers, in which overexpression of endothelin B receptor was associated with absence of tumor infiltrating lymphocytes (TILs) and short survival time. An inhibitor for endothelin B receptor increased the adhesion of T cells *in vitro* to human endothelium. This adhesion required intercellular adhesion molecule 1 (ICAM-1) and augmented tumor immunotherapy *in vivo* without increasing systemic antitumor immune response (92). Endothelial mechanisms that regulate how much and which types of T cells can infiltrate the tumor likely plays a large role in the effectiveness of immunotherapy such as cancer vaccines. This area requires much further study, particularly in the setting of lymphomas.

In a study of lymph nodes in 286 Hodgkin lymphoma patients, morphometric parameters of angiogenesis were shown to be related to poor prognosis. Morphometric microvascular parameters, like microvessel density and total vascular area were inversely related to overall disease-specific survival (93).

MECHANISMS OF TUMOR MICROENVIRONMENT MEDIATED IMMUNE EVASION AND TUMOR PROGRESSION IN NHL

The chief interactions involved in immune escape and promotion of tumor progression in NHL are illustrated in **Figure 1**. Cytotoxic T lymphocytes (CTLs), gamma delta T (γδ T) cells, natural killer (NK) cells and lymphoma associated macrophages constitute the principal antitumor immune responses in the body. The malignant lymphoma B- cells interact closely in association with the niche microenvironment elements to escape these immune responses.

Loss of lymphoma cell surface molecules/ markers, which are integral to their recognition by immune cells, leads to reduced tumor immunogenicity and immune evasion. Genetic alterations leading to loss of MHC Class I, MHC Class II, and CD58 contribute to the failure of CD 8+ T lymphocyte, CD4+ lymphocyte, and NK cell-mediated tumor cytotoxicity (52). Another mechanism of escaping T/NK cell mediated cytotoxicity is by overexpression of inhibitory lymphoma cell surface molecules, like PD-L1 and herpes virus entry mediator (HVEM), which on interaction with their counterparts on T cells lead to impaired T/NK cell activity (96). It has been shown that the use of anti CD47 antibodies lead to increased phagocytic activity of SIRP-alpha (SIRP-α) bearing macrophages (97), thereby indicating that overexpression of CD47 and SIRP-alpha is a lymphoma cell mechanism to evade macrophage-mediated destruction. The B-NHL cells also modulate the composition of microenvironment toward creation of a more immunosuppressive niche by secretion of Treg chemokine CCL22, in response to IL-4 and CD40L expression by T follicular helper cells (98). Inhibitory enzymes, like indolediamine oxidase (IDO), and phenylalanine oxidase interleukin 4-induced gene 1 (IL4I1), secreted by lymphoma associated macrophages and some B-NHL cells also contributes to immune suppression by Treg expansion and inhibition of effector T cell proliferation and activity (94, 95). Increased expression of FAS Ligand (FASL) by NHL B cell induces cytotoxic T cell apoptosis, whereas IL-12 secretion induces T cell exhaustion by LAG-3 and TIM-3 induction (99).

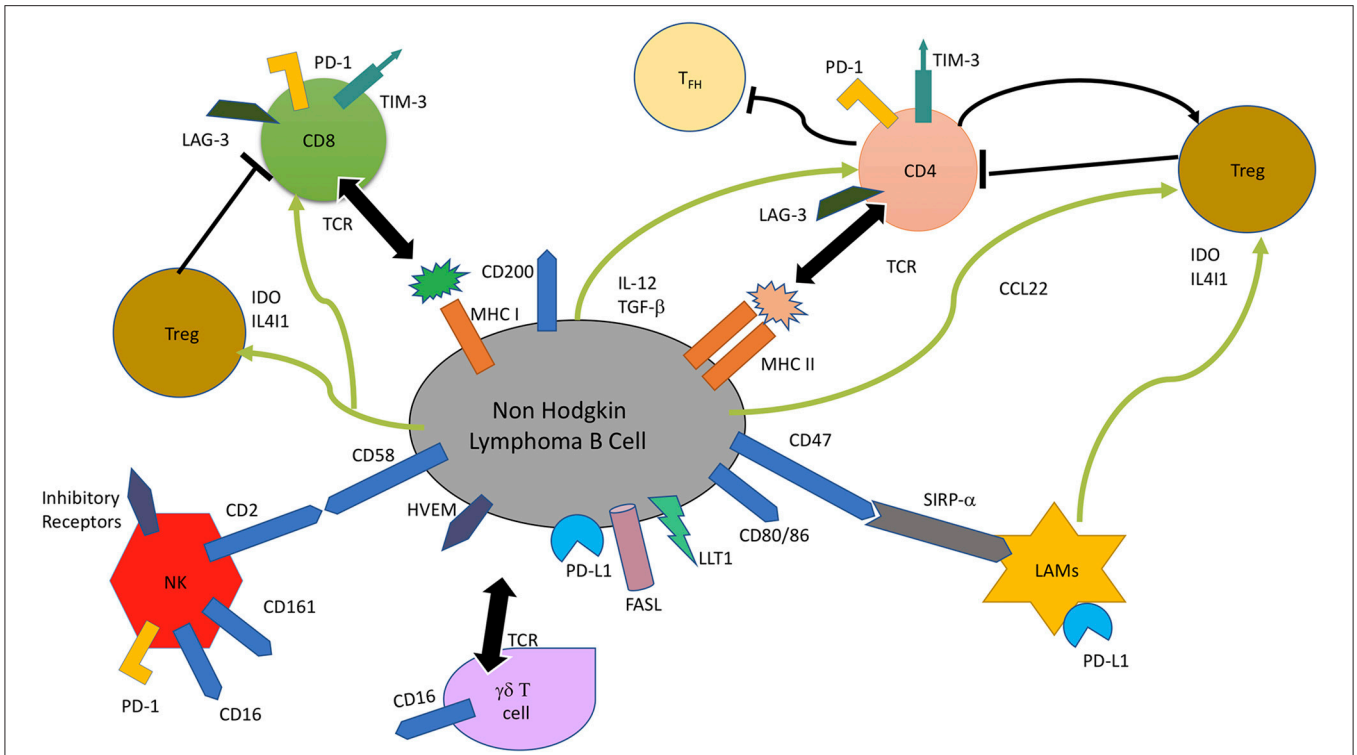

FIGURE 1 | In non-Hodgkin B-cell lymphoma, malignant B cells escape immune response by multiple mechanisms, including lack of recognition by immune cells due to loss of cell surface molecules involved in recognition, CD4+ T cells (MHC II), CD8+ T cells (MHC I), and NK cells (CD58). Overexpression of B cell surface inhibitory receptors, like PD-L1, Lectin-like Transcript 1 (LLT1), Herpes Virus Entry Mediator (HVEM), CD47, and CD200 which are the ligands for PD-1, CD161, BTLA, and SIRP-α, and secretion of inhibitory enzymes, Indoleamine 2, 3-dioxygenase (IDO) and IL4I1 leads to impaired T cell mediated cytotoxicity and T cell exhaustion. IDO and IL4I1 are also responsible for recruitment and differentiation of immunosuppressive Tregs, as well as exhaustion of T-effector cells through CCL22, TGF-β, and IL-12 secretion. FAS Ligand (FASL) induces apoptosis of CTLs (94, 95).

MECHANISMS OF TUMOR MICROENVIRONMENT MEDIATED IMMUNE EVASION AND TUMOR PROGRESSION IN CHL

The chief interactions involved in immune escape mechanism and promotion of tumor progression in CHL are illustrated in **Figure 2**. The H-RS cell orchestrates the rich polymorphous background cellularity comprising of T cells, macrophages, eosinophils, mast cells, neutrophils, plasma cells, plasma cells, stromal cells, and fibroblasts principally through secretion of cytokines and chemokines. H-RS cells secrete Colony Stimulating Factor-1 (CSF-1) and macrophage migration inhibitory factor (MIF) to recruit M2 macrophages, which in turn, secrete chemokines like, IL-8, to attract neutrophils into and eotaxin to attract eosinophils into tumor tissue (52).

It has been widely appreciated that TME and H-RS cells contribute to anti-tumor immune evasion by multiple mechanisms. Loss of MHC Class II molecules in CHL by chromosomal translocation has been linked to reduced tumor antigen presentation and hence, escape from immune cells. Aberrant expression by H-RS cells of surface molecule PD-L1, the ligand for PD- expressed on CTLs and CD4+ T cells, reduces anti-tumor immune function by T cell exhaustion. H-RS cells modify the microenvironment composition toward an immune tolerant state by inducing CD4+ T cell differentiation into immunosuppressive Tregs by secreting Galectin-1, TGF-β and CD70 and CD80 expression or by causing T-cell exhaustion through the secretion of TGF-β, IL-10, galectin-1, and prostaglandin E2 (100). Expression of FAS Ligand can induce apoptosis of CTLs, leading to reduced T cell mediated tumor cytotoxicity and tumor progression (101).

THERAPEUTIC IMPLICATIONS

A better understanding of the interactions between the lymphoma cells and the microenvironment niche has unraveled multiple new potential therapeutic targets in lymphoma treatment. The use of active and passive immunotherapy to bolster antitumor response is one such strategy and has been found to be considerably successful (102). Passive immunotherapy, based on the use of monoclonal antibodies and genetically engineered T cells has shown promising results in the treatment of relapsed/refractory NHL (103, 104). Recently, newer antibodies with multiple binding sites for tumor and T cells are being developed and early clinical trial results using bispecific

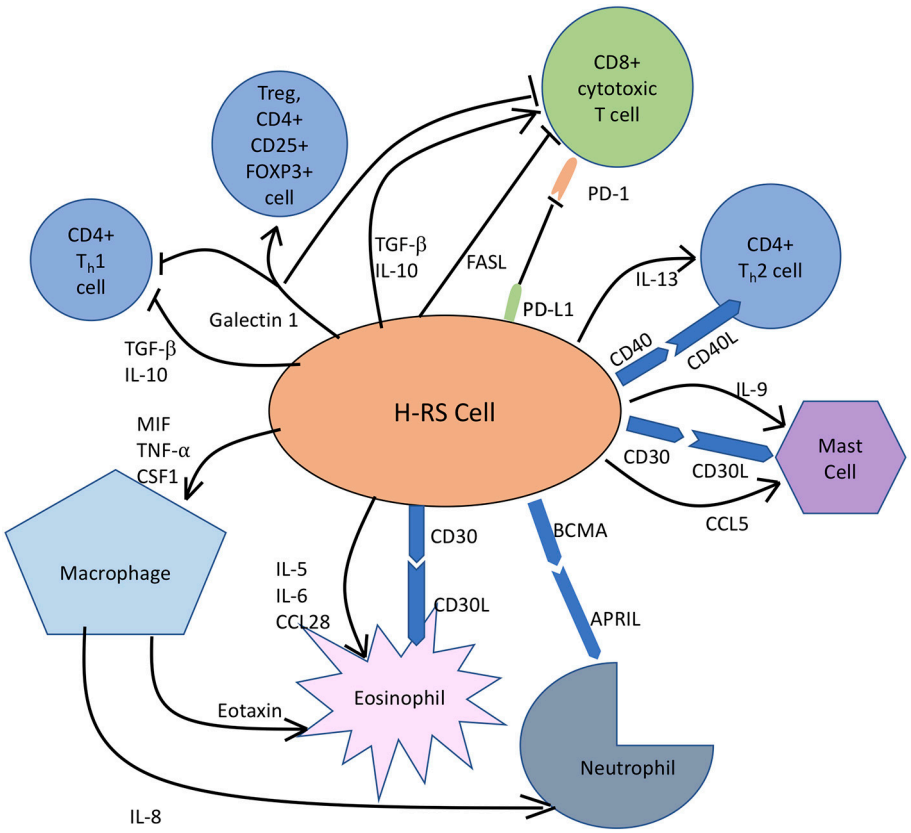

FIGURE 2 | In classic Hodgkin lymphoma, H-RS cells secrete cytokines, like IL-5, IL-9, and IL-10 to recruit eosinophils, mast cells and T cells, respectively to constitute the rich supportive tumor microenvironment. H-RS cells also produce macrophage migration inhibitory factor (MIF), which supports M2 macrophage infiltration. Galectin-1 induces differentiation of CD4+T cells toward immunosuppressive Tregs and causes apoptosis of both T_H1 cells and CTLs. FAS Ligand (FASL) induces apoptosis of CTLs. PD-L1 expression by H-RS cells helps the tumor escape immune responses by causing T-cell exhaustion. BCMA, B Cell Maturation Antigen; APRIL, Proliferation Inducing Ligand.

T-cell engager (BiTE), blinatumomab have been very promising (104).

Active immunotherapy modalities include vaccines and immune checkpoint inhibitors. The results with vaccines have been variable. Immune checkpoint inhibitors, on the other hand, have yielded excellent response rates, especially in Hodgkin lymphoma (60–80%) compared to NHL (20–40%) (105).

Improving the function of infiltrating immune effector cells, like T cells, and macrophages, has been shown to improve survival. Another major focus of upcoming lymphoma treatment strategies has been to target and diminish the microenvironment support for tumor cells, thereby limiting their survival. These treatment modalities have included targeting the pro-survival cell surface molecule signaling pathways (protumor signals), limiting tumor angiogenesis, attacking protumor microenvironment cells like mesenchymal stromal cells.

Similar to disrupting the protumor microenvironment approach is the recent focus on therapeutics aimed at mobilization of lymphoma cells away from their nourishing microenvironment. Abnormal ECM architecture, like dense collagen, has been known to be associated with poor chemotherapy response and resistance in solid tumors due to impaired drug delivery (106). In murine models, vaccine targeting tumor associated fibroblasts has been proven to decrease collagen type I expression, leading to 70% greater drug uptake (107).

CONCLUSION

The lymphoma microenvironment is a complex stage where the actors can interact with each other in varying ways depending on the context. It is becoming clear that the so-called bystander cells of the microenvironment may share the limelight with tumor cells in their contribution to disease pathogenesis and progression. Understanding their function can lead to more sophisticated methods of turning host cells effectively against the lymphoma as well as to circumvent resistance against immune checkpoint blockade and life-threatening complications from therapy.

AUTHOR CONTRIBUTIONS

DK designed, researched, wrote and revised the manuscript. MX conceived, designed, researched, wrote and edited this paper.

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REVIEW
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Cancer Immunotherapy and the Immune Response in Follicular Lymphoma

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Follicular lymphoma (FL) is the most frequent indolent lymphoma in the Western world and is characterized in almost all cases by the t(14;18) translocation that results in overexpression of BCL2, an anti-apoptotic protein. The entity includes a spectrum of subentities that differ from an indolent to a very aggressive growth pattern. As a consequence, treatment can include *watch & wait* up to intensive chemotherapy including allogeneic stem cell transplantation. The immune cell microenvironment has been recognized as a major driver of outcome of FL patients and gene expression profiling has identified a clinically relevant gene expression signature that classifies an immune response to the lymphoma cells. It is known for some time that the immune cell composition of the lymphoma microenvironment is important because high numbers of tissue-infiltrating macrophages correlate with poor outcome in patients receiving chemotherapy but not in patients receiving the combination of chemotherapy and CD20-specific monoclonal antibody rituximab. In addition, TCR signaling of tumor-infiltrating lymphocytes is dysfunctional leading to an impaired capacity to form an intact immunologic synapse. Approaches restoring local T cell function, e.g., by usage of checkpoint inhibitors has demonstrated clinical activity (ORR 40%) and can achieve long-term remissions. Ongoing trials with re-programmed autologous CART cells achieve response rates in approximately 50% of FL patients with relapsed and even refractory disease. Responses lasting for more than 6 months might be durable, indicative for a successful restoration of a functional immune system. In summary, FL is a malignant disease where the control by the immune system ultimately decides about progression and transformation rate. The advent of monoclonal antibodies has changed the way we treat FL and new approaches restoring the individual immune control will hopefully improve results further.

Keywords: follicular lymphoma, indolent lymphoma, monoclonal antibodies, bispecific antibodies, radioimmunotherapy, checkpoint blockade inhibitors, chimeric antigen receptor therapy

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INTRODUCTION

The aim of this review is to present the current therapeutic landscape of follicular lymphoma (FL) and to discuss early results of immunotherapies, e.g., checkpoint inhibitors and CAR T-cell therapies in the context of the immune system.

The clinically established therapeutic options for FL today are mainly focused on cytoreduction. Without the exception of the CD20 targeted therapies the recruitment of the patient's immune

Abbreviations: CR, complete response; PR, partial response; PD, progressive disease; FL, follicular lymphoma; OS, overall survival; CART, chimeric antigen receptor therapy.

system is not actively utilized in the conventional therapy of FL. However, for long-term benefits, it will be crucial to make progress in that direction, otherwise FL will continue to be an incurable and chronic disease. Thus, understanding the interplay between FL cells and their environment will be key for further success in this disease.

Other than in aggressive B-cell lymphomas like diffuse large B-cell lymphoma (DLBCL), the principle structure of the lymph node is more conserved in FL. In FL, the lymph node architecture is not destroyed, and the nodes boundaries are better respected by the tumor. The longevity of the malign B-cell, more than the aggressive behavior of these cells leads to abnormal large follicles. This phenotype is a result of the increase of lymphocyte numbers in the germinal centers that swell due to the sheer load. In FL, the often slow progression also leads into a long-standing relationship of the increasing B-cell numbers and their neighboring immune cells and the stromal microenvironment. This results inevitably in shifts and alterations within the immune microcosm. T-cells in FL lesions have been found to be increased (Zhang/Ansell); however, these T-cells, when examined in detail, often display features of T-cell exhaustion, for example, high expression of PD-1 or TIM-3 (1). Putative tumor supporting T-cells from T-cell subsets especially T-helper cells, T-cells (Tregs), and there most prominently follicular regulatory T-helper cells (FOXP3+) become involved in the protection of the malignant FL clone and foster its immune evasion.

The importance of an effective T-cell surveillance in the context of lymphoma has been demonstrated in various mouse models. For example, immunodeficient mice that lack T-cell or NK cell effector molecules like perforin or IFN-γ develop spontaneous lymphomas. These lymphomas when transferred on wild-type littermates are immediately rejected by CD8 positive T-cells (2, 3). In summary, suppression of a T-cell-mediated antitumor response appears to be instrumental for the initial establishment and further development of FL.

With a remarkable variability of clinical courses in FL, several efforts to better predict outcome according to biological features of the individual disease have been made. A gene expression-based model has identified two subsets of immune signatures in FL with distinct biologic attributes in FL that are associated with survival (4). These specific signatures were not expressed in the malign or benign B-cells but the genes in the immune response 1 signature were more highly expressed in T cells than in any of the B-cell or monocyte subpopulations, and genes of immune response 2 were more pronounced expressed in both T cells and monocytes but not in B-cells. Patients with immune signature 1 had a better outcome than those of immune signature 2, underscoring an important contribution of monocytes for a more dismal outcome in FL.

The role of monocytes in FL was further substantiated by a study (5) that found upregulated CCR1 and CD68-positive immune cells within FL lesions indicating a monocytes and macrophages recruitment. This pattern was apparently associated with worse survival in FL. In contrary, higher numbers of T-cells with elevated levels of CD3 and the early T-cell antigen CD7 were correlated with better survival in the examined cohort. Finally, CD4 and CD8 subsets were not significantly associated with outcome. Both findings are in line with the observation of two distinct immune profiles published by Dave et al. (4). These results confirm the role of the host immune responses for the outcome in FL and specifically demonstrate that the degree of infiltrating CD68 macrophages and CD7-positive T-cells is prognostically useful, together with identification of CCR1 as a putative novel prognostic indicator and a marker for an immune switch between macrophage and T cell-dominant response. With the advent of immune targeted therapies either against tumor supporting T-cells of lymphoma-associated macrophages, the vision of a chemotherapy free regimen for FL comes closer to reality.

BIOLOGY OF FL

Follicular lymphoma is among the most frequently occurring entities of indolent non-Hodgkin's lymphoma. Generally, FL presents as a slowly growing disease, which can be quite asymptomatic for some time. Once clinical problems are noted it is rather by compression of other structures than invasion or destruction of adjacent structures. If FL is detected in early stages (I and II) radiotherapy has curative potential. However, due to frequent bone marrow involvement (stage IV), many patients are not eligible for this curative option. Ultimately, almost all patients will experience relapse, and a proportion of patients will develop an aggressive disease with high risk of transformation. The annual rate of histological transformation in FL patients is estimated with 3% (6). Although advanced FL is considered incurable, recent advances in the treatment and management of this disease have made a significant impact on progression-free survival (PFS) and patient quality of life. Long treatment-free survival intervals in some patients suggest a possible cure in a subset of these patients, but as of today it is too early to make this claim. Even if FL is still considered incurable, affected patients generally have a long median overall survival (OS) that can reach 10 years or more. The advent of monoclonal antibody therapy in conjunction with new chemotherapeutics and the addition of radionuclides in the recent past have had a significant impact on FL management and have resulted in much better outcomes.

CLINICAL PRESENTATION AND COURSE OF FL

Most patients initially present with asymptomatic peripheral lymphadenopathy, affecting the cervical, axillary, femoral, and inguinal regions (7). Although lymph nodes are most commonly involved, the disease may also originate at or affect certain extranodal sites. These include the duodenum, skin, thyroid, salivary gland, and the breast (8). Stage IV disease is present in approximately two-third of the cases most often demonstrated by involvement of the bone marrow (9). Clinical features like night sweats and weight loss—typically associated with more aggressive forms of lymphomas such as DLBCL—might be present but are often missing even in higher stages of the disease. The ESMO recommendations appreciate the diversity of the FL subtypes, and the therapeutic options for the individual patients should be taken into consideration when planning the appropriate therapy (10).

IMMUNOCHEMOTHERAPY AND RADIOTHERAPY FOR FL

Passive immunotherapy, e.g., monoclonal antibodies against CD20, in combination with a chemotherapy backbone is currently the standard of care for patients with advanced-stage FL in need of treatment (10–13). Some patients with a low burden may be treated with CD20-specific antibodies (such as rituximab) only. However, a proportion of patients do not respond to standard treatment, and the majority will relapse after an initial response, highlighting the need for other more effective and durable therapies. An alternative approach to monoclonal antibodies with or without chemotherapy is the usage of radionuclide labeled anti-CD20 antibodies that are described in more details later in the article.

Radiotherapy has a potential to improve PFS and improves OS for FL patients with early clinical stages (I and II) by approximately 15% (14, 15). The standard radiation dose for FL is 24 Gy and has been shown to be superior to 4 Gy delivered as 2 × 2 (FORT trial) (16). However, long-term remissions in advanced FL patients receiving TBI with 2 Gy × 2 Gy and patients who had aborted the full doses for various reasons have been observed. Therefore, given the exquisite radiosensitivity of FL and the presumable added control by the immune system when applying lower doses of radiation suggest that there is a mechanism of radiotherapy beyond sheer lymphoma cell destruction.

Identification and Characterization of Potential Target Antigens

Being a more mature B-cell disorder, FL displays the immunophenotype of follicular center B-cells. Pan-B-cell markers (CD19, CD20, CD22, and IgM) are present with a co-expression of CD10. In contrast to reactive B-cells, FL cells express BCL-2. The expression of this anti-apoptotic protein due to t(14;18) (q32;q21) event, that brings the BCL-2 gene under the activity of the Ig heavy chain promoter is regarded to be pathognomonic for the disease. For therapeutic purposes, CD20 followed by CD19, CD22, and CD74 appear to be valid targets for immunotherapy (17, 18). While CD20 is a non-internalizing antigen, the latter three are internalized and they have or will be tested in trials utilizing antibody drug conjugates that rely on internalization (**Table 1** includes various contemporary approaches in FL).

Development of Monoclonal CD20-, CD19-, and CD22-Specific Antibodies

Rituximab has been the first monoclonal antibody entering clinical practice in a variety of lymphomas of the B-cell origin. Thus, it was no surprise that rituximab has found an undisputed place in the treatment of FL. Other than in aggressive lymphomas, strategies using monotherapy of rituximab with and without maintenance have been established successfully (19–21). Consequently, guidelines like ESMO recommend to start rituximab in patients in need of therapy but with low tumor burden and slow progression (10). With the advent of type II monoclonal antibodies, namely, obinutuzumab the landscape of treatment begins to shift. Obinutuzumab is a glycoengineered, afucosylated anti-CD20 antibody with increased antibody-dependent cellular cytotoxicity and increased antitumor activity by FCγRIII compared with rituximab or ofatumamab (22). In a phase III trial (GALLIUM), patients were randomized 1:1 to receive either obinutuzumab and chemotherapy or rituximab and chemotherapy, followed in responding patients by obinutuzumab or rituximab maintenance for up to 2 years. There, an advantage of obinutuzumab regarding PFS compared with rituximab was shown (23). In 2017, the Food and Drug Administration approved obinutuzumab (GAZYVA, Genentech, Inc.) in combination with chemotherapy, followed by obinutuzumab monotherapy in patients achieving at least a partial remission, for the treatment of adult patients with previously untreated stage II bulky, III, or IV FL, respectively.

Antibody-Based Radio-Immunotherapy

Bexxar (131I-tositumomab) and zevalin (90Y-ibritumomab tiuxetan) have been approved in the US and zevalin also in Europe. Both agents can achieve meaningful responses, as shown by an approximately 75% complete response (CR) rate in patients treated with 131I-tositumomab (24). In some cases, these responses lead to long-lasting remissions. Widespread use of these therapies has been hampered by challenging logistics and the restricted availability outside specialized centers. Thus, among the growing list of therapeutic options for FL, the radioimmunotherapeutics lead a shadowy existence.

Antibody-Based Immunotoxins (ITs)

SGN-CD19B, a PBD conjugated antibody, has shown its best preclinical responses in FL when compared with other B-cell malignancies (25). ITs have the advantage of increased efficacy by reduced toxicity compared with antibody chemotherapy combinations. Whether long-term control of FL like in some patients treated with rituximab monotherapy seen in SAKK 35/98 trial (26) is achievable with antibody-based ITs also has to be seen in future.

HIGH DOSE CHEMOTHERAPY FOLLOWED BY AUTOLOGOUS OR ALLOGENIC STEM CELL TRANSPLANTATION

Transplant concepts found their place in the pre-rituximab era, when relapses were more frequent and swift than after the introduction of the CD20 antibody. Beside recognition of its curative potential transplantation lost ground in the therapeutic algorithm of FL with the introduction of rituximab. Today, it can be regarded consensus to use high-dose chemotherapy followed by autologous stem cell transplantation (HDCT/ASCT) as a salvage treatment. In patients with refractoriness to first-line treatment and transformed lymphomas, this concept should be applied earlier. Allogenic stem cell transplantation has curative potential but carries a mortality risk for patients with FL (16). The benefit of a total reset of the immune system and a graft versus lymphoma effect are undeniable, but the risk involved for the patient is significant. Therefore, a careful upfront risk benefit evaluation should be done. Allogeneic transplantation should be reserved for patients failing of HDCT/ASCT [for an excellent review on

TABLE 1 | Ongoing trials in follicular lymphoma (FL) with immune interventions on clinical.trials.gov.

Spalte1	Study	Condition	Study drug	NCT-ID
1	Sequential intranodal immunotherapy (SIIT) combined with anti-PD1 (pembrolizumab) in follicular lymphoma	Fl and other NHL	Pembrolizumab	NCT02677155
2	Active specific immunotherapy for follicular lymphomas with tumor-derived immunoglobulin idiotype antigen vaccines	Fl and other NHL	Id-KLH vaccine|GM-CSF	NCT00001512
3	Cellular adoptive immunotherapy in treating patients with relapsed or refractory follicular non-Hodgkin's lymphoma	Fl and other NHL	Aldesleukin plus rituximab	NCT00182650
4	BI 695500 vs rituxan first line treatment in patients with low tumor burden follicular lymphoma	Fl and other NHL	Rituximab|BI 695500	NCT02417129
5	Monoclonal antibody CT-011 in combination with rituximab in patients with relapsed follicular lymphoma	Fl and other NHL	CT-011|rituximab	NCT00904722
6	Rituximab with or without yttrium Y-90 ibritumomab tiuxetan in treating patients with untreated follicular lymphoma	Follicular lymphoma	Rituximab|radiation: yttrium Y-90 ibritumomab tiuxetan	NCT02320292
7	Vaccine therapy plus interleukin-2 in treating patients with stage III, stage IV, or recurrent follicular lymphoma	Fl and other NHL	Aldesleukin|autologous tumor cell vaccine	NCT00020462
8	Zevalin. First line in follicular lymphoma	Follicular lymphoma	90Yttrium-ibritumomab tiuxetan + rituximab; rituximab	NCT00772655
15	Phase I dose escalation study of IMMU-114 (anti-HLA DR) in relapsed or refractory NHL and CLL	Fl and other NHL	IMMU-114	NCT01728207
16	Agatolimod (anti-toll 9 receptor), rituximab, and yttrium Y 90 ibritumomab tiuxetan	Fl and other NHL	Agatolimod sodium|radiation: indium In-111 ibritumomab tiuxetan	NCT00438880
17	Radiolabeled monoclonal antibody plus rituximab with and without filgrastim and interleukin-11	Fl and other NHL	Rituximab|yttrium Y 90 ibritumomab tiuxetan	NCT00012298
19	Epratuzumab (anti-CD22) in treating patients with non-Hodgkin's lymphoma	Fl and other NHL	Epratuzumab	NCT00022685
20	Denintuzumab mafodotin (SGN-CD19A) combined with RCHOP or RCHP versus RCHOP alone	Fl and other NHL	Denintuzumab mafodotin|rituximab|chemotherapy	NCT02855359
21	Study evaluating the efficacy and safety of PCAR-019 in CD19 positive relapsed or refractory leukemia and lymphoma	Fl and other NHL	PCAR-019 (anti-CD19 CAR-T cells)	NCT02851589
23	Treatment study of denintuzumab mafodotin (SGN-CD19A) plus RICE versus RICE alone for diffuse large B-cell lymphoma	Fl and other NHL	Denintuzumab mafodotin|rituximab|chemotherapy	NCT02592876
24	Immunotherapy with *ex vivo*-expanded cord blood-derived NK cells combined with rituximab HDCT/ASCT for B-NHL	Fl and other NHL	NK cells|rituximab|chemotherapy|ASCT	NCT03019640
25	Idiotype vaccine for low-grade non-Hodgkin's lymphoma	Fl and other NHL	FavId (Id-KLH) active immunotherapy	NCT00036426
26	Rituxan plus favid (idiotype vaccine) for low-grade non-Hodgkin's lymphoma	Fl and other NHL	Id-KLH	NCT00041730

An overview of trials in FL that are currently examining the role of conventional, e.g., non-cellular interventions (antibodies, vaccines, etc.).

this topic, see Ref. (27)]. In future, allogenic transplant will be seriously challenged by less toxic chimeric T-cell approaches described below.

CHECKPOINT BLOCKADE INHIBITING ANTIBODIES

Like in other lymphomas, PD-1-blocking antibodies have been in FL tried with varying success. To summarize the attempts from the data available at this time, we start with the curious story of pidilizumab. Pidilizumab was developed by CureTech and was later acquired by Pfizer. For the longest time, it was thought that pidilizumab is a PD-1 targeting antibody and the initial clinical trials showed efficacy and tolerablity compatible with a typical PD-1 antibody profile. In a phase II trial, pidilizumab revealed promising activity in FL. From 29 enrolled patients, 19 had an objective response with a CR in 15/29 (52%) and a partial response (PR) in 4/29 patients (14%) (28).

However, when it came to FDA filing for approval, it was found that the binding of pidilizumab was unclear, and the company had to invest further research to clarify the target. Meanwhile, DLL1 has been identified as the genuine target, and it remains to be seen how this anti-DLL1 antibody will integrate into the treatment landscape of FL.

In a pivotal basket trial of relapsed/refractory non-Hodgkin lymphomas, patients were treated with single-agent nivolumab (29). Here, FL showed the highest objective response rates (40%) followed by DLBCL (36%). Interestingly, in the translational part of the study, the malignant FL cells were mostly negative for PD-L1 and PD-L2. These two antigens, considerably the therapeutic targets, were often expressed on bystander cells in

the microenvironment. This fact demands further basic studies to elucidate the mechanism behind checkpoint inhibition in FL.

Regarding checkpoint blockade, it can be expected that a combinational approach of passive immunotherapy (anti-CD20) with anti-PD1 antibodies has potential. At the ASH meeting 2017, first data from a trial combining rituximab with pembrolizumab were shown (30). Here, 30 patients with relapsed FL received rituximab plus pembrolizumab for a total of 16 infusions. With a median observation time of 14 months, no death was noted. The ORR was 67% with a CR rate of 50%. This compares favorably to historical response rates of rituximab of 40%. Currently, a single arm study (NCT03245021) is recruiting and will explore the role of nivolumab in combination with rituximab in first-line therapy of FL. It has to be mentioned the true value of nivolumab may not be determined without a comparator arm of rituximab monotherapy. In the near future, a variety of immunotherapy combination trials will be completed, and it is very likely that the inclusion of checkpoint blockade into standard therapy of FL will improve the outcome of affected patients (for an overview of ongoing trials evaluating novel therapies from 3 to 6, see **Table 1**).

CHIMERIC ANTIGEN RECEPTOR THERAPY (CART)

Data regarding CAR therapy in patients with FL is sparse. Especially, early disease and low-grade FL have not been addressed by clinical trials yet. The CAR T cell products relevant to FL treatment are CD19 re-targeted T-cells. These products include axicabtagene ciloleucel/Yescarta ® and Tisagenleleucel/ Kymriah® that are FDA approved. The best available information for CAR therapy in FL we have at this moment is from patients participating in the Juliet trial. At ASH 2015, Schuster presented the outcome of 14 FL patients with an ORR of 73% at 3 months with 4 CRs, 4 PRs, and 3 progressive disease. Three of the four PR patients converted into CRs by 6 months and the last patient with PR remained in PR for a year before progression of the disease (31). In an updated analysis encompassing 24 patients, an ORR of 53% was published. At a median follow-up of 28.6 months, sustained remissions were observed and 89% of patients with FL who had an initial response (95% CI, 43–98) could maintain the response (20).

The most notable side effects of CART therapies are cytokine release syndromes found across trials in 50–60%, up to 10% severe (grade 4) and neurological toxicities that appear in frequencies from 25 to 30% and are severe (≥grade 4) in approximately 5%. Neurotoxicity seemed to be associated with the CAR construct itself, as JCAR015 showed higher toxicities than other constructs. In a *post hoc* analysis of the Rocket 1, trial factors associated with higher neurotoxicity were the conditioning chemotherapy (Flu/CY or not) with a higher risk odds ratio of 7.23, the bridging chemotherapy (OR 4,68), age below 30 (OR 5.16), and less or equal 2 previous line of therapies (OR 7.24) (21). No association with higher risk was found regarding prior CNS irradiation, prior IT chemotherapy, prior CNS disease, prior allogeneic

TABLE 2 | Ongoing trials chimeric antigen receptor therapy (CART) trials including follicular lymphoma (FL) on clinical.trials.gov.

	Title	Intervention	NCT no.
1	FDG-PET/CT imaging as early predictor of DP	Biological: CART-19 autologous T-cells|radiation: FDG-PET/CT	NCT02476734
2	Treatment of relapsed and/or chemotherapy refractory B-cell malignancy by tandem CAR T cells targeting CD19 and CD22	Biological: anti-CD19/22-CAR vector-transduced T cells	NCT03185494
3	CAR T cell receptor immunotherapy for patients with B-cell lymphoma	Drug: fludarabine|drug: cyclophosphamide|biological: anti-CD19-CAR PBL	NCT00924326
4	Anti-CD22 CAR-T therapy for CD19-refractory or resistant lymphoma patients	Drug: retroviral vector-transduced autologous T cells to express CD22-specific CARs	NCT02721407
5	Memory-enriched CAR-T cells immunotherapy for B cell lymphoma	Drug: CD19.CAR-T cells	NCT02652910
6	Long-term follow-up study for patients previously treated with a juno CAR T-cell product	Genetic: JCAR017|genetic: JCARH125	NCT03436771
7	Competitive transfer of Î ± CD19-TCRz-CD28 and Î ± CD19-TCRz-CD137 CAR-T Cells for B-cell leukemia/lymphoma	Biological: anti-CD19 CAR-T|drug: fludarabine|drug: cyclophosphamide	NCT02685670
8	CAR-T cell immunotherapy in CD19 positive relapsed or refractory leukemia and lymphoma	Biological: PCAR-019 (anti-CD19 CAR-T cells)	NCT02819583
9	CART19 to treat B-cell leukemia or lymphoma that are resistant or refractory to chemotherapy	Biological: CART-19	NCT01029366
10	Treatment of relapsed and/or chemotherapy refractory B-cell malignancy by CART19	Biological: anti-CD19-CAR vector-transduced T cells	NCT01864889
11	Treatment of relapsed and/or chemotherapy refractory B-cell malignancy by tandem CAR T cells targeting CD19 and CD20	Biological: anti-CD19/20-CAR vector-transduced T cells	NCT03097770
12	A safety and efficacy trial of JCAR017 combinations in subjects with relapsed/refractory B-cell malignancies (PLATFORM)	Biological: JCAR017|drug: durvalumab	NCT03310619
13	Study evaluating the safety and pharmacokinetics of JCAR017 in B-cell non-Hodgkin lymphoma (TRANSCEND-NHL-001)	Biological: JCAR017 (lisocabtagene maraleucel) single-dose schedule|biological: JCAR017	NCT02631044

An overview of the contemporary CART trials that include FL patients, with the experimental intervention and the trial accession number in the second and third row.

transplantation, higher ECOG performance status, or prior use of blinatumumab (21).

With the approval of two CART products in relapsed/refractory aggressive B-cell lymphomas and some 13 trials ongoing (**Table 2**), the value of the CART approach in FL should become clearer in the next couple of years. If long-lasting remissions can be achieved, this approach has the potential to displace autologous and allogenic stem cell transplantation in FL.

SUMMARY AND OUTLOOK

Follicular lymphoma represents in the most instances an indolent disease and tolerance of the malignant clone by the immune system is very likely. The mutational load that predicts for immune responses appears not to be exceedingly high in this disease. It is quite likely that immunotherapy with checkpoint blockade inhibitors may not find a place in the early course of the disease. However, more aggressive variants, e.g., grade IIIA and higher of FL may represent better targets and should be explored in this regard. Furthermore, during the often long course of the disease, it is reasonable to assume that the malignant clone acquires additional genomic alterations that could make it more prone to respond to checkpoint blockade inhibitors. Finally, FL that transforms into higher grade B-cell lymphoma has a poorer prognosis than *de novo* high-grade B-cell lymphomas. There, a space for immunotherapy on its own or as an adjunct to a standard therapy could be envisioned. However, with a good variety of therapeutic options at hand the role of immunotherapy in the landscape of treating FL has still to be established.

AUTHOR CONTRIBUTIONS

CR and FS contributed equally.

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REVIEW
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Targeting the Immune Microenvironment in Acute Myeloid Leukemia: A Focus on T Cell Immunity

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Immunotherapies, such as chimeric antigen receptor T cells, bispecific antibodies, and immune checkpoint inhibitors, have emerged as promising modalities in multiple hematologic malignancies. Despite the excitement surrounding immunotherapy, it is currently not possible to predict which patients will respond. Within solid tumors, the status of the immune microenvironment provides valuable insight regarding potential responses to immune therapies. Much less is known about the immune microenvironment within hematologic malignancies but the characteristics of this environment are likely to serve a similar predictive role. Acute myeloid leukemia (AML) is the most common hematologic malignancy in adults, and only 25% of patients are alive 5 years following their diagnosis. There is evidence that manipulation of the immune microenvironment by leukemia cells may play a role in promoting therapy resistance and disease relapse. In addition, it has long been documented that through modulation of the immune system following allogeneic bone marrow transplant, AML can be cured, even in patients with the highest risk disease. These concepts, along with the poor prognosis associated with this disease, have encouraged many groups to start exploring the utility of novel immune therapies in AML. While the implementation of these therapies into clinical trials for AML has been supported by preclinical rationale, many questions still exist surrounding their efficacy, tolerability, and the overall optimal approach. In this review, we discuss what is known about the immune microenvironment within AML with a specific focus on T cells and checkpoints, along with their implications for immune therapies.

Keywords: acute myeloid leukemia, T cells, microenvironment, immunotherapy, tumor antigen

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INTRODUCTION

Acute myeloid leukemia (AML) is a clinically and molecularly heterogeneous disorder. Despites its poor prognosis, the treatment of AML remains largely unchanged over the past several decades with high dose chemotherapy remaining the mainstay of therapy (1). This has created an impetus to explore novel therapeutic approaches, such as immune-based therapies or immunotherapies.

The promise of immunotherapy in AML can be traced back to the graft-versus-leukemia effect seen following allogeneic hematopoietic stem cell transplantation (HSCT) (2). This has led to an interest in other immunotherapies, such as bispecific antibodies, chimeric antigen receptor T cells, tumor vaccines, and immune checkpoint inhibitors (ICIs). The presence of a functional T cell population

is pivotal to the successful application of immunotherapies for the treatment of malignancies. Conversely, a dysfunctional T cell population may represent a novel therapeutic target for ICIs or other treatments that reinvigorate these cells. In this report, we summarize the current data regarding the functional state of T cells in the AML immune microenvironment.

TUMOR MICROENVIRONMENT (TME)

The TME can be defined as the cellular environment in which the tumor exists. This environment is made up of endothelial, stromal, and immune cells and plays a key role in the development, propagation, and survival of cancer (3). Characteristics of the TME vary greatly between cancers and can be strikingly different between patients with the same type of cancer. As an example, in renal cell carcinoma, the TME is composed of potentially dozens of types of infiltrating T cells and myeloid cells and this complex mix of cells has predictive ability on disease outcome (4). Large differences can also be observed in the TME between individual metastatic masses within the same patient, making each mass a potentially distinct environment in terms of immune recognition (5). This diversity between and within patients is likely partially responsible for the variability in outcomes.

The TME of hematologic malignancies is substantially different than that of solid tumors. For leukemia, the bone marrow serves as the sanctuary for the majority of leukemic stem cells, but secondary lymphoid organs, such as lymph nodes and the spleen, are also considered to be components of the TME. In lymphomas, the TME appears to be on a spectrum between solid and liquid cancers. The immune microenvironment has been well described in several hematologic malignancies, including Hodgkin lymphoma (HL), acute lymphoblastic leukemia, chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (6–12), but less is known about the microenvironment in AML. Compounding our lack of knowledge is the fact that many studies focused on the immune environment in AML lack sufficient patient numbers or molecular characterization to correctly interpret the reach of observations made in AML, a disease of great molecular heterogeneity. With these caveats in mind, we attempt to summarize the immune observations in AML with a focus on human observations wherever possible.

T CELLS

T cells are an abundant and important component of the immune microenvironment. Within the solid tumor literature, they are termed tumor-infiltrating lymphocytes, and the amount within the TME has been shown to be associated with prognosis (13–19). Similarly, in AML, a study of 66 patients showed an association between bone marrow T cells and clinical outcome. Patients with high percentages of total lymphocytes in their marrow (above 10% of total bone marrow cells) and high T cell percentages (>78.5% of total lymphocytes) were reported to have increased overall survival. The association between survival and T cells was independent of FLT3 and cytogenetic status. High T cell populations also correlated with leukemia-free survival [time between complete remission (CR) and relapse]. These associations were not observed for other parameters such as NK cells or peripheral blast counts (20). While this study needs to be repeated and expanded, it hints at a potential influence of T cells on AML, as observed in other tumor types.

Cytotoxic T lymphocytes (CTLs) are $CD8^+$ T cells that play an important role in antitumor immune responses. They are capable of inducing target cell apoptosis through the secretion of granzyme B and perforin. In addition, they have the capacity to secrete large amounts of IFN-gamma, which has an immunostimulatory effect.

$CD4^+$ T cells can differentiate into a variety of effector cell types depending on the cytokines present within the microenvironment. They can also act as helper cells that mediate tumor cell killing though B cells, NK cells, and CTLs. In addition, $CD4^+$ T cells can differentiate into T-regulatory cells (Tregs), which are important in maintaining self-tolerance. This is accomplished *via* immunosuppressive mechanisms that lead to the inhibition of proliferation and cytokine production of other T cells (21). Elevated numbers of Tregs in solid tumors have been associated with worse outcomes and are attributed to assisting the tumor with immune escape (22).

Numbers, Distribution, and Activation Status of Immune Cells in AML

There is a paucity of studies detailing the frequency and distribution of T cell within patients with AML, with no clear consensus from the limited number of studies available. One of the most comprehensive phenotypic analyses to date was performed by Le Dieu et al. (23). Comparing the peripheral blood and bone marrow from previously untreated patients with AML ($n = 36$) to that of healthy volunteers ($n = 17$), they were able to characterize the genotype and phenotype by immunophenotyping, T cell receptor (TCR) clonality assessment and gene expression profiling. While the T cell percentages in the bone marrow appeared analogous between the two groups ($p = 0.58$), they found a significant increase in the absolute number of total T cells circulating in the patients with AML (830×10^6 cells/L in healthy versus $1{,}900 \times 10^6$ cells/L in AML, $p < 0.05$). In addition, within this increased number of T cells, there was a higher proportion of $CD8^+$ cells demonstrated by the CD4:CD8 ratio (2.5 healthy versus 1.69 AML, $p = 0.05$), and the $CD8^+$ population was less clonal (more diverse) compared with the CD4 population (23). This increase in $CD8^+$ T cells was also observed by another group that showed a higher number of $CD8^+$ cells at diagnosis compared with age-matched healthy donors. Interestingly, this increase normalized following the administration of chemotherapy (24). Le Dieu et al. also demonstrated aberrant T cell activation *via* gene expression profiling (23). This correlates with flow cytometric data from another group that demonstrated an increase of activation markers (HLA-DR, CD69, CD71, and CD57) on T cells at diagnosis when compared with healthy controls (25). Numerous studies have documented elevated numbers of Tregs in patients with AML, which is covered more extensively later in this review (26–30).

The above results are in contrast to groups that have found no differences in the numbers of circulating lymphocytes between patients with AML and healthy individuals (31, 32). There are several explanations for these conflicting results. AML is a phenotypically and genotypically heterogeneous disease, and these studies may not have had sufficient patient numbers to address this heterogeneity. In addition, newly diagnosed patients have different past medical histories, which is likely to influence the overall balance of cells in the immune system.

Function

The concept of T cell dysfunction, and more specifically, T cell exhaustion was first detailed in chronic viral infections and can be defined as the reduced ability of T cells to proliferate and produce cytokines (33–38). Exhausted T cells can be phenotypically identified by increased expression of several inhibitory receptors [CD244, PD-1, CD160, T cell immunoglobulin domain and mucin domain 3 (TIM-3), LAG-3, and others]. This concept has been further expanded as a possible explanation for immune escape by both solid and hematologic malignancies.

Similar to the conflicting phenotypic results discussed earlier, there is currently no consensus regarding the functional status of T cells in AML. Inconsistencies in functional results may be related to different approaches in defining T cell function. In addition, most assays assess bulk T cell function and may not reveal dysfunction related to antigen-specific T cells that are more central to tumor clearance.

There is some evidence suggesting that T cell dysfunction is present at the time of disease diagnosis. One study found that T cell responses, based on proliferation and cytokine production, following both CD3 stimulation and co-stimulation with anti-CD28, appear impaired. However, this defect in T cell responses could be partially overcome following stimulation with PMA and ionomycin, suggesting dysfunction may be related to the strength of the stimulus. Even in this setting of strong stimulation, the ability of $CD4^+$ T cells to produce IFNγ was defective. This impairment of $CD4^+$ T cells to produce IFNγ was seen in samples obtained at the time of clinical diagnosis but interestingly this impairment was not present at time of relapse (39). The observed decrease in IFNγ production is in agreement with another report that found reduced levels of IFNγ circulating in the serum of patients with untreated AML compared with healthy controls (40). Using gene expression profiling, Le Dieu et al. found that T cells from patients with AML exhibited global differences in transcription compared with healthy controls (23). Some of the differentially expressed genes were involved in actin cytoskeletal formation. They further demonstrated with an *in vitro* assay that the T cells were impaired in their ability to form immune synapses that are critical for optimal T cell activation (23).

It is likely that T cell function changes based on a patient's treatment phase and disease status. Using a syngeneic murine model, Zhou et al. were able to identify a subset of $CD8^+$ T cells based on phenotype that was deficient in cytokine production and increased in frequency during AML progression (41). This supports the contribution of immune suppression in disease progression and may represent a therapeutic target, such as blocking the checkpoint molecules expressed by these cells. Lichtenegger et al. studied patients who were in CR and found that while their $CD4^+$ T cells were reduced in number; their proliferative ability was preserved (42). Conversely, another group found that during periods of chemotherapy-induced leukopenia, T cells were both low in count and also functional capacity following stimulation with an anti-CD3 antibody (43). Functional impairment could be circumvented with optimal co-stimulation through CD28 and led to proliferation values that were similar to healthy controls.

In summary, several defects in T cell function, including proliferation and cytokine production, have been associated with AML. Due to the large mutational and phenotypic variability in AML, further studies will continue to identify T cell defects in patients with AML and identify mutational profiles that result in specific immune landscapes.

MECHANISMS OF T CELL DYSFUNCTION IN AML

Tregs in AML

As mentioned earlier, numerous studies have documented elevated numbers and function of Tregs in patients with AML (26–30, 44). The majority of these studies investigated Tregs within peripheral blood, while a few compared Treg frequencies in bone marrow as well. Shenghui et al. compared the blood of patients with newly diagnosed AML ($n = 182$) to age-matched healthy volunteers ($n = 20$). They found that the frequency of Treg cells in the peripheral blood from patients with AML was higher compared with that from the healthy volunteers (9.2 versus 5.44%, $p < 0.001$). In addition, within the same patient cohort, they found a higher Treg frequency in the bone marrow compared with their own peripheral blood (11.9 versus 9.19%, $p < 0.001$). This group also observed that bone marrow-resident Tregs were more immunosuppressive than Tregs from the peripheral blood, based on Treg-induced effects on $CD4^+$ T cell division (65.3% undivided $CD4^+$ cells versus 58.85% undivided $CD4^+$ cells, $p < 0.05$) (28). These findings suggest a preferential accumulation of Tregs both in number and function in the bone marrow of patients with AML, supporting the idea of the bone marrow as an immune privileged niche. By contrast, Wang et al. found similar proportions of Tregs in the bone marrow and peripheral blood of patients with AML (30).

While the number of Tregs appears to be increased at diagnosis, their numbers vary during the course of treatment. Lichtenegger et al. showed reduced Tregs at time of remission but these numbers increased during cytotoxic maintenance therapy (42). Ersvaer et al. showed that Treg frequency decreased from diagnosis compared with after treatment but remained elevated relative to healthy controls (26). Kanakry et al. showed that Tregs are elevated during early recovery from induction therapy and that they remained functionally immunosuppressive (27).

Several studies have suggested a prognostic effect related to the presence of Tregs (28, 29). Mechanistically, this would suggest that Tregs are being utilized by the tumor to suppress normal immune cells. This is supported by a murine model of AML which

demonstrated that the frequency of Tregs was increased *in vivo* and that these Tregs had a suppressive function on effector T cells *in vitro*. When the Tregs were depleted, the *in vitro* function of the effector T cells improved, along with treatment outcomes. This suggests that the recruitment of Tregs may be a mechanism of disease persistence (45). These results mirror multiple human studies showing that not only are Tregs elevated at the time of diagnosis of AML but that this increase is associated with a worse prognosis (28, 29).

By contrast, a retrospective study investigating the presence of Tregs in AML during induction chemotherapy demonstrated that higher numbers of these cells in the early phases were associated with better CR and overall survival rates (46). Similarly, a recent systematic review revealed a beneficial effect of increased numbers of Tregs after allogeneic HSCT for AML (47). This prognostic benefit is likely related to the success of the transplant, since immunosuppressive effects of Tregs have a role in minimizing graft-versus-host disease (GVHD).

Potential Effects of Immune Suppressive Myeloid Subsets on T Cells in AML

Myeloid subsets present in tumors, including macrophages, dendritic cells, and specific immature myeloid subsets known as myeloid-derived suppressor cells (MDSC), have been shown to inhibit T cells. Macrophages can be polarized by signals from their environment into two major subsets, called M1 and M2 macrophages. M1 macrophages are activated by pathogen-associated molecules such as LPS and pro-inflammatory cytokines such as IFNγ and are involved in pathogen clearance and acute inflammatory reactions. M2 macrophages are involved in the resolution of injuries and inflammation and are associated with chronic inflammation. M2 macrophages produce soluble factors such as transforming growth factor beta (TGFβ), arginase, IL-10, and vascular endothelial growth factor that can remodel the local matrix, increase vasculature, and furthermore, inhibit T cell function. There are few studies on the impact of macrophage subsets in AML. One confounding factor for measuring macrophage subsets is that many of the markers used to commonly identify macrophages are often expressed by AML blasts, making the definitive distinction of macrophage or tumor cell difficult.

Acute myeloid leukemia blasts have been shown to differentiate monocytes from healthy donors into an M2-like phenotype in transwell coculture assays indicating that polarization can be achieved by soluble factors alone (48). A small increase in the M2 macrophage population (defined as $CD14^+CD163^+CD206^+$) in the bone marrow of patients with AML ($n = 8$) compared with healthy donors ($n = 9$) has also been reported (49). In the same study, using mouse models of retroviral oncogene-induced AML, greater numbers of macrophages that promote tumor cell line division were found compared with macrophages from control animals (49). While this study showed a potential for M2 macrophages to support tumor growth in mice, it did not measure the effects of macrophages on T cells.

Myeloid-derived suppressor cells are cells of the myeloid lineage associated with chronic inflammation and cancer. These cells can be divided into two broad categories, polymorphonuclear (PMN-MDSC) and monocyte (M-MDSC), which are phenotypically more similar to granulocytes or monocytes, respectively. These cells can suppress T cell responses though various mechanisms, including the secretion of indoleamine 2,3-dioxygnenase (IDO), arginase, TGFβ, and IL-10. MDSCs can also suppress antigen-specific T cell function by nitrosylation of major histocompatibility complex (MHC)–peptide and TCR complexes, which interrupts T cell target recognition (50). MDSC have also been shown to express immune checkpoint ligands such as PD-L1 that can suppress T cell responses *in vitro* (51). Like macrophage subsets, which have been studied in CML and B cell neoplasms, there is little information regarding MDSCs in AML (52, 53). M-MDSC (defined as $CD11b^+CD14^{+/-}$ HLA-DR^-CD15^-) and PMN-MDSC (defined as $CD11b^+CD14^-$HLA-DR^-CD15^+) are elevated in the blood of patients with AML (54). Coculture of AML cells with healthy PBMC has been shown to induce the expansion of a population of immature myeloid cells with an MDSC phenotype that can suppress T cell proliferation and cytokine production (54). There is also an association between Tregs and MDSC numbers in myelodysplastic syndrome (MDS) that correlates with a high risk of transformation to AML, indicating a potential role for MDSC in AML progression (55). While there are studies demonstrating a role for MDSCs in suppressing T cell function in AML, there are also studies showing that they may play a lesser role in this disease. As an example, depletion of MDSCs failed to restore T cell function in a cell line transfer-based mouse model of AML (56). It is clear that the potential impact of both macrophage subsets and MDSC on the function of T cells in the AML microenvironment is not fully established and should be a focus of future studies.

Soluble Factors

Soluble factors, such as enzymes and cytokines, may help tilt the TME from hostile to supportive for tumor cells by suppressing T cell function. *In vitro* studies suggest the T cell dysfunction seen in AML may be the result of blasts manipulating these soluble factors within the microenvironment (48, 57, 58). Orleans-Lindsay et al. obtained supernatants from both AML cell lines and primary patient samples and then cocultured with isolated T cells. These T cells were unable to proliferate in response to mitogenic or alloantigen stimulation but maintained their cytolytic function (48). Interestingly, when the supernatant was removed, there was partial restoration in the T cell response to mitogenic stimulation (48). Mussai et al. elucidated secretion of arginase II as a specific mechanism of AML blasts creating an immunosuppressive microenvironment (58). They first showed that arginase II activity is significantly raised in the plasma of patients with AML compared with healthy controls (9.9 versus 1.1 μmol; $p = 0.0001$). Furthermore, they showed that when T cells were cultured *in vitro* with the plasma of patients with AML, there was reduced T cell proliferation, which could be relieved *via* arginine replacement. In addition to having a directly immunosuppressive effect on T cells, they showed that AML blasts directly polarize monocytes to an M2-like phenotype, further promoting an immunosuppressive microenvironment (58).

Indoleamine 2,3-dioxygnenase is an enzyme that catalyzes the oxidation of tryptophan to *N*-formylkynurenine. This enzyme is highly expressed in macrophages and activated dendritic cells.

The breakdown of tryptophan in the local environment inhibits the proliferative capacity and differentiation of $CD8^+$ T cells (59). IDO activity has been shown to result in conversion of $CD4^+$ T cells into Treg cells as well as boosting the suppressive capacity of Tregs (60, 61). There is some evidence pointing to a potential role for IDO in AML. First, elevated systemic levels of kynurenine are detectable in patients with AML and have been shown to negatively correlate with overall survival in patients with intermediate risk disease (62, 63). IDO has been measured directly in AML blasts both constitutively and after exposure to IFNγ, indicating that the tumor itself may be responsible for the observed systemic kynurenine levels (64, 65). IDO-expressing AML cells have been shown to direct the conversion of T effector cells into Tregs and, moreover, this effect can be blocked by the addition of the IDO inhibitor, 1-methyl-tryptophan (66). Mansour et al. found a direct correlation between blast IDO expression and an increased percentage of Tregs in patients with AML (67). Therefore, it is possible that the increased Treg population and function seen in AML may be a direct result of IDO expression.

Immune Checkpoints

Naïve T cells are activated following the mediation of two signals. The first signal is through binding of the antigen-dependent TCR to the MHC molecule on antigen-presenting cells (APCs). The second signal is a co-stimulatory signal also provided by the APCs. The prototypical co-stimulatory molecule is CD28 on T cells with its cognate ligands, CD80 and CD86 expressed on APCs.

Inhibitory checkpoints are molecules in the immune system that function to fine-tune or turn off an immune response. These molecules initiate intracellular signaling events that interrupt activation cascades, thereby leading to decreased T cell proliferation and cytokine production. This process is critical for the establishment and maintenance of peripheral tolerance during normal immune responses. Cancer cells can take advantage of this system by expressing the ligands of these checkpoint receptors to turn off the immune system and avoid destruction. Therefore, blocking interactions between checkpoint molecules and ligands might potentially reverse the tumor effect. The most extensively studied checkpoint molecules are members of the CD28 family, specifically, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein 1 (PD-1). ICIs have received FDA approval for the treatment of melanoma, lung cancer, kidney cancer, head and neck cancer, bladder cancer, colorectal cancer and HL (68). HL is of particular relevance because it is a hematologic malignancy that had previously been shown to overexpress the ligands for PD-1 (68). This established a biological basis for the use of PD-1 blockade therapy in HL and now serves as a model for other hematologic malignancies. The use of ICIs after allogeneic hematopoietic stem-cell transplant has been explored with promise as ICIs may be expected to increase or reactivate a favorable graft-versus-leukemia response (69). However, this approach has also been taken with great caution, given the possibility of inducing GVHD. While careful consideration is warranted, initial results indicate that administration of CTLA-4 blockade antibody is possible with tolerable side effects in many cases (70). Importantly, clinical responses were observed, including in AML patients. Clinical trials will continue in the post-allogeneic transplant setting and will yield interesting clinical and mechanistic results [for a concise review of this topic, see Ref. (71)].

Cytotoxic T-Lymphocyte Antigen-4

Cytotoxic T-lymphocyte antigen-4 normally resides in the cytoplasm in resting T cells and is expressed on the surface of $CD4^+$ and $CD8^+$ T cells following activation. CTLA-4 shares the same ligands with CD28, CD80, and CD86 expressed on APCs, and represents a key mechanism for the immune system to halt unnecessary or inappropriate T cell activation. By simultaneously outcompeting CD28 and initiating an inhibitory signal, CTLA-4 downregulates TCR activation (72–74). Conversely, CTLA-4 is constitutively expressed on Tregs and provides an activation signal for these cells (75).

CD80 and CD86 have been shown to be upregulated on AML blasts (76–79). Direct engagement with CTLA-4 on normal T cells by these ligands may have the ability to suppress effector T cells. In preclinical models, blockade of CTLA-4 leads to enhanced T cell responses against AML (77, 80). In addition, Laurent et al. has shown that CTLA-4 is constitutively expressed on the surface of AML blasts in patients at the time of diagnosis and in patients with disease resistant to chemotherapy. Engaging CTLA-4 with CD80 and CD86 ligands was able to induce killing of leukemic cells (81, 82).

Based on data from treatment of solid tumors, this effect is likely mediated by both enhancement of effector T cell activity and inhibition of Treg function. The most compelling data to date supporting the role of CTLA-4 in the treatment of patients with AML comes from a phase 1 multicenter study exploring the role of ipilimumab, a CTLA-4 specific ICI, in patients with recurrent hematologic cancer after allogeneic HSCT (70). Twelve of the 28 patients treated had AML. Twenty-two patients received the escalated dose of 10 mg/kg of ipilimumab. Of the five patients who experienced a CR, all of them had AML. Interestingly, four of these patients had extramedullary disease (three with leukemia cutis and one with a myeloid sarcoma). In addition, the patients who responded had fewer circulating Tregs in their peripheral blood following initiation of treatment compared with those who did not respond. These data not only suggest that CTLA-4 blockade may induce a dormant graft-versus-leukemia response but also supports the concept that extramedullary AML may be immunologically distinct compared with AML isolated to the bone marrow and peripheral blood.

Programmed Cell Death Protein 1 (PD-1)

PD-1 is expressed on the surface of activated T cells. Its ligands, PD-L1 and PD-L2, are expressed on a wide variety of normal immune cells including T cells, monocytes, and dendritic cells. Similar to CTLA-4, when PD-1 and PD-L1/PD-L2 interact, an intracellular signaling cascade is initiated that inhibits T-cell activation (83). As a way of limiting inflammatory responses and preventing tissue damage, most cells are capable of upregulating PD-L1/PD-L2 in the setting of both type 1 and type 2 interferons (α, β, and γ) (84–86). In addition, similar to CTLA-4, PD-1 can be expressed on Tregs and enhance their function (87).

Many cases of AML express PD-L1 and/or PD-L2 and these ligands can be further upregulated in the presence of activated T cells, primarily by the production of IFNγ (88–90). Whether these ligands are constitutively expressed by the leukemia cells or it is an adaptive response to immune pressures, expression by AML blasts has been shown to be associated with a poor prognosis (91). Consistent with this, PD-1 expression has been found to be significantly higher in patients with AML at relapse compared with healthy controls (39). Another study investigating expression levels of activation markers on T cells showed an increase in the percentage of PD-1-positive $CD8^+$, but not $CD4^+$ T cells, in the blood at diagnosis and after attaining a remission when compared with healthy controls (92). These results establish T cell activation in AML but larger studies are required to carefully identify the functional impact of PD-1 expression on T cells in patients with AML.

Murine models have shown the immunosuppressive capabilities of PD-1 within AML. PD-1 knockout mice injected with an AML cell line had slower AML progression and longer survival compared with wild-type mice (56). Blockade of PD-1 in this model was shown to result in lower AML burden and longer survival than control mice. These data implicate PD-1 as a mechanism of immune escape and represent a therapeutic target (93).

In a phase 1b/2 trial, adult patients with relapsed or refractory AML received treatment with the PD-1 ICI, nivolumab, and azacitidine, a hypomethylating agent, in combination. Of the 53 patients treated, 11 (21%) achieved a CR or CR with incomplete hematologic recovery. The rationale for combining anti-PD-1 therapy with a hypomethylating agent originates from data showing upregulation of PD-L1 and PD-L2 mRNA in CD34-positive cells from patients with AML following treatment with a hypomethylating agent and is discussed in further detail below (94).

T Cell Immunoglobulin Domain and Mucin Domain 3

T cell immunoglobulin domain and mucin domain 3 is a negative regulatory receptor expressed on $CD4^+$ and $CD8^+$ T cells, Tregs and dendritic cells. There have been several binding ligands that have been identified for TIM-3, including HMGB1, phosphatidylserine, and galectin-9 (Gal-9) (95). Interaction of TIM-3 on Th1 $CD4^+$ T cells results in death of the T cell, thus limiting IFNγ-dependent immune reactions (96). Co-expression of TIM-3 and PD-1 on tumor-infiltrating T cells in solid tumor models marks an exhausted T cell population that can be reactivated if both PD-1 and TIM-3 are blocked (97). TIM-3 and its ligand Gal-9 have been identified as a potential target being expressed on AML blasts and leukemic stem cells (98). Focusing on T cells in AML, there is a reported increase in the percent of TIM-3 expressing $CD8^+$ T cells circulating in the blood compared with healthy donors (5.90 ± 4.91 versus $0.96 \pm 0.54\%$) (99). Although a small study, there appears to be an association with a high percentage of TIM-3 expressing T cells in patients with AML who relapse after allogeneic HSCT compared with those who remain in extended remission, indicating that there is a role for functional T cells in killing AML cells. TIM-3 and PD-1 double positive T cells isolated from the blood of patients with AML failed to produce cytokines after stimulation with wither mitogens or stimulation thought the TCR (100). In a murine model of AML, T cells co-expressing PD-1 and TIM-3 were found to have reduced production of IFNγ, TNFα, and IL-2. Blocking either of these receptors individually was not sufficient to restore function but combined blockade yielded increased tumor rejection and improved survival (41). These observations have resulted in a clinical trial with three arms; decitabine plus anti-PD-1, decitabine plus anti-TIM-3 and a combined arm with decitabine plus PD-1 and TIM-3 blockade (NCT03066648). The accumulation of data to date indicates that TIM-3 will continue to be a promising target on both AML cells and tumor-associated T cells.

T Cell Immunoglobulin and Immunoreceptor Tyrosine-Based Inhibitory Motif Domain (TIGIT)

T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain is a co-inhibitory receptor expressed on activated T cells, Tregs and NK cells. Like TIM-3, CTLA-4, and PD-1, TIGIT has multiple ligands. Engagement of TIGIT occurs by binding CD155, CD112, or CD226 (DNAM-1) (101, 102). TIGIT has been shown to be upregulated on $CD8^+$ T cells in AML and is associated with primary refractory disease and relapse post-transplant (103). A small increase in a population of $PD\text{-}1^+TIGIT^+CD8^+$ T cells has been observed in the blood of patients with AML when compared with healthy controls. This population produces slightly less IFNγ and TNFα compared with the same population isolated from healthy donors when stimulated with anti-CD3 and anti-CD28 (104). The ligands CD155 and CD112 appear to be expressed, possibly at elevated levels, in AML blasts (103, 104). Therefore, TIGIT might contribute in mediating functional abnormalities of T cells in the AML microenvironment. As with other ICIs, studies on the potential therapeutic use of TIGIT blockade are promising and will undoubtedly be the focus of future research.

When used alone, ICIs may permit the immune system to perform its normal function of tumor clearance. When used in combination, ICIs have the additional potential of enhancing the effect of other therapies, specifically other immunotherapies.

T CELL TARGETS IN AML: ANTIGENS AND VACCINES

Clinical responses to immune checkpoint blockade or vaccines require tumor antigens that can be recognized by T cells. These antigens can be from various sources, including novel epitopes from non-synonymous coding mutations in genes, developmentally regulated genes with poor tolerance such as Cancer Testis antigens (CT antigens) or virally associated epitopes.

A major class of antigens associated with tumors is derived by DNA mutations. Recognition of these "*de novo*" epitopes requires several events to occur. First, the mutation must code an amino acid change (non-synonymous mutations). This mutation must then be expressed at the RNA and protein levels, which

would not occur for all mutations detected by DNA sequencing. The new peptides then need to bind MHC molecules in order to be presented to T cells. Finally, the gene with the mutation must be expressed at sufficient levels by the tumor cells. It is currently believed that antitumor responses from the use of ICIs are related directly to the number of non-synonymous coding mutations present in the tumor. High mutational burden, including those induced by microsatellite instability, has been shown to correlate with response to immune checkpoint blockade (105–107). This is not absolute, however, as not all highly mutated tumors respond and some patients with low mutational burden are able to mount a response. This indicates that, while mutational burden is a major factor involved in response to ICI, there are other factors involved. Recent studies by Schreiber and others have revealed that even when there are hundreds or thousands of DNA mutations in a tumor, only a small number may meet all the abovementioned criteria (108–110). Numerous recurrent mutations and drivers associated with AML have been identified (111, 112). Overall, AML is thought to be of low mutational burden, falling in the lowest quarter of cancer types and therefore may be predicted to respond poorly to ICI therapy (113). There are, however, specific AML subsets that might yield high epitope expression. These subsets include a p53 loss of function with higher mutational events on average, or a complex karyotype

TABLE 1 | Ongoing clinical trials using checkpoint inhibitors in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).

Clinical trial identifier	Checkpoint target	Disease subsets	Combined therapy	Phase, status
NCT03381118	PD-1	Elderly AML	Cytarabine, haploidentical donor PBSC	2, recruiting
NCT01096602	PD-1	AML	DC vaccine	2, active not recruiting
NCT01822509	CTLA-4 or PD-1	Heme malignancy including AML post-allo transplant	None	1, recruiting
NCT01919619	CTLA-4	Post-auto SCT leukemia	Lenalidomide	1, recruiting
NCT01953692	PD-1	MDS	None in MDS arm	1, active not recruiting
NCT02117219	PD-L1 PD-L1 + CTLA-4	MDS	Azacitidine	1, recruiting
NCT02275533	PD-1	AML (remission)	None	2, recruiting
NCT02397720	PD-1 PD-1 + CTLA-4	AML	Azacitidine	2, recruiting
NCT02464657	PD-1	MDS + AML	Idarubicin + cytarabine	1/2, recruiting
NCT02530463	PD-1 CTLA-4 PD-1 + CTLA-4	MDS + AML	Azacitidine	2, recruiting
NCT02532231	PD-1	AML	None	2, recruiting
NCT02599649	PD-1	MDS	KIR2DL1/2L3 azacitidine	2, active not recruiting
NCT02708641	PD-1	AML elderly	None	2, recruiting
NCT02768792	PD-1	R/R AML	Cytarabine	2, recruiting
NCT02771197	PD-1	High-risk AML not eligible for hematopoietic stem cell transplantation (HSCT)	Fludarabine Melphalan HSCT	2, recruiting
NCT02775903	PD-L1	MDS + AML	Azacitidine	2, active not recruiting
NCT02845297	PD-1	AML + R/R AML	Azacitidine	2, recruiting
NCT02846376	CTLA-4 ± PD-1	MDS + AML	None	1, recruiting
NCT02890329	CTLA-4	MDS + AML	Decitabine	1, recruiting
NCT02935361	PD-L1	MDS, recurrent AML	Guadecitabine	1/2, recruiting
NCT02936752	PD-1	MDS	Entinostat	1, recruiting
NCT02953561	PD-L1	AML	Azacitidine	1/2, recruiting
NCT02981914	PD-1	AML, MDS	None	1, recruiting
NCT02985554	PD-1	Post-allo SCT leukemia	None	1, recruiting
NCT02996474	PD-1	R/R AML	Decitabine	1, recruiting
NCT03059485	PD-L1	AML, remission	DC/AML fusion vaccine	2, recruiting
NCT03066648	PD-1 TIM-3 PD-1 + TIM-3	MDS + AML	Decitabine	1, recruiting
NCT03092674	PD-1	AML or high risk MDS	Azacitidine, cytarabine, decitabine, and midostaurin	2/3, recruiting
NCT03094637	PD-1	MDS	Azacitidine	2, recruiting
NCT03146468	PD-1	Post-allo SCT leukemia	None	2, recruiting
NCT03154827	PD-L1	AML	CXCR4	1b/2, recruiting
NCT03259516	PD-1	MDS	Azacitidine Cytarabine Sildenafil Melphalan	1/2, recruiting
NCT03286114	PD-1	MDS, AML, ALL	None	1, recruiting
NCT03291353	PD-1	refractory AML	None	1, recruiting
NCT03358719	PD-1	MDS, AML, chronic myelomonocytic leukemia, and refractory anemia	DEC-205/NY-ESO-1 CDX-1401 decitabine poly OC:C	1, recruiting
NCT03390296	PD-L1 OX40 4-1BB	AML	Azacitidine Gemtuzumab Ozogamicin Glasdegib	2, recruiting
NCT03395873	PD-L1	AML	Decitabine	1, recruiting

with multiple translocations. Another important issue regarding mutations as targets in AML is the diverse clonality of the disease (114). This diversity at the mutational level in a single patient has been associated with resistance to chemotherapy and may present a similar issue for immunotherapies because one clone expressing an immunogenic epitope may not represent the entire tumor.

Expression of developmental antigens has long been recognized in AML. These proteins are not expressed or are expressed at low levels in normal adult tissues. Expression of antigens such as RAGE, MAGE, WT1, and NY-ESO-1 has been identified and studied in AML, and their expression has been associated with improved clinical outcome (115). These proteins are often regulated at the level of promoter methylation and are often expressed in cancers due to defects in epigenetic regulation (116). DNA methyltransferase inhibitors, such as azacitidine and decitabine, may increase expression of these antigens and therefore can potentially be used as a method to increase the antigenicity of AML. In AML cell lines, treatment with either of these agents resulted in increased RNA and protein expression of NY-ESO-1, WT1, and MAGE A1, A2, and A3 (117, 118).

The goal of vaccine strategies is to boost the number and activity of tumor-reactive T cells. Vaccines based on tumor-associated antigens have shown immunological responses to the tumor and in the remission setting may result in more prolonged remissions (119). One target for AML vaccines is WT1. WT1 is a zinc finger containing transcription factor important in the development of the kidney and other organs and is named after its association with Wilms tumor (120). WT1 is highly expressed by AML blasts in approximately 90% of patients and WT1-specific T cells have been shown to be present (121–123). A number of vaccine trials using WT1 peptide vaccines [(124) and summarized in Ref. (125)] demonstrated both safety and clinical responses, but larger studies are necessary to identify the scope of these responses. It is important to consider that even if T cells successfully expand through vaccination, they will still face the same suppressive mechanisms as naturally primed T cells. For this reason, strategies combining vaccination with other therapies such as immune checkpoint blockade are more likely to be effective.

INCREASING T CELL RESPONSES TO AML BY TARGETING DNA METHYLATION

Hypomethylating agents are a form of epigenetic therapy and have been used to treat hematologic malignancies such as MDS, chronic myelomonocytic leukemia (CMML) and AML for years. The mechanism of action on tumor cells has been assumed to be due to de-methylation and thus increased expression of tumor suppressor genes (126). While these drugs were first appreciated for their ability to suppress leukemia cell counts, recently their effects on the immune system have gained more attention [reviewed in Ref. (127)]. As described earlier, there has been a substantial body of research showing that hypomethylating agents can increase expression of developmental antigens. There is also evidence indicating direct consequences of hypomethylating agents on T and NK cells. The indiscriminate nature of their effects on gene promoters results in a complex series of positive and negative effects on T cells. FoxP3 is the lineage-defining transcription factor for Tregs and is regulated at the level of the promoter *via* methylation. Treatment of T cells with azacitidine results in stable expression of FoxP3 (128–131). Furthermore, azacitidine treatment has been shown to suppress T cell proliferation and cytokine production, resulting in suppression of GVHD in mice (132). A similar result was shown in patients following HSCT where treatment with azacitidine was associated with an increase in Tregs (133). While this increase in Treg frequency was observed in patients with MDS being treated with azacitidine, the Tregs isolated from these patients had reduced suppressive capacity (134). Treatment of patients with AML or MDS with azacitidine also led to elevated PD-1 expression on T cells by demethylating the PD-1 promoter (135). Likewise, azacitidine treatment increased the expression of a series of other checkpoint molecules, including PD-L1 and PD-L2 in cells from patients with MDS or AML (94).

Additional mechanisms for hypomethylating agents to promote generalized inflammation include reactivation of endogenous retroviruses that are then recognized resulting in an IFN response. This response can then synergize with CTLA-4 blockade to induce antitumor immunity (136, 137). Similarly, expression of costimulatory molecules such as CD80 has been shown to be increased by hypomethylating agents as well, resulting in enhanced antitumor immunity in a mouse model of lymphoma (138). Azacitidine treatment results in expanded antitumor T cell recognition in patients with AML and increased TCR repertoire in patients with MDS (139, 140). As a result of this information several clinical trials have been initiated to investigate the potential of hypomethylating drugs and immune ICIs for AML (**Table 1**).

CONCLUSION

All of the recent advances in tumor immune therapy come from decades of basic immunology research focusing primarily on autoimmunity. In fact, autoimmunity and tumor immunity represent opposite sides of the same coin, one representing an overactive immune system and the other a suppressed immune system. By understanding the immunologic mechanisms that lead to disease, we will be better equipped to treat or prevent these diseases. This is highlighted in solid tumors, where immune therapies such as ICI have led to dramatic cure rates in universally fatal diseases. There is also great interest in using ICIs in AML, as evidenced by the number of clinical trials currently underway (see **Table 1**) (141). Furthermore, by being able to substitute these therapies for traditional cytotoxic therapies, there is a hope of reducing the acute and chronic toxicities associated with chemotherapy. With the development of drugs targeting specific signaling pathways and mutations in AML along with epigenetic modifiers there is great potential in the near future to develop strategies that combine the high rates of response of targeted agents with the

durability of immune therapies. Immunotherapies are just now starting to infiltrate the world of AML and the more we learn about the immune microenvironment, the more successful these therapies will become.

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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Past, Present, and Future of Rituximab—The World's First Oncology Monoclonal Antibody Therapy

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Rituximab is a chimeric mouse/human monoclonal antibody (mAb) therapy with binding specificity to CD20. It was the first therapeutic antibody approved for oncology patients and was the top-selling oncology drug for nearly a decade with sales reaching $8.58 billion in 2016. Since its initial approval in 1997, it has improved outcomes in all B-cell malignancies, including diffuse large B-cell lymphoma, follicular lymphoma, and chronic lymphocytic leukemia. Despite widespread use, most mechanistic data have been gathered from *in vitro* studies while the roles of the various response mechanisms in humans are still largely undetermined. Polymorphisms in Fc gamma receptor and complement protein genes have been implicated as potential predictors of differential response to rituximab, but have not yet shown sufficient influence to impact clinical decisions. Unlike most targeted therapies developed today, no known biomarkers to indicate target engagement/tumor response have been identified, aside from reduced tumor burden. The lack of companion biomarkers beyond CD20 itself has made it difficult to predict which patients will respond to any given anti-CD20 antibody. In the past decade, two new anti-CD20 antibodies have been approved: ofatumumab, which binds a distinct epitope of CD20, and obinutuzumab, a mAb derived from rituximab with modifications to the Fc portion and to its glycosylation. Both are fully humanized and have biological activity that is distinct from that of rituximab. In addition to these new anti-CD20 antibodies, another imminent change in targeted lymphoma treatment is the multitude of biosimilars that are becoming available as rituximab's patent expires. While

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Abbreviations: ABC, activated B-cell-like; ADCC, antibody-dependent cell-mediated cytotoxicity; ADP, antibody-dependent phagocytosis; BCR, B-cell receptor; BL, Burkitt lymphoma; BR, rituximab with bendamustine; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukemia; CDC, complement-dependent cytotoxicity; CR, complete remission; Cr, chromium; DLBCL, diffuse large B-cell lymphoma; EFS, event-free survival; EU, European Union; FCR, fludarabine, cyclophosphamide, and rituximab; FDA, Food and Drug Administration; FFS, failure-free survival; FL, follicular lymphoma; GCB, germinal center B-cell-like; HCL, hairy cell leukemia; HIS, human immune system; INN, International nonproprietary names; IR, ibrutinib plus rituximab; LPL, lymphoplasmacytic lymphoma; mAb, monoclonal antibody; MAC, membrane attack complex; MALT, mucosa-associated lymphoid tissue; MCL, mantle cell lymphoma; MR, maintenance rituximab; MZL, marginal zone lymphoma; NHLs, non-Hodgkin lymphomas; NK, natural killer; OB, ofatumumab with bendamustine; OKT3, muromonab-CD3; ORR, overall response rate; PD, pharmacodynamics; PK, pharmacokinetics; PMBCL, primary mediastinal large B-cell lymphoma; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; SLL, small lymphocytic lymphoma; WHO, World Health Organization; WM, Waldenstrom macroglobulinemia.

the widespread use of rituximab itself will likely continue, its biosimilars will increase global access to the therapy. This review discusses current research into mechanisms and potential biomarkers of rituximab response, as well as its biosimilars and the newer CD20 binding mAb therapies. Increased ability to assess the effectiveness of rituximab in an individual patient, along with the availability of alternative anti-CD20 antibodies will likely lead to dramatic changes in how we use CD20 antibodies going forward.

Keywords: rituximab, lymphoma, cancer, immunotherapy, monoclonal antibody

INTRODUCTION

Immunotherapies represent a broad and rapidly growing group of therapies having a substantial impact on cancer outcomes. Their strength is in their potential to activate the immune system to specifically target cancer cells without the broadly damaging side effects of many conventional chemotherapeutics. Monoclonal antibodies (mAbs) were among the initial types of immunotherapy approved for anti-cancer treatment and continue to play a pivotal and growing role in current treatment regimens. Newer therapies have built upon the initial success of mAb therapy. An exciting recent example was the Food and Drug Administration (FDA) approval of two chimeric antigen receptor (CAR)-T cell therapies. These therapies provide high complete remission (CR) rates in patients with otherwise untreatable hematologic malignancies and hold great promise for future advancements. CAR-T cell therapies offer a novel strategy involving *ex vivo* modification and subsequent activation of a patient's T-cells, but the specificity of the CAR recognition site and subsequent targeting to tumor cells is enabled by mAb technology. Therefore, CAR-T cell therapies and most other immunotherapies rely on mAbs directly or indirectly to target specific antigens on cancer cells. Understanding how best to apply and monitor mAbs and mAb technology is therefore critical for the future success of immuno-oncology.

The first mAb implemented in oncology, and still the most widely used, is the CD20-targeting mAb rituximab. Rituximab is recommended to treat nearly all B-cell non-Hodgkin lymphomas (NHLs). It is most commonly given with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), but also with other chemotherapeutic combinations, with small molecule targeted therapies, as a monotherapy, or as maintenance therapy. Despite its widespread use, there is still much uncertainty regarding the mechanism(s) of action of rituximab *in vivo*. We also lack effective predictive biomarkers to identify which patients will respond to rituximab, and when it is given in combination with other chemotherapeutics, we cannot identify patients who are specifically benefiting from its inclusion. This has become more of an issue now that alternative anti-CD20 mAbs have been FDA approved, adding to the impetus to determine when a patient will not respond or has become resistant to rituximab. Furthermore, as rituximab was the first immunotherapeutic used in oncology, it is also the first to have its patent expire, ushering in a swell of competition from biosimilars. Unlike chemical compounds whose efficacy is more easily compared with the originally approved drug, the intrinsic complexity of biologicals is increased by variability that can arise during manufacturing. This complexity, combined with our currently incomplete understanding of the mechanisms behind rituximab efficacy, means that we will need improved methods for determining if these emerging anti-CD20s are as efficacious as the original. This review covers what is known about rituximab's mechanism(s) of action, activity in various B-cell malignancies, and future directions to optimize the clinical utility of this agent as alternative anti-CD20 antibodies become more prevalent in clinical practice.

The History of Ritixumab

While the general concept of immunotherapies has been around for over a century, effective antibody therapies were not feasible before the ability to generate mAbs using continuously growing cell lines (**Figure 1**). In 1975, Köhler and Milstein generated the first hybridoma cell lines capable of producing mAbs by immunizing mice against sheep red blood cells followed by isolation of B-lymphocytes from the murine spleens and subsequent fusion of those cells with a myeloma cell line (1). The medical and industrial potential of their achievement was quickly realized and has rapidly become a booming biotechnology industry (2).

In 1986, the FDA approved the first mAb for use in a medical application, Muromonab-CD3 (OKT3). OKT3 was developed to treat acute kidney transplant rejection by targeting the CD3 antigen on the T-lymphocytes responsible for the rejection and inducing the death of those cells (3). Oncology mAb therapeutic development is faced with additional challenges, most notably target choice. Optimal targets are universally present on tumor cells but can lead to significant toxicity if their normal cellular counterparts are also targeted.

CD20 is a glycosylated transmembrane phosphoprotein expressed on the surface of developing B-cells, as well as many B-cell malignancies. Because mature plasma cells and B-cell progenitors do not express the protein, depleting B-cells at these intermediate developmental stages generally does not cause permanent side effects. With the limited expression of CD20 among other cell lineages, it was identified as a potential B-cell NHL target for mAb therapy early in the field. Nadler et al. demonstrated a historic proof of principle for mAb immunotherapy in oncology with a preliminary serotheraputic trial in 1980 using an antibody targeted against CD20, designated as Ab 89. The patient, N.B., presented with what was categorized at the time as diffuse poorly differentiated lymphocytic lymphoma that was resistant to standard chemotherapeutics. Although N.B. did not achieve CR, a transient response, measured by a decrease in circulating tumor cells along with an increase in dead circulating tumor cells,

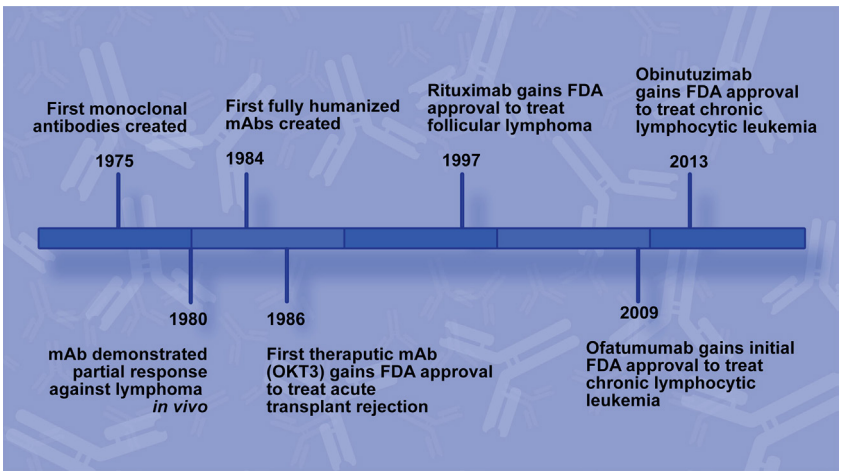

FIGURE 1 | Rituximab development timeline. Key milestones leading to the development of rituximab and additional CD20 monoclonal antibodies (mAbs) for use to treat B-cell non-Hodgkin lymphoma.

provided the first evidence for CD20 as a mAb therapy effective against at least some B-cell lymphomas (4).

Two years before OKT3 approval, another major development in mAb technology was reported. Groups elucidated molecular biology methods for ligating the murine variable region of mAbs with human IgG which generated hybridoma cell lines that produced functional mouse/human chimeric antibodies by retaining the murine variable region but possessing a human Fc region (5, 6). Swapping the murine Fc region for a human one overcame many of the side effects associated with patients developing an adaptive immune response against the therapeutic mAb itself, and it facilitated a more robust immune response against the target due to better binding at the Fc region with human immune effectors. This chimeric technology was the basis for rituximab production, and in 1997 the FDA approved rituximab, brand name Rituxan, for use to treat follicular lymphoma (FL) (7).

Rituximab was created by Ronald Levy for the express purpose of targeting malignant B cells. In 1982, it was made public that his first mAb cancer patient was successfully treated with the mAb, which rapidly lead to the creation of the pharmaceutical company IDEC. Maloney et al. reported the first phase I clinical trials of rituximab, initially named IDEC-C2B8, in 1994, after it had proved effective at killing CD20 expressing cells *in vitro* and in the blood and lymph of macaques (8). Fifteen patients with relapsed NHL were given one of five dosage ranges from 10 to 500 mg/m^2, and six of those patients experienced tumor regression (8). In 1997, results from a phase I/II trial of 20 patients receiving 125, 250, or 375 mg/m^2 of rituximab weekly for 4 weeks were published. This study was the basis for FDA approval of rituximab as well as the now standard 375 mg/m^2 dosage that was used for phase II trials (7). One year later, McLaughlin et al. reported equally impressive benefits of rituximab treatment for patients with relapsed indolent lymphoma, with half of the 166 patients responding to the same four-dose regimen (9).

Due to both its high degree of success as well as its relatively high price, it remained the highest grossing anti-cancer therapeutic through 2016 (10).

Ritixumab Target: CD20

The hematopoietic stem cell lineage has been well studied. A subset of cells from this hierarchy make the commitment to B-cell development with the transition to pro-B cells within the bone marrow. Following this, they mature into pre-B cells, and then immature B-cells possessing a mature B-cell receptor (BCR) region expressed from VDJ rearranged heavy-, and VJ rearranged light-chain genes capable of recognizing specific antigens. It is at this point that the immature B-cells are negatively selected against for self-reactivity (11). Following this, the remaining immature B-cells move from the bone marrow, maturing into follicular, or marginal zone B cells (11). CD20 expression begins at the early immature B-cell stage, but is not expressed before that point, and is not known to be expressed on other normal cells of the body which has made it a relatively safe and effective anti-cancer target (12).

CD20 is a tetra-transmembrane protein with an intracellular N- and C-terminal region and two extracellular loops, generally referred to as the small and large loop, and are the portion of the peptide which is targeted by current therapeutic mAbs (**Figure 2**) (13). Aside from the fact that CD20 is expressed as part of B-cell development, very little is known about its actual biological function. It is known to be involved in store-operated calcium influx, and loss of a cytoplasmic portion of CD20 inhibits activated BCR mediated intake of calcium (14). Also, ectopic expression of CD20 in fibroblasts causes calcium conductance, similar to that of B lymphocytes (14). While it is believed to play a role in B-cell development and activation through calcium influx, it remains unclear if the protein itself is a calcium ion channel, or what other signaling pathways it activates to bring about this function. Despite evidence of its importance in B-cell function, CD20$^{-/-}$ mice harbor no gross phenotype, have normal lifespans,

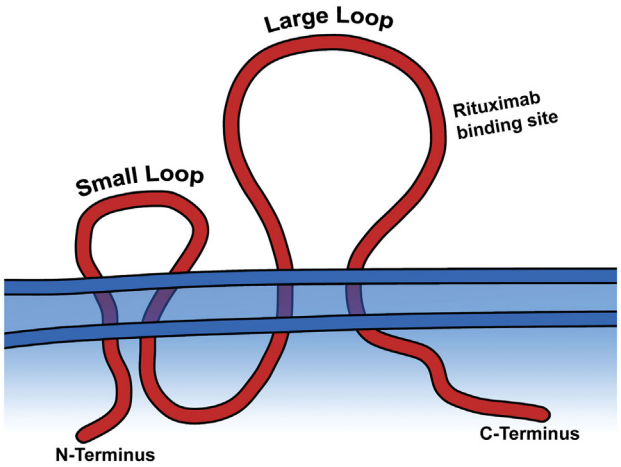

FIGURE 2 | CD20 is a transmembrane protein. The large and small extracellular loops and the general binding site of rituximab are depicted.

reproductive success, and normal infection susceptibility (15). Surprisingly, even B-cell development was mostly normal in $CD20^{-/-}$ mice, with the main finding being reduced calcium response following IgM ligation (15). These animal model data strengthen the theory that CD20 is involved in calcium intake in B-cells, but the biological significance of that role and the mechanisms employed that facilitate the associated calcium influx remain unsolved.

CLINICAL IMPACT OF RITUXIMAB IN B-CELL NHL TREATMENT

Despite the incomplete details on the biological role of CD20, targeting it with rituximab has proven effective for treating a subset of patients in nearly all forms of B-cell NHL. It is frequently given as an initial treatment, either in combination with traditional chemotherapeutics or as a monotherapy. It is also given as maintenance therapy, although benefits of maintenance rituximab (MR) are still unclear for many NHLs. It is rare for B-cell NHLs to be CD20-negative at initial diagnosis, representing only 1–2% of all B-cell lymphomas (16). However, it is more common among B-cell NHL that have relapsed following rituximab treatment, suggesting a selective process toward increased resistance (16). The following section contains current standards of care for the various lymphoma subtypes with historical context from select clinical trials. Current trials and the remaining questions still surrounding the immunotherapy for each specific cancer are also highlighted.

Diffuse Large B-Cell Lymphoma (DLBCL)

Diffuse large B-cell lymphoma is the most common type of NHL, representing 30–40% of all lymphoma diagnoses in Western countries (17, 18). Since the World Health Organization's (WHO's) consensus in 2008, DLBCL has been categorized as germinal center B-cell-like (GCB), activated B-cell-like (ABC), or as unclassifiable lesions which do not fit either profile (19). This classification is based on gene expression profiles that most closely represent the likely B-cell of origin. Along with transcriptional markers, malignancies within these categories share similar genetic aberrations, signaling pathway activation, and clinical outcomes. For example, patients diagnosed with GCB-DLBCL have higher survival, while ABC-DLBCL is more likely to be refractory or relapse. Retrospective studies have shown the benefit of rituximab in both ABC- and GCB-DLBCL, and prospective studies have shown these subtypes to be prognostic for patients treated with either CHOP or R-CHOP (20).

The first phase II single-arm trial for treating DLBCL with rituximab was reported in 2001, studying patients who had aggressive, untreated NHL. Thirty-three patients were included in this trial; the majority of patients (67%) had DLBCL (17). The study showed a 94% overall response rate (ORR) compared with historical CHOP controls (80–90%) and 61% CR compared with historical CHOP controls (44–55%), with only two patients experiencing disease progression by week 24 (21). The increased response rates in this trial were promising, and the use of R-CHOP for these lymphomas demonstrated the feasibility and safety of the regimen in DLBCL treatment.

Approximately 50% of DLBCLs occur in patients over 60, and within that group CR is achieved in only 40–50% of cases when treated with CHOP alone (18, 22). The first phase III trial demonstrating the superiority of R-CHOP in DLBCL, over CHOP alone, was carried out in elderly patients and reported by the GELA group in 2002. This study included patients from 60 to 80 years old who were given CHOP every 3 weeks for eight cycles as tolerated ($n = 196$), or were treated with the same CHOP regimen plus rituximab on day 1 of each cycle ($n = 202$). A CR rate of 76% was achieved with R-CHOP vs. 63% with CHOP (22). A median follow-up of 24 months resulted in an event-free survival (EFS) of 77% for the R-CHOP group and 39% for the CHOP group, reflecting an impressive 42% reduction in risk of events with R-CHOP (22).

An additional study among elderly DLBCL patients was reported in 2006 to look closer at early and late treatment failures and whether MR therapy was beneficial following the successful initial treatment with CHOP or R-CHOP. The study included 415 patients among the four treatment groups with a median follow-up of 3.5 years (23). One important finding from this study was that MR following CHOP resulted in an increased failure-free survival (FFS) compared with only observation following CHOP (23). However, MR following R-CHOP was not significantly different than R-CHOP alone, showing no benefit from MR if rituximab was given during the initial treatment (23).

The phase III MInT trial in 2006 demonstrated the benefits of R-CHOP over CHOP in younger patients, aged 18–60 years, who had a good prognosis. The study involved 824 patients from 18 countries. Individuals who were given R-CHOP had increased EFS (79%) compared with those who received only CHOP (59%) at a median follow-up of 34 months. The R-CHOP group also attained a better three-year OS of 93% compared with 84% with CHOP (24). A 6-year follow-up report by the same group found a 74% EFS among the R-CHOP group compared with 55% with

CHOP alone indicating the addition of rituximab to CHOP provides a durable improvement in response for younger DLBCL patients (25).

While GCB and ABC are the more common DLBCL subtypes, primary mediastinal large B-cell lymphoma (PMBCL) is another important subtype of DLBCL specified by the WHO classification of lymphoid malignancies (19). Although it is uncommon, it constitutes approximately 2–3% of all NHL (26). Like all DLBCL, the addition of rituximab has improved both CR and OS over combination chemotherapy alone, and rituximab is now a part of treatment regimens used to treat PMBCL (26).

It is surprising that there is no definitive consensus on optimal dosage of rituximab, despite 20 years of use (27). This is the case for DLBCL, as well as other lymphomas, but efforts are being made to determine if the standard 375 mg/m^2 is ideal for all patients. Recent findings suggest that dosage may need to be tailored as precision medicine or perhaps increased overall. The SEXIE-R-CHOP-14 trial sought to address the problem that elderly male DLBCL patients had worse outcomes compared with females by increasing the dose of rituximab for elderly males (28). The study showed that increasing the rituximab dose from 375 to 500 mg/m^2, given every 14 days for six cycles, led to a 32.5% increase in PFS and a 30% increase in OS, although OS increase did not achieve significance (28). Interestingly, these survival rates were slightly better than the elderly female patients treated with 375 mg/m^2 who were used as the control group in this study which suggests further dosage improvements could have significant impacts on that population as well (28).

In addition, a recent meta-analysis discovered maintenance therapy with rituximab in DLBCL patients improved EFS and PFS, although OS was not significantly improved. However, there was a sex-based difference found in that study as well, with males receiving more benefits from the MR (29).

These findings highlight the need for a better understanding of how rituximab works, its optimal dose and schedule, and the factors that modulate its efficacy, especially between sexes. With a better understanding of those factors, we can optimize rituximab usage by employing a precision medicine strategy.

Burkitt Lymphoma (BL)

Burkitt lymphoma accounts for 1–5% of adult NHL and is characterized as aggressive lymphoma that is associated with extremely short doubling time caused by MYC dysregulation (17). The disease is usually treated with short-intensive regimens of high-dose cyclophosphamide and methotrexate in combination with vincristine, doxorubicin, and cytarabine, and this has achieved high cure rates in pediatric BL, but a less ideal OS of 64% in adults with the disease (30).

The largest prospective study to date, published in 2014, which spanned from 2002 to 2011 and included 363 patients ranging from 16 to 85 years old demonstrated that the combination immunotherapy was efficacious and feasible, and while the CR rate was not significantly higher than comparable studies without rituximab, OS and PFS were substantially improved (30). Several retrospective studies have attempted to determine rituximab benefits for these patients but were unable to achieve significance (31). However, a recent meta-analysis concluded that there was a significant increase in overall survival when rituximab was given with various chemotherapy regimens compared with chemotherapy alone (31). Also, a 2016 single-arm randomized phase III trial comparing short-intensive chemotherapy alone ($n = 66$) or the same treatment in combination with rituximab ($n = 70$) on BL patients over 18 years of age found that inclusion of rituximab indeed improved 3 years EFS (75 vs. 62%) (32).

Although beneficial, the benefits of rituximab in BL are less clear than for other lymphomas. Indeed, there is some *in vitro* and xenograft model derived evidence that type II anti-CD20 mAb obinutuzumab may work better on BL than rituximab, suggesting mAbs of CD20 with differential binding to either CD20 or immune effectors, may lead to better results for some lymphomas (33). This would be clinically important, but could also elucidate the mechanism(s) of therapeutic response of rituximab and other anti-CD20 mAbs.

Mantle Cell Lymphoma (MCL)

Mantle cell lymphoma is a moderately aggressive lymphoma that comprises 2–4% of all NHL and has a median OS of 3–5 years (17). MCL is technically classified as an indolent lymphoma; but it usually has an aggressive clinical course and is incurable, despite an initial response to either dose-intense chemotherapy or combination therapy (34). Although rituximab has proven beneficial as a maintenance therapy, R-CHOP achieves a relatively short median PFS of 16–17 months (17, 34, 35). Several chemotherapeutic regimens are recommended to treat MCL, including bendamustine, CHOP, high-dose cytarabine, or fludarabine-based regimens (34). Rituximab is also generally used in combination, despite few studies directly evaluating the efficacy of rituximab in treating MCL, and retrospective analyses have concluded addition of the immunotherapeutic does indeed improve OS (34, 36).

Rituximab maintenance has demonstrated an OS benefit in a phase III randomized MCL clinical trial, which has not been shown in other lymphoma subtypes (37). The study compared MR ($n = 120$) after autologous stem cell to observation only ($n = 120$) and found a 4-year PFS of 83 and 61%, respectively (37). The MR group also had a significantly increased OS (37). Interestingly, retreatment with rituximab when molecular relapse occurs has also proven a successful strategy to regain molecular remission status, and likely to prolong clinical remission time (38, 39). This could provide a strategy for more cost-effective maintenance of remission in MCL.

Indolent Lymphomas

Unlike the more aggressive NHLs, indolent NHLs progress more slowly. Following diagnosis, the disease can be treated immediately or treatment may be delayed until symptoms appear. Because of this, indolent lymphomas have a longer median survival; but while they progress slowly and often respond to initial treatment, they also relapse and ultimately tend to be incurable. Rituximab monotherapies and rituximab in combination with chemotherapeutics have had a significant impact on the survival of patients with these lymphomas.

One important question that remains to be fully answered is the benefit of maintenance therapy in treating indolent lymphomas,

which is where rituximab and other mAbs may play a pivotal role in increasing FFS or OS since they can be more safely given long term due to their lower toxicity (40). Despite extensive research, it remains uncertain how helpful maintenance therapies are for most indolent lymphomas. Outlined below are important historical trials, as well as recent advances using rituximab as part of initial therapies and MR on specific indolent NHLs.

Follicular Lymphoma

Follicular lymphoma arises from malignant transformation of follicle center B-cells and accounts for approximately 20% of adult NHLs in the Western countries (17). FL has an indolent clinical course with an average OS rate of 73% at 10 years with modern treatments, but the majority of cases are ultimately still incurable (41). The median age at diagnosis of FL is 55–60 years old, and it occurs slightly more frequently in females (42).

Follicular lymphoma was the first cancer for which the FDA approved rituximab use. The milestone phase II study evaluated 37 FL patients with low-grade relapsed disease and treated them with four weekly doses of 375 mg/m^2 as a monotherapy (43). Clinical remission was achieved in 17 patients (46% response rate, 3 patients achieved CR) with a median time to progression of 10.2 months among those responders (43). The results of this study showed not only the safety and feasibility of treating FL with rituximab; it demonstrated clear efficacy which led to its approval. A phase II/III multicenter trial published in 1998 included 166 patients with recurrent indolent FL patients from 31 centers (9). Rituximab was given as a monotherapy on the same dosage schedule as the 1997 study and again achieved a 48% response rate (6% achieved CR) with a median time to progression of approximately 12 months among responders (9). The remission rates of these studies were comparable to response rates achieved by standard chemotherapeutics (9). In 1999, another milestone study was published which was aimed at testing the safety and feasibility of combination CHOP and rituximab (44). The study included 40 indolent NHL patients given R-CHOP and achieved an impressive 95% ORR (55% CR) and helped solidify R-CHOP and other rituximab combination therapies as the current standard of care for most CD20 expressing NHLs. A recently published phase II trial composed of 66 FL patients determined lenalidomide in combination with rituximab may be a reasonable R-CHOP alternative as it yielded similar CR and PFS rates current therapies with low toxicity (45). This is being evaluated in the phase III RELEVANCE study, and interim results have not demonstrated superiority of either regimen (46). Half of all FL patients are 60 or above, and treatment choices in these groups can be more difficult due to overall health and comorbidities. Still, the low toxic side effects of rituximab compared with chemotherapies indicate mAb as a safe and effective treatment in elderly FL patients both in combination or often as a monotherapy (47).

Because of its indolent nature, FL often does not require immediate treatment. There is uncertainty surrounding what the best treatment is, if any, during asymptomatic periods following diagnosis. This is a point of contention for both the time before initial treatment, as well as optional maintenance therapy following remission, with the alternative option being "watchful waiting" in which treatment begins only once symptoms or impending organ failure occurs. In retrospective studies and several clinical trials, there was no significant survival benefit to starting treatment early compared with watchful waiting (48). Likewise, maintenance strategies are similarly not well established to have an overall survival benefit. The PRIMA study of MR enrolled 1,217 patients and, following induction therapy, randomized them into groups receiving either observation or 2-year MR (375 mg/m^2 rituximab every 8 weeks) (49). In a 6-year follow-up report, the group concluded a significant benefit to PFS, but not OS (50). Another phase III trial published in 2014 enlisted 379 patients with low-tumor-burden FL for either watchful waiting, rituximab induction (375 mg/m^2 weekly for 4 weeks), or rituximab induction followed by MR consisting of 12 additional infusions given every 2 months over 2 years (48). The key endpoint for this study was time until the disease progressed to the point of needing treatment. Within the watchful waiting group, only 46% of patients had not yet required treatment by 3 years while 78% of patients within the rituximab induction group and 88% of patients within rituximab induction plus MR group did not require treatment by the same timepoint (48). Interestingly, quality of life metrics were significantly higher in the group receiving MR than in the other two groups. These data argue that rituximab may significantly delay the need for chemotherapy in FL, and given the relatively low toxicity, could be considered as initial therapy in this group of patients. Since both induction rituximab and induction followed by MR produce similar response rates, it is unclear what mechanisms provide the durable remission considering that continued dosage had a minimal additional benefit in disease response. Although the immune effectors of rituximab are not associated with memory, there is growing evidence to support memory–natural killer (NK) cells with cytotoxic capacities and these cells, or possibly some unknown effector mechanism, may be responsible for the durable delayed disease progression (51). A recent meta-analysis found MR may also provide improved overall survival in all FL patients based on findings across seven trials including 2,315 patients, although OS benefit to the subgroup of patients receiving R-chemo in the first-line setting was not demonstrated. These findings have not been replicated in phase III trials, and importantly did not include patients treated with bendamustine, which has subsequently become a standard frontline regimen for FL (52).

Marginal Zone Lymphoma (MZL)

Marginal zone lymphoma is an indolent lymphoma that comprises 5–10% of all NHL (17). Randomized trials are lacking to demonstrate the efficacy of rituximab in this lymphoma subtype specifically, but rituximab is usually included in treatment regimens, and single-agent activity has been demonstrated (53, 54). There are three main categories of MZL, with the majority being classified as extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT). These cancers are often associated with an infectious agent (e.g., gastric MALT is associated with *H. pylori*) and can sometimes be eradicated with successful treatment of the underlying infection. If further treatment is needed for localized disease, radiation treatment often leads to long-term remissions. Systemic treatment for widespread disease consists of a combination of chemotherapy

(e.g., bendamustine or chlorambucil) and rituximab, or with either reagent alone. Efforts are being made to identify the ideal treatment based on a prognostic index in the post-rituximab era (55).

Splenic MZL is a rarer form of MZL. There are no standardized treatments for splenic MZL due to a lack of randomized trials. However, rituximab or rituximab with chemotherapy is often used (56). Unlike reported for DLBCL and FL, rituximab combined with chemotherapy has not yet been demonstrated to improve survival, while rituximab monotherapy is reported to achieve a 69% 7-year PFS (56).

Nodal MZL is another indolent lymphoma that is thus far incurable but has a 5-year survival rate of 70–90% with current treatments (57). This disease also has no standard treatment, but when localized is usually treated with radiotherapy, while high tumor burden disseminated disease is treated with rituximab in combination with various chemotherapy regimens including bendamustine, fludarabine, or fludarabine with cyclophosphamide (57).

Lymphoplasmacytic Lymphoma (LPL)

Lymphoplasmacytic lymphoma follows an indolent clinical path and is incurable but rare. Due to its indolent nature, it has a median survival of 5–10 years in symptomatic patients (58, 59). The disease comprises cells most similar to those intermediate between small lymphocytes and true plasma cells, with features of both, including secretion of an IgM paraprotein (17). Multiple chemotherapy treatment regimens exist, including those based on alkylators (e.g., bendamustine), proteasome inhibitors (e.g., bortezomib), nucleoside analogs (e.g., fludarabine), or mAb ibrutinib. Since LPL is CD20-positive (unlike plasma cells) and rituximab has shown activity as a single agent in this disease (60), rituximab is often added in combination with chemotherapy regimens (61, 62).

The vast majority of cases of LPL are classified as Waldenstrom macroglobulinemia, which has pathophysiology in part determined by the two key mutations, such as *MYD88*^{L265P} and *CXCR4*^{WHIM}. While the disease is considered incurable, asymptomatic patients are not treated until symptoms appear, like other indolent lymphomas. There is no single recommended treatment for this disease, but it is treated with combination regimens including rituximab and fludarabine, oral cyclophosphamide with cladribine or fludarabine, as well as fludarabine, cyclophosphamide, and rituximab (FCR) as the first-line therapies for the disease (59). Since rituximab can cause an IgM flare, it should not be used until the IgM paraprotein levels are below 4,000 mg/dL. MR in rituximab-responsive patients was shown to improve OS in an observational study, but no randomized studies have proven the effectiveness of this strategy (63).

Hairy Cell Leukemia (HCL)

Hairy cell leukemia is a lymphoma of mature B-cell origin, despite its name. It is a rare chronic disease with a good prognosis, with a small percentage (~10%) not requiring immediate treatment but instead observation until treatment becomes necessary (64). It is regarded as one of the few cancers that were once generally fatal but is now almost always curable or maintainable, usually allowing patients to reach normal life expectancy (65).

The disease is effectively treated with nucleoside analogs, but patients relapse. A recent phase II study found that cladribine followed by rituximab achieved a durable remission of nearly 100% 5-year FFS in HCL patients (66).

Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia, also referred to as small lymphocytic lymphoma depending on where the primary presentation of the disease occurs, which can be in the peripheral blood, bone marrow, or solid lymphoid organs. Despite the different names and primary locations, the two diseases comprise the same type of lymphocyte and share similar pathogenesis and prognosis (67). The disease is indolent with a relatively high median survival. Importantly, CD20 expression in CLL patients tends to be lower compared with other B-cell lymphomas (68). Although rituximab does have clinical relevance in CLL, it is thought this lower expression of CD20 may be why the mAb is not as beneficial in these lymphomas and is the reason newer, possibly more potent, anti-CD20 drugs were first tested in CLL (68, 69).

In CLL patients who are young and fit and lack deletion of 17p or TP53, current treatment guidelines recommend chemotherapy, commonly fludarabine, cyclophosphamide in combination with rituximab (FCR) as initial treatment based on the proven effectiveness of rituximab from several clinical trials (70). A recent Canadian study confirmed the tangible benefits in CLL by evaluating patients treated in the pre- and post-rituximab era (71).

The currently ongoing FLAIR phase III trial includes 754 CLL patients given either the current standard of care FCR, ibrutinib plus rituximab, ibrutinib plus venetoclax, or ibrutinib alone, potentially eliminating the need for more harmful chemotherapeutics in favor of more targeted therapeutics as the new standard of care (70). This study should also assess the benefit of the addition of rituximab to small molecule targeted therapies (in this case ibrutinib), which has been relatively understudied.

Rituximab Depletion of Non-Malignant B Cells to Treat Autoimmune Diseases

Because rituximab depletes normal B cells, it has also been effective in treating a wide variety of autoimmune diseases by reducing the adaptive immune response against self. The FDA approved it for treating rheumatoid arthritis (RA) in 2006, and it has shown promise in treating some other autoimmune disease as well (72). Both case reports and meta-analyses indicate rituximab helps alleviate symptoms, even in refractory patients, of pemphigus (73), pemphigoid (74), myasthenia gravis (75), and neuromyelitis optica (76). However, despite successful clinical trials for RA, not all autoimmune diseases respond as well to rituximab. Systemic lupus is one unfortunate example where recent randomized, double-blind phase II/III trials found no significant benefit of adding rituximab to the standard of care (77, 78).

The increasing use of rituximab to treat RA since 2004 has told us a lot about normal B cells' response to the mAb (79). Four weekly doses of 375 mg/m^2 rituximab depletes B cells from the peripheral blood for approximately 6 months in RA patients, although response duration varies between individuals (80). Surprisingly, B-cell depletion is well tolerated among most patients and has limited negative health effects. Increased risk of infections and late-onset neutropenia are two of the most common problems, while reduced vaccine efficacy is also thought to be an issue (81). The vast majority of information on rituximab response comes from monitoring of peripheral blood. However, there is only a modest drop in antibody production in RA patients treated with rituximab, suggesting incomplete depletion of B cells in the spleen, lymph nodes, and bone marrow (81).

SHORTFALLS OF RITUXIMAB AND ALTERNATIVES

Although rituximab as a monotherapy, or in combination with chemotherapeutics, has greatly improved the prognosis of all B-cell NHL, there are still many cases in which it fails. In the case of DLBCL, 30–50% of patients are not cured by R-CHOP, with about 20% being initially refractory and another 30% relapsing after CR (82). The majority of indolent NHLs will eventually relapse and are incurable. This high rate of failure has spurred on the search for improved methods of treating refractory or relapsed patients, emphasizing the need for new biomarkers that identify those who will, and those who will not, be effectively treated by rituximab-based regimens (83).

Subcutaneous Rituximab

A relatively recent development in rituximab therapy was the FDA approval of a subcutaneous formulation of the mAb which combines it with recombinant human hyaluronidase. Recombinant human hyaluronidase is used to increase the dispersion and absorption of molecules and thus allow very small, highly concentrated volumes to be injected subcutaneously while retaining efficacy (84). In 2014 a randomized phase III study, SABRINA, evaluated the pharmacokinetics (PK) and safety of subcutaneous rituximab in FL. The study compared 48 patients who received subcutaneous rituximab to 54 who received intravenous rituximab and found that subcutaneous delivery was non-inferior (85). The subcutaneous delivery was also preferred by nearly all patients, and the benefits include less time in the clinic with anticipated reduced workloads for clinical staff, lower health-care cost, and increased accessibility of rituximab therapy (86). Following the 2014 study, similar trials have found subcutaneous rituximab to be non-inferior in treating CLL and DLBCL as well (84). The subcutaneous formulation was approved by the FDA in 2017 to treat FL, DLBCL, and CLL.

Radiolabeled and Toxin Conjugated Anti-CD20

Rituximab is a powerful antitumor reagent with relatively low side effects but, as discussed, its mechanisms of action are still not well understood, hampering efforts toward further improving patient survival. One method of modifying rituximab and other anti-CD20 mAbs is by conjugation of a radiolabel or cytotoxic drugs, delivering the toxic payload directly to the targeted B-cell malignancies.

Radiolabeled anti-CD20 antibody tositumomab (Bexxar) and ibritumomab tiuxetan (Zevalin) have produced higher CR rates compared with unlabeled mAbs, and one course is approximately as effective as six to eight cycles of combination chemotherapy (87). Both were approved by the FDA in 2002 and 2003, respectively, to treat several relapsed or rituximab-refractory NHL subtypes. Logistical obstacles have prevented them from being widely used, and despite the success of these first-generation radiolabeled mAbs they both suffered from poor sales, which ultimately lead to Bexxar being pulled from the market (88).

Ritixumab conjugated to doxorubicin is one example of toxin conjugated anti-CD20 therapy. This strategy has been further modified to improve efficacy, including attempts to generate reduction-sensitive micellar nanoparticles for better delivery, although neither these nor any similar anti-CD20 conjugate with toxins, have been approved by the FDA to date (89).

Additional CD20 mAbs for Lymphoma

Several additional therapeutic anti-CD20 mAbs have been generated since the advent of rituximab. Each features "next generation" modifications: an alternate binding epitope, additional humanization, altered glycosylation, or another combination of modifications (**Figure 3**). Two have already been approved by the FDA, ofatumumab, and obinutuzumab, while many others are in various phases of development of both type I and type II anti-CD20 mAbs. Type I mAbs translocate CD20 to lipid rafts, preferentially activate complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), have a weak homotypic adhesion, and have a caspase-dependent apoptosis induction (90). On the other hand, type II mAbs do not rearrange CD20 to lipid rafts, have a higher affinity toward ADCC induced death, and caspase-independent induced by a lysosome-mediated mechanism (90).

Ofatumumab

Ofatumumab (trade name Arzerra) became the first fully humanized mAb targeted to CD20 to gain initial approval for anti-cancer therapies by the FDA in 2009, and full approval in 2014. This mAb binds to a different epitope than rituximab, which binds the large extracellular loop of CD20 (91). Ofatumumab, on the other hand, can bind both the small and large extracellular loop of CD20 (91). This unique binding, which is more proximal to the cell membrane, is suspected to be the source of increased CDC activity compared with rituximab (92). Being fully humanized, ofatumumab should cause less anaphylaxis, and a recently released case study reported it was successfully administered without reaction to a patient who had previously presented with anaphylaxis in response to rituximab (93).

Ofatumumab is approved to treat CLL that is refractory to fludarabine and alemtuzumab therapies (94). The study included 59 patients refractory to fludarabine and alemtuzumab and 79 patients with bulky lymphadenopathy refractory to fludarabine alone (95). Patients were given an initial 300 mg of ofatumumab

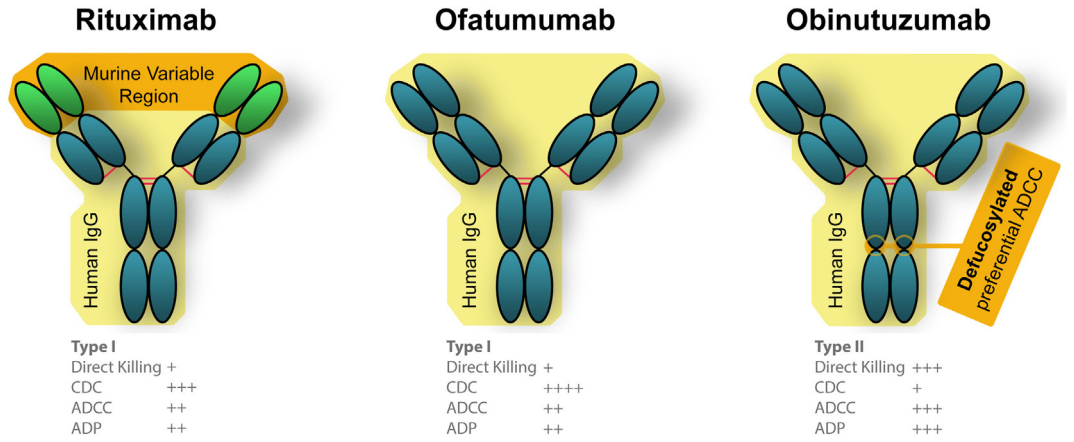

FIGURE 3 | Engineered differences between Food and Drug Administration approved anti-CD20 monoclonal antibodies (mAbs). Rituximab is a chimeric mAb that is partially humanized, that has a human Fc portion but retains the murine variable region which recognizes CD20. Both ofatumumab and obinutuzumab are fully humanized mAbs, which reduces unintended immune responses against the therapies. Ofatumumab also has a glycoengineered Fc region which results in better binding with immune effector cells (106, 190).

dose followed by 11 additional doses at 2,000 mg over 24 weeks (95). The ORRs were 58 and 47%, respectively, among the two groups with an OS of 13.7 and 15.4 months, respectively (95). While this study showed ofatumumab to be efficacious in refractory CLL, it was not directly compared with rituximab. It is worth noting that a retrospective follow-up study examined response based on prior rituximab exposure and found ofatumumab achieved an ORR of 44% in patients who were refractory to rituximab (96). A 2001 rituximab study found doses of 2,250 mg/m^2 achieved a 75% overall response, making the higher doses of ofatumumab a confounding factor for comparison of efficacy between the two mAbs (97). A 2015 phase II trial treated 49 indolent NHL patients with bendamustine and ofatumumab and found the ORR comparable to historical treatments with bendamustine and rituximab (98). A 2017 study by the Alliance found PFS was comparable between ofatumumab with bendamustine (OB) and historical rituximab with bendamustine in previously untreated FL, despite an initially improved CR with OB (99). Given conflicting reports of increased benefits, additional randomized phase III trials and more biologically representative *in vitro* assays are needed to fully assess the differences in efficacy between these CD20 mAbs.

Obinutuzumab

In 2013, obinutuzumab (trade name Gazvya) became the first glycoengineered antibody approved in the US as the next generation of anti-CD20 mAb for cancer treatment. The glycoengineering is accomplished by overexpressing two glycosylation enzymes, MGAT III and Golgi mannosidase II which resulted in antibodies that are mostly non-core-fucosylated and possess unique properties distinct form regular IgG1 (100). mAbs of this particular subisotype are also referred to as IgG(1E5) (100). These modifications create better binding of effector immune cells and a more efficacious response compared with rituximab, although the clinical benefits have been variable. A phase II trial which tested obinutuzumab in combination with chlorambucil for previously untreated CLL patients found similar response rates compared with rituximab and ofatumumab in similar patient groups (101). FDA approval was based on a subsequent phase III trial (102). It is important to note that obinutuzumab (and ofatumumab, as discussed above) were given at substantially higher doses compared with rituximab, making a direct comparison of efficacy difficult (95, 101).

In the phase III GOYA study of DLBCL patients who compared G-CHOP (n = 706) and R-CHOP (n = 712) followed out to a median observation of 29 months, Vitolo et al. found no improvement in PFS after treatment with obinutuzumab vs. rituximab plus CHOP (103). A recent phase III trial treated FL patients with either R-CHOP (n = 601) or G-CHOP (n = 601) and followed their progression for a median of 34.5 months (104). Unlike the similarly powered DLBCL study, this group found that G-CHOP with maintenance therapy provided an increased PFS (104). In February 2016, obinutuzumab was approved to treat patients with FL who relapsed or have refractory disease to any rituximab-containing regimen (105).

Unlike in DLCBCL, but similar to FL, recent CLL clinical trials comparing G-CHOP to R-CHOP appear to show a better response to obinutuzumab combined with CHOP rather than rituximab (106). Although the data are preliminary and based on higher doses of mAbs given for both obinutuzumab and ofatumumab, it suggests different CD20 antibodies may work better for specific lymphomas, and clinical trials for each mAb may result in more personalized medicines. However, better methods for rapid screening of efficacy of specific anti-CD20 mAbs against an individual's lymphoma are needed to achieve effective precision medicine that would be clinically most useful.

Ublituximab

Ublituximab is a type I glycoengineered anti-CD20 mAb that binds to an epitope unique from rituximab, ofatumumab, or

obinutuzumab and contains a low-fucose Fc region that facilitates enhanced ADCC activity *in vitro* (107). A recent phase I/II trial included 45 patients with relapsed or refractory CLL who were treated with a combination of ublituximab and ibrutinib (107). The treatment achieved an ORR of 88%, with a 5% CR but the durability of the response is not yet known, and while the safety and feasibility of ublituximab have been established, an ongoing phase III study will determine if the anti-CD20 increases the efficacy above ibrutinib monotherapy (107).

MECHANISMS OF RITUXIMAB RESPONSE

The binding of rituximab to CD20 facilitates cell death in four main ways, three of which rely on recruiting effector mechanisms from the patient's own immune system. Because of this reliance on the human immune system (HIS) to mediate antitumor effects, the exact *in vivo* mechanisms remain challenging to study. Based on a combination of *in vivo*, *ex vivo*, and *in vitro* work, we know that rituximab-mediated killing occurs by triggered cell death *via* binding of rituximab to CD20, CDC, ADCC, and antibody-dependent phagocytosis (ADP) (**Figure 4**). One major barrier to fully understanding the mechanisms of the immune system in immunotherapies is the lack of an ideal animal model. Because rituximab is targeted against human CD20, it can be evaluated in immunocompromised mice xenografted with human lymphoma, but these mice do not possess human immune cells or human complement proteins. Much work is being done to better model human NK cells in mice to provide more biologically relevant animal models, mainly through the development of HIS mice (108). One major issue is achieving normal NK cell development in a murine body. Recent findings suggest knocking in human *SIRPA* and *IL15* to replace the wild-type copies in HIS mice resulted in normal tissue distribution circulation of NK cells. Furthermore, the NK cells in these HIS mice can facilitate ADCC, providing a crucial next step toward research tools for understanding the role of rituximab-mediated ADCC *in vivo* (109). Still, the complexities of rituximab response are further complicated by potential competition and synergy between all other immune effector responses, including direct cell killing.

Direct Signaling Induced Cell Death

Although presumed to have limited contribution to the *in vivo* antitumor effects of rituximab, many *in vitro* studies have demonstrated that binding of the mAb can trigger cell death without immune system effector mechanisms. Two main pathways for this direct cell killing have been identified which are caspase-dependent, and -independent (**Figure 4**, top left). Surprisingly, despite over 30 years of intensive study, no CD20 ligand has been discovered, making it difficult to predict and understand how anti-CD20 binding alone might trigger cell death. Rituximab binding to CD20 causes rearrangement of lipid rafts and alters CD20 localization; defining it as a type I CD20 antibody (110). It is not entirely known how this rearrangement triggers cell death, it is known that the process is src family kinase-dependent and results in caspase-mediated apoptosis (111). Although relatively little is known about the molecular pathways of cell death *in vivo*, Akt, ERK1/2, NF-κB, and p38 MAPK are pathways shown to be involved in rituximab-mediated apoptosis (112). Ivanov et al. found that type II CD20 antibodies primarily induce cell death without lipid raft formation, through actin reorganization leading to lysosome-mediated cell death, independent of caspase pathways (113). No direct evidence of human *in vivo* killing by this mechanism has been found, but one compelling study demonstrated a reduction in CNS lymphoma after rituximab was injected directly into the cerebrospinal fluid, where limited immune responses are available, arguing for a direct cell killing mechanism (114).

Complement-Dependent Cytotoxicity

Complement-dependent cytotoxicity is mediated by the classical pathway of the complement system. The C1 complex binds to rituximab opsonized cells and triggers the complement cascade which results in the insertion of the membrane attack complex (MAC) into the target cell membrane, thus compromising the membrane and triggering cell lysis (**Figure 4**, top right). CDC is known to play some role in the *in vivo* killing of B-cell malignancies, potentially having the largest effect on circulating tumor cells and contributing to the recruitment of immune cells, although the true extent of its contribution to response is still unknown (115).

There is evidence that CDC is not as effective as ADCC *in vivo* and an effective CDC response may have a negative overall impact on rituximab efficacy as both processes compete for access to the bound mAb (116). Different anti-CD20 antibodies have different propensities to activate CDC (115). Studies have also shown a competitive relationship between ADCC and CDC *in vitro* (117).

Other studies that suggest the importance of CDC *in vivo* centers around the frequent observation of complement-regulatory proteins CD55 and CD59 were expressed on circulating tumor cells (118). When tested *in vitro*, high expression of these proteins were associated with increased resistance to rituximab, but their neutralization overcame that resistance (118). In addition, one study utilizing sera collected from CLL patients demonstrated patients were more frequently deficient in C1q, C3, and C4 complement proteins and that their sera was more readily exhausted of complement activity following anti-CD20 mAb treatment, resulting in lowered CDC activity (119). On the other hand, in some mouse studies with genetic deficiencies for either FcR common γ chain-deficient or complement components C3, C4, or C1q, it was found that CDC does not play a role in the killing of circulating tumor cells utilizing murine anti-CD20s to target murine lymphoma (120, 121). Therefore, the impact of CDC on rituximab-mediated anti-cancer effects *in vivo* is still not fully defined, interactions between ADCC and ADP with CDC have yet to be addressed, and additional *in vitro* methods for characterizing those interactions need to be further developed.

Antibody-Dependent Cell-Mediated Cytotoxicity

Antibody-dependent cell-mediated cytotoxicity is thought to be a significant contributor to the *in vivo* antitumor activity of rituximab. Binding of the variable region of the mAb to CD20 facilitates the binding its Fc region to FcγRIII receptors on NK cells, thus leading to the formation of the immune-synapse that consists of

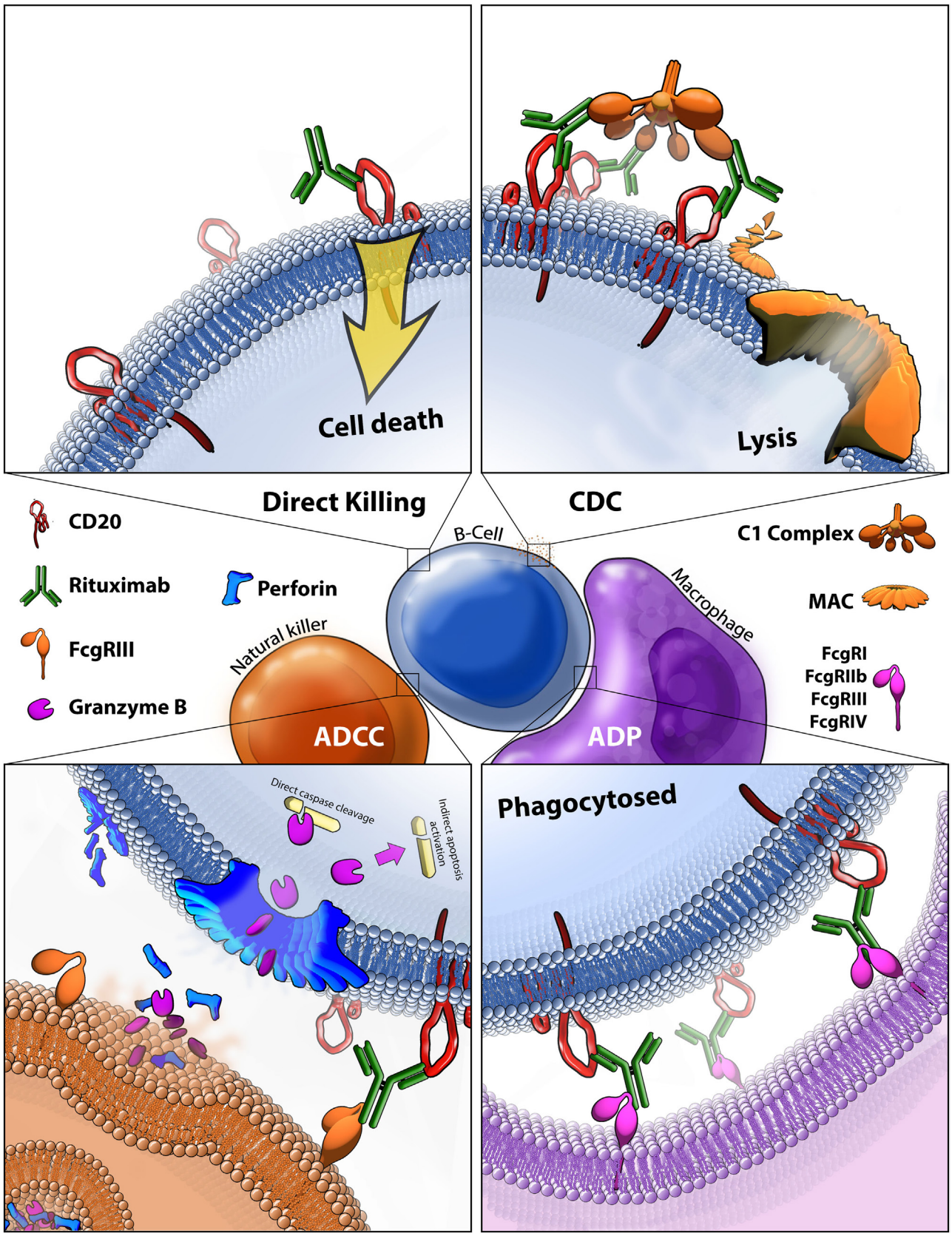

FIGURE 4 | Continued

FIGURE 4 | Rituximab-mediated cell killing of CD20 expressing B-cells. (Top left) Binding of rituximab to CD20 can directly trigger apoptosis through both caspase-dependent and -independent mechanisms that are still not fully characterized. (Top right) Bound rituximab can recruit the C1 complex triggering the classical complement cascade which leads to insertion of the membrane attack complex (MAC) and ultimately leads to cell lysis, also known as complement-dependent cytotoxicity (CDC). (Bottom left) Bound rituximab can recruit natural killer cells *via* recognition by the FcγRIII leading to antibody-dependent cell-mediated cytotoxicity (ADCC). This facilitates release of perforin, which assembles into membrane compromising pores in the target cell, and granzyme B, which enters the target cell and triggers apoptosis by cleaving caspases and potentially by other methods. (Bottom right) Macrophages recognize CD20 bound rituximab through various Fcγ receptors which leads to antibody-dependent phagocytosis (ADP) of the target cell.

the region where the two cells make contact (**Figure 4**, bottom left). This binding triggers a response in cytotoxic NK cells to release granules containing perforin, which self-compiles in a Ca^{2+}-dependent manner into a non-selective pore which embeds into and permeabilizes the membrane (122). The NK cells also release granzyme B at the immune-synapse, which infiltrates the permeabilized membrane of the target cell and induces programmed cell death, through various ways including caspase-dependent mechanisms, having the ability to cleave caspase 3, 6, 7, 8, 9, and 10 directly, as well as activate caspase 2, 6, and 9 indirectly (123).

Detecting and quantifying rituximab-mediated ADCC *in vivo* is challenging for the same reasons as CDC, in that it largely requires a functional HIS and therefore makes animal model data more difficult to interpret. Nonetheless, a mouse study demonstrated FcγRs were necessary and sufficient for anti-CD20 depletion of various cancers in both xenografted and syngeneic models (124).

Quantifying ADCC *in vitro* has proven challenging due to the necessity of combining NK effectors and target cancer cells into the final reaction which makes it difficult to separate NK cell death from that of the target cells. Originally, ^{51}chromium (^{51}Cr) was used to measure lysis by NK cells by first having the target cells uptake the ^{51}Cr, then combining the cells and measuring the amount of ^{51}Cr released into the supernatant, thus indirectly measuring the percentage of cells lysed. Similarly, fluorescence assays were developed using calcein-acetoxymethyl which is taken in and cleaved by living cells to generate a hydrophilic fluorescent molecule that is trapped within intact membranes (125). Both methods are indirect, can be influenced by factors unrelated to actual cell death, and are often hard to reproduce which makes them difficult to use for highly sensitive measurements (126). A luciferase assay was recently published as an alternative method to the release assays by creating novel effector cells expressing variants of FcγRIIIa believed to impact ADCC activity (127). This also relies on the indirect measurement of cell killing and requires using specific effector cell lines (127). Recently, a flow cytometry-based assay was published using is a small molecule, CFSE, that binds to proteins of live cells thus labeling target cells fluorescent green before combining in the ADCC assay and then directly measuring the percentage of dead target cells *via* flow cytometry. This proved more accurate than release assays and required only 5,000 target cells for sufficient consistency while providing an ideal system for answering additional questions through co-staining with additional antibodies (126).

Antibody-Dependent Phagocytosis

Antibody-dependent phagocytosis is the least studied of the four known rituximab effector mechanisms. It is facilitated by macrophage recognition of bound rituximab through various Fcγ receptors (**Figure 4**, bottom right). *In vitro* measurement of ADP carries the same challenge as ADCC, but phagocytosis can be observed in real time. Microscopy and flow cytometry-based methods are most commonly relied on to quantify the amount of opsonized cancer cells that are phagocytosed. Although no *in vivo* evidence of rituximab-mediated ADP in humans exists, some evidence of ADP in knockout mouse models has been demonstrated based on a reliance on macrophage-specific FcγRIV to achieve rituximab anti-cancer effect (115).

Trogocytosis

Trogocytosis is not thought to be a mechanism of rituximab-mediated cell death, but rather a response that occurs when other mechanisms have become exhausted and that may contribute to the reduced efficacy of rituximab. Trogocytosis, also referred to as shaving, is a process by which monocytes, neutrophils, or macrophages remove rituximab bound to CD20 by transferring plasma membrane, which has unknown contributions to rituximab resistance through an Fc receptor-mediated response (128, 129). Importantly, although trogocytosis is potentially helping cancer cells escape from mAb therapies, there is also evidence that macrophage-mediated trogocytosis can lead to target cell death rather than escape (130). These findings suggest the interplay between the immune effector-mediated responses to rituximab may be more complex than is currently known.

Rituximab Resistance

As mentioned above, SNPs affecting the Fc receptor of NK cells have been correlated with survival. Other innate rituximab resistance mechanisms have been identified for CDC, for example, CD55 and CD59 (membrane complement-regulator proteins which prevent insertion of the MAC) are known to be expressed on some resistant lymphoma cells and reduction of those proteins *in vitro* overcomes that resistance (118). In addition, one study utilizing sera collected from CLL patients demonstrated patients were frequently deficient in C1q, C3, and C4 complement proteins and that their sera were more readily exhausted of complement activity following anti-CD20 mAb treatment, resulting in lowered CDC activity (119). In an effort to determine mechanisms of resistance to rituximab, Czuczman et al. exposed CD20 expressing lymphoma cell lines to escalating doses of rituximab exclusive of any immune effectors. From these studies, a global decrease in CD20 through pre- and post-transcriptional controls occurred in the resistant lines (131). Similarly, Small et al. observed reduction of CD20 in the sublines with acquired rituximab resistance, emphasizing antigen expression as a key mechanism of resistance

(132). Reduction in pro-apoptotic factors Bax and Bak were also observed following chronic *in vitro* exposure to rituximab, which highlights potential therapies to re-sensitize resistant cells (133). Efforts are being made to circumvent resistance, either through sensitizing resistant cells or developing combination therapeutics that synergize with rituximab.

Synergy Between Rituximab and Conventional Therapeutics

Very is little is known about how rituximab and CHOP interact *in vivo*, and this has not yet been well-studied *in vitro*. Still, there is evidence that rituximab and at least some cytotoxic chemotherapeutics have synergistic mechanisms mediating anti-cancer effects *in vitro* (27). For instance, rituximab downregulates anti-apoptosis factor Bcl-xL and sensitizes some B-cell cancers to drugs that induce cell death through cytotoxic mechanisms, thus creating synergistic effects (133, 134). CD20 binding by rituximab is also reported to increase uptake of other antibody–drug conjugates (135). Although radiation primarily functions through induction of DNA damage, there is evidence that it also recruits an immune response that may synergize with mAb therapy (136). Furthermore, DNA damage itself promotes ADCC. Fine et al. found that loss of Clr-b expression in cells under chemotherapeutic-induced genotoxic stress allowed attack by NK cells expressing NKR-P1B, which usually prevents killing of self through recognition of Clr-b on the target cell (137).

POTENTIAL BIOMARKERS

Rituximab has been in use for more than 20 years, benefiting ~15% additional DLBCL patients compared with CHOP alone, and around 50% of patients when given as a monotherapy. Despite its widespread use and variable benefits, we continue to lack biomarkers to predict or measure rituximab response beyond CD20 expression and tumor burden, although the search for additional biomarkers of response is ongoing.

One type of candidate for such a predictive biomarker are SNPs in the Fc receptor genes which code for the proteins that recognize bound rituximab. These have been interrogated in several studies and may have a clinically relevant impact on rituximab efficacy, although reported conclusions are variable (138). Most reports indicate that FcγRIIIa–V158F has a poorer response compared with homozygous valine genotypes among adult patients. Indeed, a study by Weng et al. consisting of 139 FL patients showed that homozygous V/V genotypes and humoral immune response to immunoglobulin idiotype vaccines were both independent positive predictors for PFS (139). It is worth nothing that a small study including adolescents and children with mature B-cell lymphoma or leukemia, reported in 2016 by Burkhardt et al. found a response rate of 59% in children with homozygous FcγRIIIa-V158F SNP, but only 32% among patients with the major allele coding for valine (140). A recent meta-analysis of publications from searches in the PubMed and EMBASE databases up to July 2014 concluded FcγRIIa-H131R SNP, but not FcγRIIIa-V158F, is associated with inferior response to rituximab (141). Both SNPs have been implicated to affect the ability of the receptor to bind to rituximab in various studies, and the variable data on their effects on clinical response likely reflect the complicated nature of rituximab's effect *in vivo* (115). It is possible that the complexity of the immune effector response mediated by rituximab confounds attempts at confirming a direct variable that modulates only one portion of the response. This may be why, despite better binding of obinutuzumab due to fucosylation designed to overcome decreased binding Fc-binding affinity due to the FcγRIIIa-V158F SNP, the improvement in clinical outcomes are not as dramatic as expected.

While glycoengineering of anti-CD20 is thought to improve response, variation in glycosylation of the FcγR may also be important for response. Recent findings based on *in vitro* results show that FcγRs also have glycosylation variation, and the effect of those differences is not well studied with respect to rituximab-mediated ADCC assays. Recent findings provide evidence that FcγR glycosylation has a significant impact on binding kinetics with rituximab (142). While the potential effects on ADCC were not investigated, it suggests that there are more factors that modulate binding beyond mAb fucosylation and FcγR SNPs (142).

There is some evidence that SNPs affecting CDC can predict rituximab response as well, either by direct effects on CDC or indirectly by interfering with ADCC. Indeed, in a retrospective study, a homozygous A SNP in $C1qA_{276}$ was also correlated with improved OS in patients with DLBCL treated with R-CHOP (143). Because the polymorphism is a synonymous SNP, the effector mechanism is unclear and requires further validation. Studies looking at the epistatic or combinatorial effects of the SNPs that affect various methods of rituximab-mediated killing may also be useful for determining their *in vivo* roles. A recent study found that a SNP that correlated with reduced expression of complement-regulatory proteins such as CFHR1 and CFHR3 was associated with patient outcome (144). Interestingly, the effect appeared to vary based on the specific anti-CD20 used (144).

A comprehensive review by Di Rocco et al. enumerates numerous molecular markers for DLBCL that are associated with prognosis and response to current therapies and could be used as biomarkers for personalized medicine (145). However, few predictive biomarkers for identifying which specific patients will benefit from rituximab are reported (145). In a unique approach to identifying biomarkers, researchers performed a screen of 1,140 paired potential biomarkers in FL patients to determine if any pairs could be used to predict outcomes and thus advise new patient treatments. One pair from their screen, low CD68 expression presenting in combination with a G/G or C/G SNP in the PSMB1 gene was associated increased PFS of patients treated with bortezomib and rituximab compared with rituximab alone. A similar approach could also be used to identify patients who would benefit from rituximab monotherapy alone (146).

Because germline genetic markers can be easily probed with current technologies, they remain the most attractive potential biomarkers to facilitate personalized medicine choices. However, somatic mutations that arise in cancer tend to make more accurate predictions, although limitations such as biopsy requirements and tumor heterogeneity as well as distinguishing driver and passenger mutations, still need to be fully overcome (147).

TABLE 1 | List of rituximab biosimilars around the world including the manufacturer and their corporate location, clinical trial status and for respective disease, status, and cost relative to the rituximab.

Biosimilar (reference)	Manufacturer	Clinical trials ongoing or completed	Disease	Status	Relative[a] cost to rituximab; $3,693 (500 mg) (191)
1B8 (192, 193)	Center of Molecular Immunology (Cuba)	Phase I	DLCBL	Pharmacokinetics and Safety in Progress	N/A
ABP 798 (194, 195)	Amgen (USA)	Phase III	NHL	Recruiting	N/A
BCD-020 (Acellbia) (173, 196–198)	Biocad (Russia)	Approved	INHL	Launched	72% less
BI 695500 (167, 168, 170–175, 199)	Boehringer Ingelheim (Germany)	Phase III	LTBFL	Terminated	N/A
CT-P10 (Truxima) (177, 200)	Celltrion (South Korea)	Approved	ASFL	Launched	72% less
GP2013 (Rixathon) (201)	Novartis Pharmaceuticals (Switzerland)	Phase III	ASFL	In progress	N/A
HLX01 (182)	Shanghai Henlius Biotech (China)	Phase III	DLBCL	In progress	N/A
JHL1101 (202, 203)	JHL Biotech (Taiwan) and Sanofi (France)	Phase I and III	NHL	In progress	N/A
Kikuzubam (204, 205)	Probiomed (Mexico)	Phase I	NHL	Withdrawn	N/A
Maball (206, 207)	Hetero (India)	Approved	CLL, DLCBL, and FL	Launched	87% less
MabionCD20 (208)	Mabion SA (Poland)	Phase III	DLBCL	Recruiting	N/A
MabTas (209–211)	Intas Pharmaceuticals (India)	Approved	NHL	Launched	76% less
MK8808 (212)	Merck Sharp & Dohme Corp. (EU)	Phase I	FL	Terminated	N/A
Novex (213, 214)	Laboratorio Elea (Argentina)	Approved	NHL	Launched	9% less
PF-05280586 (215)	Pfizer (USA)	Phase III	LTBFL	Recruiting	N/A
Reditux (155, 187, 216)	Dr. Reddy's Laboratories (India)	Approved	DLBCL	Launched	50% less
Rituxirel (217, 218)	Reliance Life Sciences, Torrent Pharma (India)	Approved	NHL (DLBCL and FL)	Launched	84% less
RTXM83 (219)	mAbxience (Switzerland)	Phase III	DLBCL	Completed	N/A
SAIT101 (220)	Samsung BioLogics (South Korea) and AstraZeneca (UK)	Phase III	LTBFL	Completed	N/A
TL011 (221)	Teva Pharmaceuticals (Israel)	Phase III	DLBCL	Terminated	N/A
Zytux (Ristova) (222, 223)	AryoGen Biopharma (Iran)	Approved	NHL	Launched	50% less

ASFL, advanced stage follicular lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; INHL, indolent non-Hodgkin lymphoma; LTBFL, low tumor burden follicular lymphoma; NHL, non-Hodgkin lymphoma; EU, European Union.

[a]*Prices vary depending on the market and the country where the product is sold. N/A, not available.*

TP53 mutations are the most common *de novo* mutation in nearly all cancer types and are also common in lymphomas. TP53 is considered the master regulator of the DNA damage response and defects in this gene can cause tumors to be more resistant to the genotoxic chemotherapeutics which are a key part of most lymphoma treatments. A retrospective study evaluating data from the RICOVER-60 trial found that TP53 mutations occurred in 23.85% of the patients in the study and were independent predictors of patient survival (148). These findings highlight the need for studies able to analyze multiple key biomarkers at once, as focusing on only one could reduce significance and result in false negatives. It is known that TP53 is still a valuable prognostic marker in the post-rituximab era, but it is still unknown what role these mutations may have on rituximab efficacy specifically (149). Overexpression BCL2 is also known to be a biomarker of poor prognosis in DLBCL and is also a key factor of the rituximab direct killing pathway, although its effect on rituximab monotherapy outcomes has also not been tested (150).

Markers to monitor actual response, rather than predict response, are even more lacking. One study concluded that degranulation of NK cells following mAb treatment might be a marker of response, while granzyme B release levels was suggested in a trastuzumab study (151, 152).

The degree of CD20 expression levels among DLBCL may also be correlated to overall patient survival. It is variable both between patients and heterogeneous within an individual's malignancy. Johnson et al. reported that a lower overall expression of CD20 is correlated with reduced survival, based on a retrospective study of DLBCL patients treated with CHOP ($n = 82$) or R-CHOP ($n = 181$). They found individuals with the low CD20 expressing disease had a median OS of 1.2 (CHOP) and 3 (R-CHOP) years, while patients with higher CD20 expression did not reach median survival in either treatment group (153).

BIOSIMILARS

As the first therapeutic mAb in oncology, rituximab is also one of the first to encounter competition from biosimilar products as its patent expires. The recent patent expiration (2013 and 2016 in Europe and the US, respectively), and the economic significance of rituximab as the top-selling oncology drug has spurred the development of a multitude of rituximab biosimilars. Biosimilar regulatory approval pathways have been established in both the US and Europe, offering a pathway to marketing approval designed to decrease price and increase drug accessibility while maintaining safety and efficacy standards. Increased availability of biosimilars will drive prices down, provide better accessibility to anti-CD20 mAbs worldwide, and stimulate further research that may lead to better and more widespread treatment options (154). Current pricing for rituximab biosimilars worldwide is often less than half the price of rituximab (**Table 1**). In the US, use of biosimilars is expected to bring a savings of $9–12 billion

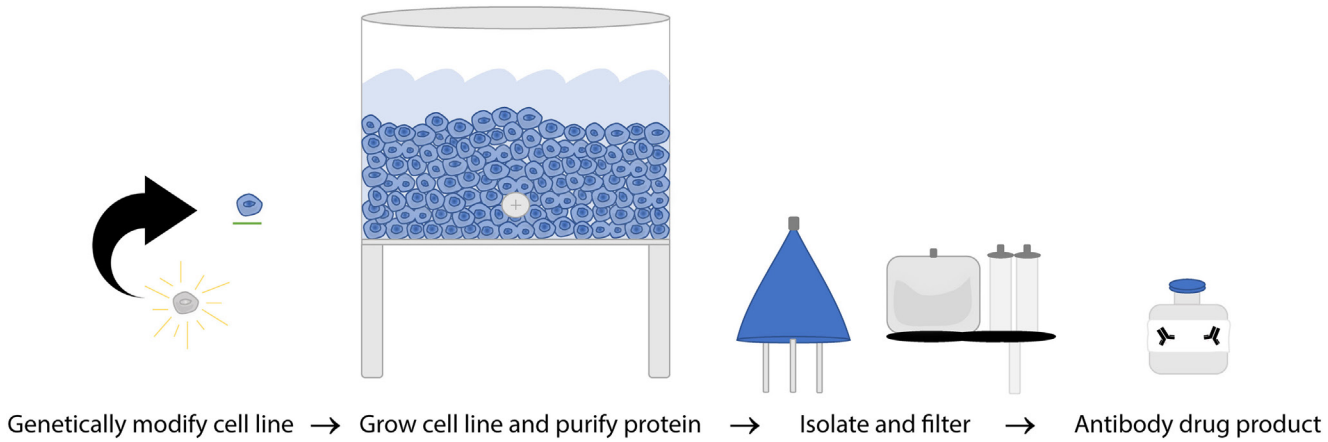

FIGURE 5 | A simplified overview of rituximab manufacturing process. Once hybridoma cell lines are established from a single clone, the cultures are expanded to produce a single specific monoclonal antibody (mAb) on a massive scale. That mAb is then collected, purified, analyzed, and certified on a per lot basis.

to the Medicare system in the next decade (155). In **Table 1**, we provide a summary of anti-CD20 biosimilars emerging into the marketplace; most are still in clinical trials or pending approval. However, evaluation of these biosimilars for equivalence to rituximab raises new challenges.

The FDA and European Union (EU) have subtly different definitions of biosimilars but share the concept that they must be biological therapeutics that are highly similar to the original product in structure and function. WHO established guidelines in 2016 which include a few current shortfalls in assays used to compare mAb biosimilars due to variation between target and effector cells used to evaluate response as well as the challenges of reproducing results in different laboratories (156). Structural evaluation of amino acid sequences and higher order structure, as well as glycosylation state are all evaluated to ensure they are identical while functional assays including binding affinity, cell killing efficacy of *in vitro* CDC and ADCC separately, and direct killing are all evaluated as part of the path to being granted biosimilar approval (156, 157). Exact replication of a biological product is impossible, but biosimilars are designed to be as close as possible to the parent molecule. The process of making nearly identical biosimilars can be affected by two main factors: variability in the biological processes involved in manufacturing and variability in the details of the manufacturing procedures themselves. Unlike generic drugs, antibody production depends on a biological process, introducing more variables that can affect the final product. Second, producing mAbs is a proprietary process and companies do not share all manufacturing practices meaning each biosimilar company has to develop independent best practices, standard protocols, raw material sources, and equipment to utilize (158). Because of these variable factors in biosimilar production (**Figure 5**), it is essential to validate that the new mAb produced has the same efficacy as the original, but the protocols for doing so are hindered due to incomplete knowledge of *in vivo* effectors of rituximab response. The current requirements for the regulatory approval pathway are outlined in **Table 2**.

TABLE 2 | Biosimilars and their respective approved regulatory standards.

Rituximab biosimilar	Approved regulator standards	Reference
BCD-020	Ministry of the Russian Federation, Department of Biotechnology and the Central Drugs Standard Control Organization (under review)	(173, 224)
CT-P10	European Medicines Agency, Korean Ministry of Food and Drug Safety, & FDA (under review)	(225, 226)
Maball	Department of Biotechnology and the Central Drugs Standard Control Organization	(227)
MabTas	*Central Drugs Standard Control Organization*	(210)
Novex	National Drugs, Foods and Medical Technology Administration (ANMAT)	(228)
Reditux	Department of Biotechnology and the Central Drugs Standard Control Organization	(210)
Rituxirel	Department of Biotechnology and the Central Drugs Standard Control Organization	(210)
Zytux	Food and Drug Organization	(229)

Although biosimilars emulate the parent antibody's function and clinical effects in small patient trials, they are not an identical replicate for the reasons described above (154). Several initial analytical tests are used to compare biosimilars to their originator product (159). Initially, the amino acid sequence can be compared to assure identity. Other factors to be assessed are homogeneity, glycosylation state, and antibody binding to the correct antigen. SDS-PAGE characterizes homogeneity, mass spectrometry is used to determine the glycoform patterns, and the antibody crystal structure is utilized to verify binding to CD20 (159–162). In addition, there are different functional tests to assess rituximab-mediated cell death *in vitro*. As mentioned above, rituximab can induce cell death by CDC, direct apoptosis through direct signaling, and antibody-dependent cellular cytotoxicity (ADCC), as well as ADP (163–165). Biosimilar developers can confirm their product has the same effect for each mechanism *in vitro*, although no comprehensive test to evaluate interactions of effector mechanisms, which

might be more representative of the *in vivo* situation, has been developed.

Once the antibody is determined to be highly similar to the parent antibody based on molecular characteristics, the effectiveness of the biosimilar is tested in small clinical trials. Unlike for their original predecessor, it is not necessary for biosimilars to go through full clinical trials to compare the efficacy in a relevant patient population (166, 167). Therefore, phase I and phase III non-inferiority trials are conducted to ensure safety, equivalent potency, and non-inferior efficacy. Post-marketing surveillance is also required by some regulatory agencies, to ensure there is no increased rate of immunogenicity (159).

The level of scrutiny a biosimilar receives is dependent mainly on the regulatory standards of the country in which it is being marketed. The rigor and standards for comparability with the originator product (in this case, rituximab) may differ depending on the approval guidelines followed (e.g., FDA, European Medicines Agency—EMA, or others). Rituximab biosimilars are produced all over the world (**Table 1**), and manufacturing standards are location dependent.

Nomenclature

The development of biosimilars created a need to develop a new nomenclature. The purpose is to serve as a means of distinguishing drugs so that users know they are getting a drug that is not identical to rituximab. The typical method of drug naming through the International Nonproprietary Names is not utilized for biosimilars (168). There is currently no universal global naming system for biosimilars, but standards and drafts to establish this have been initiated. Methods of distinction include, but are not limited to adding a prefix, suffix, or color to the label (169).

Less Financial Risk

Biosimilars provide an opportunity for less expensive therapeutic development (**Table 3**). Bringing a novel drug to market is rapidly increasing in cost, and currently costs more than a billion dollars (170). Biosimilars generally require smaller and fewer clinical trials, and therefore pose a lesser financial risk with a shorter timeline to approval. This is especially favorable for countries that have limited access to the originator compounds or have product shortages. With the rituximab patent expired, biotechnology and pharmaceutical companies are now legally able to participate in an 8-billion-dollar per year niche market that does not require expensive, high-risk *de novo* drug creation (171). Rituximab biosimilars have thus become an appealing development opportunity for companies in countries such as India and South Korea (172).

TABLE 3 | Comparison of rituximab and biosimilars: years, phases of research, estimated costs, and market.

Considerations	Rituximab	Biosimilars
Time (years) (170, 230)	7–12	3–5
Phases of research (231)	Discovery, development, preclinical, and clinical trial phases I–III consecutive	Development, preclinical, and phase I and III
Estimated cost (232)	1 billion	100 million
Total market (233)	85.4 billion	

BCD-020

BCD-020 is a biosimilar with the trade name of AcellBia. It is the first mAb biosimilar developed in Russia (173). Data reportedly suggest BCD-020 is comparable to the parent drug with regard to PK/pharmacodynamics (PD), safety, and efficacy. However, these results and those regarding the clinical studies are not publicly available (174). Regardless of the lack of transparency, biosimilar production companies are emerging and increasing competition on a global scale. BCD-020 development has created increased competition between biotech companies in Russia (Biocad) and the US (Genentech/Roche). Although Russia is less established in the biotechnology market, they have a financial advantage, with a highly educated workforce and low employment costs relative to the US (175). Competition such as this may cause the price of parent and biosimilar products to decrease, although regulatory standards ensuring a high-quality biosimilar product must also be considered.

CT-P10

Also known as Truxima™, this is the first biosimilar to be granted marketing authorization by the EU, in 2016 (159). A phase I and phase III trial of CT-P10 was done to confirm safety, similar PK/PD, and efficacy in RA patients (176). There were no significant differences between CT-P10 and rituximab, and CT-P10 was also tested in untreated advanced stage FL patients. Patients were randomized to either R-CVP ($n = 70$) or CT-P10-CVP ($n = 70$) for eight cycles, and the primary endpoint was response rate. PK/PD was also monitored in a subset of patients and safety was assessed in all patients (177). The ORR was 97.3% in the CT-P10-CVP group and 92.6% in the R-CVP group, meeting the endpoint for non-inferiority (177). PK/PD and safety measures were also similar between the two groups (177). These studies led to the approval to market CT-P10 by the EMA for all rituximab indications. It is important to note that extrapolation of treatment indications beyond the tested patient populations is permissible by the EMA and FDA, based on the totality of the data and the diversity of disease populations tested in clinical trials used for the approval application. This is likely why one autoimmune and one oncologic disease population were studied in CT-P10 clinical trials. Application for FDA approval of CT-P10 has been submitted and is pending.

GP2013

Also known as Rixathon™, this biosimilar is also approved for use in the EU, and is the second anti-CD20 biosimilar for which an FDA application has been submitted in the US (along with CT-P10, above). Clinical studies have included a PK/PD study in RA, a phase III study in RA (178), and a confirmatory safety and efficacy phase III study in FL (179). In the ASSIST-FL study, 629 untreated, advanced FL patients were randomly assigned to either R-CVP or GP2013-CVP for eight cycles, followed by 2 years of monotherapy mAb maintenance in responders. ORR, the primary endpoint, was 87% with GP2013 and 88% with rituximab. Safety profiles were similar in the two groups as well.

It is noteworthy that both GP2013 and CT-P10 were approved without PFS efficacy results being reported, indicating that response rate is a sufficient surrogate endpoint in rituximab biosimilar studies.

HLX01

HLX01 is the biosimilar closest to approval in China and has been tested in clinical trials both in DLBCL and in severe RA. The first clinical trial in 2015 determined the PK and PD of this biosimilar relative to rituximab (180). Afterward, the effects of HLX01 and rituximab were compared in patients with CD20-positive B-cell lymphomas (181). In 2016, CHOP with HXL01 was compared with CHOP and rituximab in DLBCL patients, to ensure similar efficacy (182). Lastly, a fourth clinical trial in phase I/II testing the efficacy of HLX01 in patients with severe RA is scheduled to be completed in 2018 (183).

Reditux

This is the world's first biosimilar and was launched in 2007, before the rituximab patent expiration date (184). Like Russian biosimilar companies, India is also contributing to affordable pricing and reduced dependence on foreign imports in their country by producing their own biosimilars. The combination of the WHO publishing standards for the biosimilar evaluations (185) and the need for studies on post marketed products (186) led to a retrospective study in 2013. Response rates, toxicity, progression-free survival, and overall survival for 173 DLBCL patients (101 treated with R-CHOP; 72 treated with Reditux-CHOP) were compared, and were similar in all respects (187). In 2016, another study was reported assessing the PK in 21 DLBCL patients treated with Reditux-CHOP, and results suggested that Reditux has a similar PK relative to rituximab (188). However, data from that study demonstrated a decrease in the estimated central volume of distribution relative to rituximab by 68–76% (189). Tout et al. hypothesized there could be one of two reasons for this: either there was an alteration due to differences in tumor burden or to a dissimilarity in the methods used to compared PK in rituximab and Reditux. Further prospective studies will likely be required to establish equivalent potency and efficacy prior to approval in the US or Europe, but Reditux is already increasing accessibility in Asia, Latin America, and the Middle East (155).

Many other rituximab biosimilars, including BI 695500, Kikuzubam, SAIT101, and TL011, halted development prematurely due to either changes in regulatory standards, strategic marketing decisions, and/or the health of the economy (**Table 1**).

Transparency

There is a lack of public information available for some of the biosimilars listed above, particularly regarding how data is collected, analyzed, and compared with rituximab. Increasing the transparency of biosimilar development may help support the overall claim that these biosimilars are equivalent in efficacy to rituximab while still being a cheaper treatment option. Given that there are biosimilars produced all over the world, it would be helpful if international regulatory standards be aligned as much as possible. More universal biosimilar drug development and approval processes may result in further decreasing the price of biosimilar mAbs by increasing global access to them, along with comfort in the approval process. Educating prescribing physicians about biosimilars and the approval process is another important component that will determine the level of biosimilar uptake in various markets.

CONCLUSION

As the first mAb approved for oncology treatment, rituximab is an important milestone in the age of immunotherapeutics and is currently used to treat the majority of B-cell NHL as a monotherapy or in combination with conventional lymphoma therapies. Its use has substantially improved the outcome among all B-cell lymphoma patients. Rituximab has paved the way for immunotherapy biologic discovery, regulatory pathways, and clinical practices; and it is now indirectly outlining how the world deals with biosimilar development in the field of oncology.

Despite rituximab's long history of successful application, much remains to be discovered. Like other mAb therapies, rituximab facilitates cell killing through various mechanisms including direct signaling of cell death as well as immune-mediated responses such as CDC, ADCC, and ADP. However, we do not yet know which of these mechanisms play the most significant role *in vivo*, nor do we understand why only a subset of patients achieve a durable response. Furthermore, we do not yet know the ideal dosage schedules, and many *de novo* anti-CD20's have been approved for different dosing, which makes direct comparison more difficult. We also lack biomarkers to reliably predict which patients will benefit from rituximab, or even which patients are benefiting from its inclusion in combination therapies. These gaps in knowledge surrounding rituximab make assessing next generation anti-CD20 therapies and rituximab biosimilars a challenging goal, providing opportunities for improvement as the relative efficacies of those new mAbs are evaluated.

The field of anti-cancer immunotherapies continues to deliver powerful new treatment options beyond mAb therapies. However, the areas that are still poorly understood are being actively studied and represent a potential to improve rituximab, and possibly all mAb therapies, with the end goal of making them cheaper, more accessible, and improving their efficacy for the largest number of patients possible.

AUTHOR CONTRIBUTIONS

TP, CL, and KR all contributed to the writing and editing of this manuscript.

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REVIEW
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Cancer Immunotherapy and the Immune Response in Hodgkin Lymphoma

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Patients with classical Hodgkin lymphoma (cHL) have an impaired cellular immune response as indicated by an anergic reaction against standard recall antigens and a diminished rejection reaction of allogeneic skin transplant. This clinical observation can be linked to the histopathological feature of cHL since the typical pattern of a cHL manifestation is characterized by sparse large CD30$^+$ tumor-infiltrating Hodgkin–Reed–Sternberg (HRS) cells that are surrounded by a dense inflammatory immune microenvironment with mixed cellularity. Despite this extensive polymorphous inflammatory infiltrate, there is only a poor antitumor immune response seen to the neoplastic HRS cells. This is primarily mediated by a high expression of PD-L1 and PD-L2 ligands on the HRS cell surface which in turn antagonizes the activity of programmed death-1 (PD-1) antigen-positive T cells. PD-L1/L2 overexpression is caused by gene amplification at the 9p24.1 locus and/or latent Epstein–Barr virus infection present in around 40% of cHL cases. The blockade of the PD-L1/L2–PD-1 pathway by monoclonal antibodies can restore local T cell activity and leads to impressive tumor responses, some of which are long lasting and eventually curative. Another feature of HRS cells is the high CD30 antigen expression. Monoclonal antibody technology allowed for the successful development of CD30-specific immunotoxins, bispecific antibodies, and reprogrammed autologous T cells with the first one already approved for the treatment of high risk or relapsed cHL. Altogether, the discovery of the described pathomechanism of immune suppression and the identification of preferential target antigens has rendered cHL to be a prime subject for the successful development of new immunotherapeutic approaches.

Keywords: Hodgkin lymphoma, monoclonal antibodies, bispecific antibodies, immunotoxins, check-point blockade inhibitors, chimeric antigen receptors

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BIOLOGY OF CLASSICAL HODGKIN LYMPHOMA (cHL)

Hodgkin lymphoma (HL) is a rare lymphoma entity with 3–5 new cases/100,000 inhabitants. The histopathological picture is unique as usually a few (1% or less of all cells) malignant cells called Hodgkin–Reed–Sternberg (HRS) cells are surrounded by a strong inflammatory cellular component (1, 2). The survival of HRS is highly dependent on the interaction with surrounding inflammatory cells since they do not survive as single cells when taken into cell culture (3, 4). The composition of the inflammatory cell compartment can vary substantially and defines the four histopathological subtypes of cHL (1). The origin of HRS cells was debated controversially and it is believed nowadays that they originate from germinal center B cells although they lack most B cell

markers (5, 6). The recognition of HRS cells by the immune system such as cytotoxic T cells or T helper (Th) cells is dampened as they frequently downmodulate MHC-I and MHC-II molecule expression (7). In addition, they produce cytokines such as CCL5 and MIF that attract macrophages and mast cells. HRS cells stimulate M2 macrophages to produce MIF which in turn stimulates HRS cells through binding to the constitutively expressed CD74 antigen to even increase MIF production (4). In addition, HRS cells secrete CCL17 and CCL22 that recruit immunosuppressive Tregs into the cHL microenvironment which support the evasion of an immune attack (8). These cellular and soluble factors contribute to the special immune-evasive phenotype of cHL that is orchestrated to a large extent by HRS cells and could explain why attacking HRS cells might restore immunological control.

IMMUNE DEFICIENCY AND IMMUNE EVASION IN HL

It has been known for a long period of time that viral and fungal infections are increased in patients with cHL (9). In parallel, cHL patients have a decreased delayed type hypersensitivity reaction and were shown to be anergic against standard recall antigens including a diminished rejection reaction for allogeneic skin transplants (10, 11). A vast body of literature has accumulated on different aspects of a depressed T lymphocyte response *in vitro* to phytohemagglutinin, concanavalin A (Con A), and pokeweed mitogen (12, 13). These functional abnormalities correlate with the severity of the disease and are of prognostic relevance. The T-cell deficiency in cHL was assumed to be caused by qualitative defects as lymphocyte counts in cHL patients did not differ significantly from healthy controls. The qualitative defects were detected by a decreased proliferative response on stimulation with standard mitogens and the secretion of significantly lower amounts of interleukin-2 (IL-2) (14–17). The reduced IL-2 levels could not be explained by reduced IL-2 receptor expression in T cells from cHL patients. The observation of a decreased activity of the enzymes adenosine deaminase and 5′ nucleotidase in Hodgkin T cells, both essential for adequate T-cell proliferation, supported the hypothesis that cHL patients have an intrinsic defect for enzymes with relevance for T-cell function (18, 19). The same enzyme defect was found in other Epstein–Barr virus (EBV)-positive tumors, and it was speculated that the EBV infection was the common causative link for the observed immunodeficiency. Already at that time, researchers believed that HRS cells would express certain molecules, either as soluble factors or membrane bound that hampers the efficacy of T cell-mediated antitumor immune responses. For example, HRS cells express the immunoregulatory glycan-binding protein, galectin-1, which supports a Th2 regulatory immunosuppressive tumor microenvironment (20). Nowadays, we believe that the detection of variable amounts of programmed cell death-1 ligand 1 (PD-L1, also known as B7H1 or CD274), and later on of PD-L2 (B7DC or CD273) expression on primary HRS cells with high level of expression of the counter-receptor, programmed death-1 (PD-1) on surrounding T cells is the clinically most relevant finding explaining the immunosuppressive tumor environment (21). Once the link of PD-1/PD-L1-mediated immunosuppression in cHL was established, a potentially effective immunologic strategy for the treatment of cHL was postulated. The hypothesis was supported by laboratory evidence as bulk cHL tumor cells cultured in the presence of anti-PD-L blocking antibodies produced increased amounts of IFN-γ. Furthermore, PD-L blockade was accompanied by the inhibition of SHP-2 phosphorylation known to be a mediator of the PD-1 signaling pathway (22). In turn, depletion or enrichment of T-cell subsets from cHL cell suspension indicated that PD-L blockade restored primarily the function of $CD4^+$ T cells of cHL which were already known to be the primary cells of contact surrounding HRS cells in cHL tissue. Following theses data, it was postulated that the antitumor activity of HL-infiltrating T cells was inhibited *via* the PD-1–PD-L signaling pathway, and that this inhibition could be successfully overcome by the use of PD-1/PD-L blocking antibodies.

High PD-L1 expression on HRS cells is caused by a structural amplification on chromosome 9, locus 9p24.1 which leads to a higher expression of PD-L1 and, to a lesser extent, PD-L2 protein (23). The high expression level of PD-L1 is explained by increased JAK2 signaling which further augments PD-1L expression in cell lines with 9p24.1 amplification. Therefore, JAK2 inhibition might be the next rational therapeutic target alone or in combination with PD-1 blockade. This hypothesis is supported by laboratory evidence using commercially available JAK2 inhibitors, demonstrating an excellent correlation between the doses required to inhibit phospho-JAK2 and decreased PD-L1 transcription which reduces the proliferation of cHL cell lines (21). These data may explain the cellular immunodeficiency seen in cHL patients and, moreover, support the further evaluation of PD-1 blockade and JAK2 inhibition, alone and in combination, in patients with cHL characterized by 9p24.1 amplification and its associated targets.

IMMUNOTHERAPY OF HL

Identification and Characterization of Potential Target Antigens

The search for target antigens in cHL has resulted in the identification of different molecules with most of them belonging to either the group of lymphocyte (activation) antigens (e.g., CD25, CD30, CD40, and CD80) (24, 25) or molecules of unknown function at the time of discovery (e.g., IRac) (26). Although CD25 antigen and IRac were used in initial studies as potential target molecules, the CD30 antigen is nowadays accepted as probably the best and most reliable marker for the identification of Reed–Sternberg cells. As a consequence, the CD30 antigen is used as target molecule for the treatment of cHL despite its expression in other malignant diseases such as some subtypes of non-HLs, embryonal carcinomas, malignant melanomas, and mesenchymal tumors (24). In addition, CD30 antigen expression is upregulated in some autoimmune diseases as well. The molecular cloning of the extracellular domain of CD30 antigen was done more than 20 years ago (27), and the sequence indicates that it belongs to the nerve growth factor receptor (NGFR)

superfamily (28). The CD30 antigen constitutes a 120 kDa type I transmembrane glycoprotein of 578 amino acids and shares common features with TNFR-I, TNFR-II, and NGFR factors, respectively. Biochemical studies of the CD30 molecule provided strong evidence for a signal-transducing role since all CD30 forms are phosphorylated at serine and/or tyrosine residues and its intracellular component possesses kinase activity (29). CD30 signaling activates NF-κB and ERK1/2 and, in some studies, supports the survival of cHL cells (30). Following this observation, it has been speculated for some time that CD30 plays a key role in antiapoptosis and cytokine expression leading to the characteristic histopathological pattern of cHL (31). However, how CD30 contributes to the intracellular signaling network of HRS cells has not been thoroughly investigated. A link between chaperone proteins such as heat shock proteins (HSPs) and CD30 antigen was recently established. Signaling through the CD30 antigen facilitated the phosphorylation of heat shock factor 1 and activated the heat shock promoter element which in turn induced HSP 90 expression (30). The authors could demonstrate that CD30 repression and subsequent inhibition of HSP90 suppressed NF-κB, extracellular signal-regulated kinase, AKT, and STAT pathways in some cHL cell lines. Thus, CD30-mediated induction of HSP90 might serve as a central hub for the integration of intracellular signaling in cHL cells (30).

External CD30 antigen stimulation by soluble recombinant CD30 ligand seems to have a counteractivity on cell survival since the growth of human T-cell lymphoma cell lines *in vitro* is inhibited by apoptosis (32, 33).

Development of Monoclonal CD30-Specific Antibodies

The CD30 antigen was originally identified on cultured HRS cells using the monoclonal antibody (Mab) Ki-1 (34). Since overexpression of the CD30 antigen has first been described for cHL, CD30 antigen-specific Mabs were originally raised against cell lines from this entity and most Mab-based studies have been performed in this entity (35). The first-generation CD30 antigen-specific Mabs raised in the 1980s (e.g., Ki-1, BerH2, and HRS1–4) (36) had no effect on cultured HRS cell lines and showed no signs of activity in early clinical trials. However, they demonstrated rapid cellular internalization after binding to the CD30 antigen. Their potential therapeutic role was believed to be outside of the so-called unconjugated antibody field and more in the area of delivery vehicle for cytostatic drugs (37), plant toxins (38), or a number of chemically linked immunotoxins (ITs) with some of them being developed and evaluated for clinical application (39–41) as delineated in the following paragraphs. The clinical development of these antibodies was supported by biodistribution studies in cHL patients (42), performed in the early 1990s. In these trials, specific tumor targeting with positive imaging could be confirmed for the first-generation CD30-specific antibody HRS-3. As a consequence, this antibody was used by our group as the tumor-targeting backbone for the development of different constructs.

However, at the same time, other groups had developed second-generation CD30-specific antibodies recognizing different CD30-epitopes and could demonstrate *in vitro* activity by growth inhibition of cultured cell lines and, in some instances, direct *in vivo* efficacy by reduced growth of tumor xenografts in SCID mouse models. This effect was not observed with first-generation CD30-specific antibodies such as BerH2. The precise mechanism underlying this inhibition remained unknown, and the authors speculated that the second-generation CD30-specific Mabs were directed against the CD30-ligand-binding site and, therefore, might directly affect antigen–ligand interaction resulting in impaired cell growth (43). However, conflicting data were published subsequently since some second-generation Mabs such as 5F11 activated the NF-κB pathway and the antiapoptotic protein cellular FLICE (Fas-associating protein with death domain-like interleukin-1β-converting enzyme) inhibitory protein (c-flip) causing apoptosis resistance and, thus, limiting the potential clinical use of 5F11. To overcome this resistance, 5F11 had to be combined with proteasome inhibitors such as bortezomib and this combination demonstrated a synergistic cytotoxic effect *in vitro* and in a human cHL xenograft model provided that 5F11 preceded bortezomib treatment (44).

Nevertheless, the data on the second-generation CD30-specific Mabs sparked renewed interest in the clinical use of unconjugated CD30-specific antibodies and resulted in multiple clinical trials treating relapsed and refractory patients with $CD30^+$ lymphomas with CD30-specific Mabs. These trials have evaluated primarily chimeric or even fully human antibodies such as cAC10 (SGN-30) or 5F11 (MDX-60), respectively.

Clinical Development of SGN-30

The chimeric CD30-specific cAC10 Mab (SGN-30) was tested in a pivotal phase II study for efficacy after having passed classical dose-escalation phase I protocols without dose-limiting toxicity (45). In this trial, the objective response rate (ORR) was 17%, reaching 25% for the 28 anaplastic large cell lymphoma (ALCL) patients who had received at least one full course of SGN-30. Although a meaningful number of cHL patients (29%) achieved a stable disease (SD), no objective responses (ORs) to SGN-30 were observed. The dose of SGN-30 was increased to 12 mg/kg on weekly administration following an interim analysis of the safety data. Again, with a limited number of patients in each group, no relationship between antitumor activity or safety and dose level in either cHL or ALCL patients was seen, respectively. The authors speculated that the modest antitumor activity of SGN-30 in ALCL with almost no effect in cHL patients may reflect the limited number of $CD30^+$ HRS cells accessible in cHL tumors which are significantly less per tumor volume when compared with the homogeneous expression of $CD30^+$ lymphoma cells in ALCL tumors. The collective results of this phase II study demonstrated that SGN-30 administered to patients with relapsed or refractory cHL and systemic ALCL was well tolerated since only three of 79 patients (4%) presented with hypersensitivity or allergic reactions, respectively. Therefore, the acceptable safety profile of SGN-30 and the modest observed antitumor activity supported the use of this agent as antibody drug conjugate.

Clinical Development of MDX-060

The fully human CD30-specific Mab MDX-060 (5F11, iratumumab) was tested in a similar setting, and doses up to 15 mg/kg

were administered without dose-limiting toxicity (46). Although clinical responses were seen in both patient subgroups (cHL and ALCL) at most dose levels ≥1 mg/kg, the ORR was only 8%. Two (28%) of the seven ALCL patients had a response, compared with 6% of patients (4 of 63) with cHL. Disease stabilization was observed in 35% of patients, similar to one seen in SGN-30 trials. Four of six responding patients had received corticosteroids while on study making it difficult to attribute the efficacy observed to the antibody alone. Once again, the results of this study indicate that MDX-060 administration to patients with relapsed or refractory CD30 expressing lymphomas was well tolerated. However, due to its limited clinical value, the future development of MDX-060 was abandoned.

Antibody-Based Radio-Immunotherapy (RIT)

As described earlier, biodistribution trials using radiolabeled first-generation CD30-specific HRS-3 Mab had demonstrated favorable uptake in cHL tissue, and the development of radioimmunoconjugates (RICs) was a logical next step (47). The rational for RIC is supported by the well-known clinical experience of high sensitivity of cHL to ionizing radiation. In a pivotal phase I/II clinical trial, 22 patients with biopsy proven $CD30^+$ cHL were included. Most patients presented with advanced-stage disease (19 of 22 patients) and were heavily pretreated with a median of four different prior chemotherapy regimes (range, 2–6) including high-dose chemotherapy (HDT) and autologous stem-cell transplantation (ASCT) in 16 of 22 patients. Iodine-131 was selected as radioisotope since it is readily available and allows for dosimetry after trace doses of the RIC (day 1) followed by a therapeutic dose on day 8. As reported by the authors, results were disappointing since visualization of tumor masses was seen only in a minority of patients (23%). Moreover, measurable tumor responses were limited and included one CR, five PRs, and three MRs, which lasted for a median of 4 months. In general, acute toxicity was mild with transient fatigue in 86% and nausea in 23% of patients. The most relevant toxicity in this heavily pretreated patient population was severe myelosuppression as seen in 33% of all patients. Against expectations, there was neither a correlation between toxicity observed and number of prior treatment lines, administered whole-body dose or laboratory values prior to treatment preventing the definition of a most optimal dose for further studies. As a consequence, RIT in HL has not been further explored.

Antibody-Based ITs

Delivery of highly cytotoxic reagents by an antibody construct at the tumor site has been an attractive concept for quite some time and was supported by the availability of first-generation CD30-specific antibodies with favorable tumor-targeting properties in cHL patients. As part of early laboratory studies, Engert and colleagues had analyzed five CD30-specific Mabs antibodies and two derived Fab′ fragments linked to deglycosylated ricin A chain (dgA) for their potential to act as ITs for the treatment of cHL (48). Once again, the first-generation CD30-specific Mab HRS-3 turned out to be the most optimal candidate based on its high tumor antigen affinity (K_d 15 nM) and high activity as measured by inhibition of protein synthesis of L540 cHL cells by 50% [$0.9 \times 10(-10)$ M]. HRS-3.dgA was chosen as the preferred IT as it was only 15 times less toxic than the toxin ricin itself (49). HRS-3.dgA was later replaced by an even more potent IT (Ki-4.dgA) which was five times more potent *in vitro* and displayed high efficacy in the treatment of disseminated human cHL when studied in SCID mice xenografts (50). Thus, Ki-4.dgA was selected for a clinical phase I trial in 16 patients with refractory $CD30^+$ lymphoma (25). The maximal tolerated dose (MTD) was lower than expected and established at 5 mg/m^2. The authors speculated that binding of the IT to sCD30 and prolonged persistence of sCD30/IT complexes in the blood might have been a factor contributing to higher toxicity. Dose-limiting toxicities were hypoalbuminemia, weight gain, tachycardia, hypotension, dyspnea, weakness, and fatigue. Additional side effects included myalgia, nausea, and vomiting. Response rates were moderate with one PR, one MR, two SD, which is similar to other studies using, for example, CD25.dgA constructs in a similar patient population. More importantly, 7 of 17 patients (one patient with ALCL) developed human-anti-Ricin-A antibodies (HARA) and in 1 of 17 patients human-anti-mouse-antibodies (HAMA) against the antibody backbone were detected. Both, HAMA and HARA might limit the number of applicable IT courses and prevent further treatment cycles. At that time, it was clear that the future development of antibody-based ITs for cHL treatment needed improvement in three major areas:

1. Less immunogenic antibodies (or their fragments) of either chimeric or human/humanized nature
2. Less immunogenic but still very potent toxin compound
3. Optimal conjugation (the so-called linker) between the antibody and toxin moiety. The linker should be stable enough to prevent unwanted toxin release from the antibody in blood circulation but still allow for rapid toxin release once the antibody construct had been internalized by the HRS cells.

Brentuximab Vedotin (BV)

It took a long time and huge effort to achieve the three aforementioned goals until a clinically successful IT construct was established: the antitubulin agent monomethyl auristatin E (MMAE) was attached to the already mentioned CD30-specific Mab cAC10 by an enzyme-cleavable dipeptide linker generating the antibody–drug conjugate BV (SGN-35) (51). The antibody–drug conjugate is rapidly internalized after binding to the CD30 antigen and transported to lysosomes, where the peptide linker is selectively cleaved. The toxin MMAE is then released into the cell, binds to tubulin, and prompts cell cycle arrest between the Gap 2 phase and mitosis (G2/M) leading to cell apoptosis (51).

BV Treatment of Relapsed and/or Refractory HL Patients

After successful preclinical tests demonstrating high and selective activity against $CD30^+$ tumor-cell lines *in vitro* and *in vivo* xenograft models, a phase I, open-label, dose-escalation trial was initiated (52). Of the 45 patients treated, 42 had cHL, 2 systemic

ALCL, and 1 CD30+ angioimmunoblastic T-cell lymphoma. As characteristic for cHL patients with relapsed and/or refractory disease, patients were of young age (36 years; range, 20–87) and had undergone multiple lines of prior treatment (median of three previous chemotherapy regimens with a range from 1 to 7). In addition, 33 patients (73%) had undergone previous HDT followed by ASCT. In contrast to previous IT trials in cHL, tumor responses were observed in the majority of patients treated with BV with tumor regression in 86% of patients. Tumor-related symptoms ameliorated in 81% of those in whom such symptoms were present at the time of treatment initiation. Seventeen patients achieved an OR including 11 CRs. Six of 12 patients (50%) receiving the maximum tolerated dose had an OR suggesting a potential relationship between administered dose and efficacy. Remissions were durable in this patient population who had relapsed or refractory disease at study entry. The median duration of response (DOR) was at least 9.7 months.

Side effects included mainly grade one or two fatigue, pyrexia, diarrhea, nausea, neutropenia (with one grade three event), and peripheral neuropathy at the MTD. Standard supportive care controlled most adverse events that were typically of grade 1 or 2. Clinically relevant is the cumulative, dose-related grade 1 or 2 peripheral neuropathy caused by the MMAE toxin as potent antitubulin agent. This toxicity is known to be a class effect of microtubule inhibitors (53).

The phase I trial was followed by a multicenter phase II trial of BV monotherapy in a total of 102 patients with relapsed or refractory cHL (54). Patients were treated with BV 1.8 mg/kg by intravenous infusion every 3 weeks. Patients received a maximum of 16 cycles in the absence of disease progression or prohibitive toxicity. Tumor reductions were common and seen in 94% of all patients treated. As confirmed by independent review, 75% of patients achieved an OR with 34% obtaining a CR. The median progression-free survival (PFS) was 5.6 months, and the median DOR for CR patients was 20.5 months. Thirty-one patients were still alive and free of documented progressive disease after a median observation time of more than 1.5 years. The cohort of patients included in the trial was particularly refractory to prior treatments as evidenced by the fact that 71% of patients did not achieve a CR or had experienced a relapse within 3 months following frontline therapy. Furthermore, these patients had a poor prognosis because the median time to relapse after HDT + ASCT was only 6.7 months. In this context, the OR rates and durable CRs are quite impressive for a single-agent therapy considering the failure of prior combination chemotherapies including HDT plus ASCT. It is a common observation that each successive treatment delivered to a patient with relapsed lymphoma results in diminishing remission times, usually cut by half with every additional line of treatment. Therefore, it was encouraging to see that the PFS achieved with BV was significantly longer than the one achieved with the most recent prior therapy in the subset of patients who had received a systemic therapy after HDT plus ASCT.

In this study, BV was administered for a maximum of 16 cycles; the actual median and mean durations of treatment were 9 and 10 cycles, respectively. Although the majority of responses occurred early in the course of treatment, one CR was initially documented after approximately 1 year of therapy. Eighteen patients received all 16 treatment cycles. One has to admit that the optimal treatment duration is unknown and not answered by the present trial. However, peripheral neuropathy is usually the most frequent dose-limiting side effect making it in general quite unlikely that treatment is continued even beyond 16 cycles. Peripheral neuropathy typically develops after prolonged BV exposure with a median onset of grade 2 at 27.3 weeks (eight to nine treatment cycles). Peripheral neuropathy was largely reversible since dose reductions or even cessation of treatment was done promptly (54). Twenty-three percent of all patients entering the trial had already existing peripheral neuropathy as they had been exposed to neurotoxic drugs in their previous lines of treatment.

In summary, these two pivotal phase I/II trials were a major achievement in the process of establishing IT for the treatment of relapsed/refractory cHL since they had demonstrated and confirmed the safety and efficacy for the chosen compound. Moreover, they had a significant impact beyond cHL treatment since they laid the ground for the development of additional ITs in other tumor entities. From this trial onward, efficacy and to some extent dosing for BV was established and drug development to find the most optimal setting for clinical use of BV was started.

BV Consolidation in cHL Patients After High-Dose Chemotherapy and Autologous Stem Cell Support

Since BV is effective in detecting and eliminating CD30+ cells, concepts for an early use of BV at the stage of minimal residual disease (MRD) were developed. One approach of using BV as consolidation treatment in cHL patients at high risk of relapse after HDT plus ASCT was tested in the AETHERA trial (55). As generally seen in aggressive lymphoma, relapse or progression after front-line or even second-line treatment including HDT plus ASCT happens generally early. After HDT plus ASCT, 71% of progression events are observed within 1 year of transplant, and 90% will happen within 2 years. Therefore, patients passing the 2 years' time period without relapse have usually a high change of being cured (55).

The results from the AETHERA trial demonstrated that consolidative treatment with BV compared with placebo provided a statistically and clinically significant improvement in PFS. By independent review, the estimated proportion of patients who were alive, and progression free was 63% with BV vs. 51% with placebo at 24 months. By investigator assessment, the estimated 24 months PFS data were very similar with 65 and 45%, respectively. Overlooking 108 patient-years of follow-up, only four PFS events were detected after the 24 months assessment period. In addition to the sustained clinical benefit of BV consolidation, more patients needed subsequent antitumor therapies in the placebo group than in the BV group, including nearly twice as many allogeneic stem-cell transplantations (55). These data suggest that the early use of BV after HDT plus ASCT can control MRD and might be beneficial in the long term since it might spare subsequent, sometimes quite intensive treatments.

The PFS benefit for BV treated patients was seen across all prespecified subgroups, including primary refractory patients

and patients who had relapsed less than 12 months after frontline therapy (55). These two patient cohorts are generally seen as the ones with the worst overall prognosis and low chances of being cured by subsequent therapies. Compared with historical survival data for high-risk patients with Hodgkin's lymphoma undergoing HDT plus ASCT, the 3-year OS rate in this study was remarkable exceeding 80% and underlined the clinical benefit of BV treatment as consolidation therapy and as rescue therapy, respectively. However, the study did not conclusively answer the question if BV should be used in all cHL patients after HDT plus ASCT to control MRD or if it should be spared for relapsing patients as salvage therapy. It is tempting to speculate that early BV consolidation will result in a reduced number of progression events and more patients might be cured with consolidation therapy. We think it is fair to agree with the authors that reduced numbers of patients will need subsequent toxic therapy for active disease including allogeneic stem-cell transplantation.

BV First-Line Treatment in Patients With Advanced cHL

Very recently, data from the randomized phase III ECHELON-1 trial were presented at the ASH meeting 2017 and published (56). The trial compared BV as part of a combination chemotherapy regimen (BV plus doxorubicin, vinblastine, and dacarbazine; A + AVD) against the standard ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine) chemotherapy in cHL patients with advanced stage (III or IV) disease and had not been previously treated with systemic chemotherapy or radiotherapy. The ECHELON-1 trial met its primary endpoint and showed a statistically significant improvement in a so-called modified progression-free survival (mPFS) endpoint. The 2-year rate of mPFS was 82% in the A + AVD group [95% confidence interval (CI), 78.7–85.0] compared with 77% (95% CI, 73.7–80.4) in the ABVD group. The hazard ratio for progression, death, or modified progression was 0.77 (95% CI, 0.60–0.98; $P = 0.03$).

From today's perspective, it is obvious that BV will soon be incorporated into standard first-line chemotherapy regimen of cHL. However, it is not yet clear if the combination partner will be AVD as in the present trial or other regimens such as BEACOPP variants. cHL patients with advanced-stage disease in need of a frontline chemotherapy are currently studied with BV in the BrECADD HD21 regimen as part of the German HL study group (NCT02661503).

Bispecific Antibodies

Bispecific antibodies (BiMabs) are typically designed to bind simultaneously to the tumor cells and to a trigger receptor on immune effector cells (57), for example the FcγRIII (CD16) on natural killer (NK) cells or CD3 receptor on T cells, respectively. For the treatment of cHL, we had established NK- and T cell stimulating BiMabs and demonstrated their efficacy *in vitro* and *in vivo* using different xenograft mouse models (58–62).

The CD30/CD16 BiMab HRS-3/A9 was chosen for clinical development and produced under GMP conditions confirming the general applicability of this approach. Fifteen patients with refractory cHL were treated in a phase I/II trial with the BiMab HRS-3/A9 (62). The BiMab was administered four times every 3–4 days, starting with 1 mg/m^2. The treatment was well tolerated, and the MTD was not reached at the highest dose administered (64 mg/m^2) because of limited amounts of available antibody. BiMab HRS-3/A9 induced no DLT but only short-lasting mild to moderate side effects occurred in a minority of patients. Nine patients (60%) developed an HAMA response as determined by ELISA 4 weeks after treatment. At that time, a second treatment cycle was intended in four patients, of whom three were HAMA-positive. As defined in the protocol, HAMA-positive patients were challenged with intracutaneous BiMab HRS-3/A9 application, and one patient who had presented with an allergic skin rash after the first BiMab treatment cycle developed a marked skin reaction with erythema and induration and, therefore, was excluded from further treatment. The remaining three patients with negative skin tests received a second BiMab infusion at the dose level that they had tolerated during the first treatment cycle. In all retreated patients (including one who had been HAMA negative), moderate systemic reactions such as shivering, hypotension, low back pain, and chest tightness occurred despite pretreatment with anti-histamins and prednisone leading to the termination of treatment after one to three additional BiMab infusions. In total, the ORR was 33% with one CR and one PR (lasting 6 and 3 months, respectively) as well as three MRs lasting for 1–15 months. Our results emphasized the necessity to reduce the immunogenicity of the murine BiMab construct but at the same time encouraged us to develop this novel immunotherapeutic approach further.

Based on these data, the objectives of a second phase I trial were the evaluation of a modified BiMab application schedule with a prolonged infusion time designed to provide a higher antitumor efficacy and/or a better tolerance of retreatment attempts, respectively (63). Finally, because patients with advanced cHL generally show a severe qualitative and quantitative immunosuppression as outline before, and because the number and degree of activation of NK cells are crucial for this immunotherapeutic approach, the influence of additional cytokine co-stimulation was evaluated. Therefore, patients achieving an SD after the first course of BiMab treatment were scheduled for a second BiMab course adding concomitant IL-2 and GM-CSF as immune stimulation. Infusions were given either as continuous infusion over 24 h on four consecutive days or as a standard 1 h infusion, respectively. In summary, patients had received a total of 27 BiMab courses, including six courses with co-administration of cytokines. At re-evaluation after the first treatment cycle, two PRs and six cases of SD were observed, whereas treatment was stopped in the remaining eight patients because of progressive disease. The cumulative ORR after BiMab treatment was 25%, with one CR lasting for 6 months and three PRs lasting for 3, 5, and 9 months, respectively. Continuous infusion seemed to be the superior application regimens with three of the four OR in this treatment arm. In addition, four disease stabilizations (after documented preceding PD) lasting for 3 to more than 6 months (the latter in a patient finally undergoing allogeneic bone marrow transplantation with a fatal outcome) were observed. Toxicity proved again to be very low, with transient mild to moderate fever as the major side effect occurring in about one-third of

the patients. A BiMab-directed HAMA response occurred in 37.5% of our patients within 4 weeks after treatment. This is in line with incidences between 40 and 84% observed in other clinical trials with murine BiMabs (64, 65) and our previous study (46%) (62). As a result of the two studies, we postulated that redirecting NK cells by CD16-specific BiMab was a promising approach but needed significant technical improvement to obtain an antibody construct with lower or ideally missing immunogenicity, high activity, and good productivity under GMP conditions.

These prerequisites might be fulfilled by a bispecific, tetravalent chimeric antibody construct (TandAb) called AFM13 (66, 67). This antibody construct specifically recruits NK cells since it only recognizes the CD16A isoform on NK cells and does not cross-react with granulocytes. TandAbs have two binding sites for each antigen, but no Fc domains and can be produced at large scale in mammalian cells. Preclinical data have demonstrated a specific and efficient antitumor activity against $CD30^+$ target cells by the engagement of NK cells. After passing extensive preclinical tests, a phase I study with AFM13 in heavily pretreated cHL patients who had received all standard therapies was initiated (66). Since no appropriate *in vivo* model for safety and efficacy was available, AFM13 dosing started at very low levels and was then gradually escalated by 700-fold. Treatment with AFM13 was well tolerated at all dose levels and the MTD not reached. Side effects were generally mild with moderate AEs. A PR (11.5%) was seen in 3 of 26 evaluable patients, and 13 patients achieved disease stabilization (50%) leading to an overall disease control rate (DCR) of 61.5%. A dose–response dependency could be seen since 13 patients treated with AFM13 doses of ≥1.5 mg/kg had an ORR of 23% and the DCR was 77%, respectively. Important regarding the most optimal scheduling of IT and BiMab therapies was the observation that AFM13 was also active in BV-refractory patients. As expected, AFM13 treatment resulted in a significant NK-cell activation and a decrease of sCD30 in the peripheral blood. As stated by the authors, AFM13 treatment was safe and demonstrated reasonable activity in this heavily pretreated patient cohort. The results obtained so far warrant further development of this construct at earlier stages of disease or even in combination with other immunotherapeutic approaches as described below.

Check-Point Blockade Inhibiting Antibodies

Check-point blockade inhibiting antibodies have changed the treatment paradigm of many solid organ cancers and revived our belief that the immune system can control and even eradicate cancer cells (68). CTLA-4 and/or PD-1/PD-L1 blocking antibodies have established themselves in the first-line treatment of so far difficult to treat cancers such as advanced-stage melanoma or lung cancer, respectively. cHL was not the prime target for check-point blockade inhibiting antibodies, mainly because effective treatment options at diagnosis and relapsed were available. That changed when the abovementioned association between chromosome 9p24.1 amplification and enhanced expression of the PD-1 ligands on HRS cells was detected. This created a rational link between the immunosuppressive state observed in cHL tissue and a potential therapeutic option by using Mabs against PD-1/PD-ligands blocking the interaction of these molecules and unleashing the immune response against HRS cells. Nowadays, cHL is known as the disease with the highest response rates toward check-point blockade inhibitor treatment and time will tell, if cHL patients at relapsed can be cured by this treatment (69). A first landmark study using the PD-1 blocking antibody nivolumab was published in 2015 (70). In this study, 23 patients with relapsed or refractory cHL had been enrolled. The median age of patients at stud entry was 35 years (range 20–54 years), and 17 patients (74%) had an ECOG performance-status score of 1e. Since first- and second-line treatment in cHL is well established, all patients entering the trial had been extensively pretreated with 87% having received three or more previous treatment regimens. Moreover, 78% of the patients had received BV, and the same number of patients had undergone HDT plus ASCT. The DCR was 87% (95% CI, 66–97), with 4 patients (17%) reaching a CR, 16 patients (70%) a PR, and 3 patients (13%) with disease stabilization. The response rate was unchanged when only patients ($n = 15$) with disease recurrence after HDT plus ASCT and BV treatment were analyzed. All three patients who had not received prior HDT + ASCT but BV treatment achieved a PR leading to a response rate of 100% (95% CI, 29–100). Besides the high ORR, DOR was impressive as well with a PFS of 86% at 24 weeks. Adverse events were similar to the known toxicity profile as seen in solid organ tumors and mainly of grade 1 or 2. The high efficacy of PD-1 blockade in cHL is not restricted to nivolumab alone but has been confirmed for the alternative PD-1 blocking antibody (pembrolizumab) and was shown by a large phase II trial in 210 patients with relapsed and/or refractory cHL (71). The study focused more closely on responses and DOR in three different patient subpopulations as defined by relapse after HDT plus ASCT and subsequent BV treatment (cohort 1); salvage chemotherapy and BV but ineligible for ASCT because of chemoresistant disease (cohort 2); and HDT plus ASCT but without BV after transplantation (cohort 3). Cohort 1 is the more classical group of patients who receive all available treatments but relapse over time. Patients received a flat dose of pembrolizumab 200 mg once every 3 weeks. Overall, the ORR was slightly lower than in the nivolumab trial with 73%. However, the patient composition in the pembrolizumab trial was more unfavorable and more patients with refractory ($n = 170$) or even primary refractory disease ($n = 73$) were included. From a clinical perspective, patients with primary refractory disease need special attention since they can hardly be rescued by any subsequent treatment. It was encouraging to see that the ORR was 79.5% (95% CI, 68.4–88.0) in this patient subgroup. This was even higher than the ORR in patients from cohort (64.2%; 95% CI, 52.8–74.6). With still short follow-up, median OS continued at time of analysis and was not reached. The authors reported a 9 month OS and PFS rates of 97.5 and 63.4%, respectively (71). So far, none of the PD-1 antibody trials could reveal a robust predictive biomarker identifying either those patients who might have the greatest benefit or those where PD-1 blockade is not sufficient and further support by other approaches is needed. One retrospective analysis addressed this issue for cHL patients after

nivolumab therapy and identified MHC class II expression beside PD-L1 as potential marker (72). Positive MHC class II expression on HRS cells was predictive for prolonged PFS in patients who had received the PD-1-blocking antibody >12 months after ASCT. These data would argue for an important role of $CD4^+$ T cell activation by PD-1 blockade as mechanism of action in cHL and, potentially, other PD-L1-positive tumors.

In summary, the success of PD-1 blockade by Mabs has spurred the search for other lymphoma entities with constitutive PD-L overexpression and will change the treatment paradigm in cHL, certainly for patients with primary refractory disease and those with multiple relapse. PD-1 blockade can be combined with other established or novel therapies, and **Table 1** summarizes the ongoing studies of PD-1 blocking antibodies in cHL treatment

TABLE 1 | Ongoing trials in classical Hodgkin lymphoma with programmed death-1 blocking antibodies as listed on http://clinicaltrials.gov.

	Title	Study drug(s)	NCT no.
1	A Study of Safety and Efficacy of Nivolumab and Bendamustine (NB) in Patients With Relapsed/Refractory Hodgkin's Lymphoma	Nivolumab Bendamustine	03343652
2	Treatment With Nivolumab at the Fixed Dose 40 mg (Nivo40) in Patients With Relapsed/Refractory Hodgkins Lymphoma	Nivolumab	03343665
3	A Study of Brentuximab Vedotin Combined With Nivolumab for Relapsed or Refractory Hodgkin Lymphoma	Nivolumab Brentuximab vedotin (BV)	02572167
4	Ibrutinib and Nivolumab in Treating Patients With Relapsed or Refractory Classical Hodgkin Lymphoma	Nivolumab Ibrutinib	02940301
5	Study of Nivolumab in Patients With Classical Hodgkin's Lymphoma (Registrational)	Nivolumab Doxorubicin Vinblastine Dacarbazine	02181738
6	Nivolumab and AVD in Early-stage Unfavorable Classical Hodgkin Lymphoma	Nivolumab Adriamycin Vinblastine Dacarbazine	03004833
7	A(B)VD Followed by Nivolumab as Frontline Therapy for Higher Risk Patients With Classical Hodgkin Lymphoma	Nivolumab Doxorubicin Bleomycin Vinblastine Dacarbazine	03033914
8	A Study of Nivolumab Plus Brentuximab Vedotin in Patients Between 5 and 30 Years Old, With Hodgkin's Lymphoma (cHL), Relapsed or Refractory From First Line Treatment	Nivolumab BV Bendamustine	02927769
9	A Study of Nivolumab Plus Brentuximab Vedotin Versus Brentuximab Vedotin Alone in Patients With Advanced Stage Classical Hodgkin Lymphoma, Who Are Relapsed/Refractory or Who Are Not Eligible for Autologous Stem Cell Transplant	Nivolumab BV	03138499
10	Brentuximab Vedotin and Nivolumab With or Without Ipilimumab in Treating Patients With Relapsed or Refractory Hodgkin Lymphoma	Nivolumab BV Ipilimumab	01896999
11	Nivolumab, Ifosfamide, Carboplatin, and Etoposide as Second-Line Therapy in Treating Patients With Refractory or Relapsed Hodgkin Lymphoma	Nivolumab Carboplatin Etoposide Ifosfamide	03016871
12	Nivolumab and Brentuximab Vedotin After Stem Cell Transplant in Treating Patients With Relapsed or Refractory High-Risk Classical Hodgkin Lymphoma	Nivolumab BV	03057795
13	Nivolumab and Brentuximab Vedotin in Treating Older Patients With Untreated Hodgkin Lymphoma	Nivolumab BV	02758717
14	Nivolumab and Ipilimumab in Treating Patients With HIV Associated Relapsed or Refractory Classical Hodgkin Lymphoma or Solid Tumors That Are Metastatic or Cannot Be Removed by Surgery	Nivolumab Ipilimumab	02408861
15	Safety of Allogeneic Hematopoietic Cell Transplantation (HCT) For Patients With Classical Hodgkin Lymphoma (CHL) Treated With Nivolumab	Non-interventional	03200977
16	Study to Assess the Safety of Nivolumab in the Treatment of Metastatic Melanoma, Lung Cancer, Renal Cancer, Squamous Cell Carcinoma of the Head and Neck, and Chronic Hodgkin Lymphoma in Adults in Mexico	Non-interventional	03161613

(*Continued*)

TABLE 1 | Continued

	Title	Study drug(s)	NCT no.
17	A Study of Brentuximab Vedotin in Adults Age 60 and Above With Newly Diagnosed Hodgkin Lymphoma (HL)	Nivolumab BV Bendamustine	01716806
18	Brentuximab Vedotin With or Without Nivolumab in Treating Patients With Relapsed or Refractory CD30+ Lymphoma	Nivolumab BV	01703949
19	Nivolumab With Epstein Barr Virus Specific T Cells (EB-VSTS), Relapsed/Refractory EBV Positive Lymphoma (PREVALE)	Nivolumab EB-VST cells	02973113
20	Nivolumab With or Without Ipilimumab in Treating Younger Patients With Recurrent or Refractory Solid Tumors or Sarcomas	Nivolumab Ipilimumab	02304458
21	Ipilimumab or Nivolumab in Treating Patients With Relapsed Hematologic Malignancies After Donor Stem Cell Transplant	Nivolumab Ipilimumab	01822509
22	Trigriluzole With Nivolumab and Pembrolizumab in Treating Patients With Metastatic or Unresectable Solid Malignancies or Lymphoma	Nivolumab Pembrolizumab Trigriluzole	03229278
23	Pembrolizumab and Involved Site Radiation Therapy for Early Stage Relapsed or Primary Refractory Hodgkin Lymphoma	Pembrolizumab Involved site Radiation therapy	03179917
24	Study of the Combination of AFM13 and Pembrolizumab in Patients With Relapsed or Refractory Classical Hodgkin Lymphoma	Pembrolizumab AFM13	02665650
25	Study of Pembrolizumab (MK-3475) vs. Brentuximab Vedotin in Participants With Relapsed or Refractory Classical Hodgkin Lymphoma (MK-3475-204/KEYNOTE-204)	Pembrolizumab BV	02684292
26	Study of Pembrolizumab (MK-3475) in Participants With Relapsed or Refractory Classical Hodgkin Lymphoma (MK-3475-087/KEYNOTE-087)	Pembrolizumab	02453594
27	Safety & Efficacy Study of Combination of Pembrolizumab and Lenalidomide, in Patients With Relapsed Non-Hodgkin and Hodgkin Lymphoma	Pembrolizumab Lenalidomide	02875067
28	Pembrolizumab After ASCT for Hodgkin Lymphoma, DLBCL and T-NHL	Pembrolizumab	02362997
29	PET-Directed Therapy With Pembrolizumab and Combination Chemotherapy in Treating Patients With Previously Untreated Classical Hodgkin Lymphoma	Pembrolizumab Dacarbazine Doxorubicin Vinblastine	03226249
30	Pembrolizumab and Combination Chemotherapy in Treating Patients With Relapsed or Refractory Hodgkin Lymphoma	Pembrolizumab Carboplatin Etoposide Ifosfamide	03077828
31	Pembrolizumab and Vorinostat in Treating Patients With Relapsed or Refractory Diffuse Large B-Cell Lymphoma, Follicular Lymphoma, or Hodgkin Lymphoma	Pembrolizumab Vorinostat	03150329
32	Pilot Study of Pembrolizumab Treatment for Disease Relapse After Allogeneic Stem Cell Transplantation	Pembrolizumab	02981914
33	A Study of Pembrolizumab (MK-3475) in Pediatric Participants With an Advanced Solid Tumor or Lymphoma (MK-3475-051/KEYNOTE-051)	Pembrolizumab	02332668
34	Pembrolizumab in Treating Patients With HIV and Relapsed, Refractory, or Disseminated Malignant Neoplasms	Pembrolizumab	02595866
35	ACP-196 (Acalabrutinib) in Combination With Pembrolizumab, for Treatment of Hematologic Malignancies	Pembrolizumab Acalabrutinib	02362035
36	A Trial of Pembrolizumab (MK-3475) in Participants With Blood Cancers (MK-3475-013/KEYNOTE-013)	Pembrolizumab Lenalidomide	01953692
37	Pembrolizumab and Ibrutinib in Treating Patients With Relapsed or Refractory Non-Hodgkin Lymphoma	Pembrolizumab Ibrutinib	02950220
38	Safety Study of SEA-CD40 in Cancer Patients	Pembrolizumab SEA-CD40	02376699

as listed on http://clinicaltrials.gov. These trials cover the full spectrum of combinations with first/second-line chemotherapy, ITs (BV), bispecific antibodies (AFM13), immunomodulators, radiotherapy, and novel TKIs. Hopefully, new combinational therapies will secure the high cure rates seen in cHL with less long-term toxicity and, eventually, replace classical chemotherapy.

CELLULAR THERAPIES: CD30-SPECIFIC CHIMERIC ANTIGEN RECEPTORS (CAR) T CELL CONSTRUCTS

Today, it is impossible to conclude a review on new immunotherapeutic approaches in malignant hematological diseases without addressing new cellular therapies. The reprogramming of (autologous) T cells with CAR has received FDA approval for the treatment of relapsed juvenile B-ALL and DLBCL and is currently evaluated in additional diseases including cHL (73, 74). Again, the CD30 antigen is the most promising target antigen for CAR T cell approaches in cHL, and preliminary *in vitro* (75) and *in vivo* (76) experiments have revealed promising data.

Recently, data from a phase I dose-finding trial with nine patients suffering from relapsed/refractory EBV-negative cHL and ALCL using autologous CD30scFv-CAR T cells were published (77). Clinical responses were seen in three of nine patients (two CR, one continued CR) with additional three patients achieving an SD. CAR T cells were well tolerated, and the highest degree of expansion of circulating CAR T cells was detected within the first week after infusion in a dose-dependent manner achieving the highest values at the third dose level. Seven patients received a second infusion of CAR T cells with one patient receiving a total of four infusions. However, this resulted only in a modest expansion of CD30-specific CAR T cells in the peripheral blood. One explanation for the modest expansion and low persistence of CAR T cells could be the omission of a lymphodepleting chemotherapy before CAR T cell infusion. This procedure is routinely used in CAR T cell studies for leukemia or lymphoma treatment and has increased CAR T cell persistence quite substantially (74). Since single cases have shown a synergistic effect of PD-1 blockade and CAR T cells in PD-L$^+$ lymphoma patients, the combination of CD30-specific CAR T cells and PD-1 blocking antibodies in cHL patients is intriguing and might reveal synergistic activity.

HL: A RARE DISEASE WITH UNIQUE FEATURES

Classical Hodgkin lymphoma is a rare but unique disease and research in cHL has been the pioneer for many medical breakthroughs: cHL was first described in 1832 as deadly disease until the mid-twentieth century. Then, radiotherapy and later on polychemotherapy changed the course of the disease dramatically with nowadays the majority of patients being cured. ITs such as BV were first established for the treatment of relapsed cHL and paved the way for ITs to be accepted as treatment modality in various tumors. Most recently, the immunosuppressive nature of HRS cells was identified to be caused by the constitutive high expression of PD-L, and the rational use of PD-1/PD-L1 blocking antibodies has shown the highest activity in all tumor entities studied so far. With a plethora of therapeutic options, the challenge for future trials will be to design novel study protocols that maintain the high efficacy of their predecessors and abolish the short- and long-term side effects of contemporary standard therapies. Especially, fertility preservation, cardiac and pulmonary toxicity, and lastly neuropathy are issues that need to be addressed. In this regard, collaboration of international study groups will be key to advance treatment in cHL and support clinicians to choose wisely for their patients. Such an effort to combine classical treatment procedures with all the abovementioned immunotherapeutic approaches shall lead to a cure of cHL in all patients with minimal side effects. That would be the next milestone to focus on in the history of cHL.

AUTHOR CONTRIBUTIONS

CR and FS have contributed equally to this work.

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REVIEW
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The Role of Macrophage/B-Cell Interactions in the Pathophysiology of B-Cell Lymphomas

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Macrophages (MPs) are heterogeneous, multifunctional, myeloid-derived leukocytes that are part of the innate immune system, playing wide-ranging critical roles in basic biological activities, including maintenance of tissue homeostasis involving clearance of microbial pathogens. Tumor-associated MPs (TAMs) are MPs with defined specific M2 phenotypes now known to play central roles in the pathophysiology of a wide spectrum of malignant neoplasms. Also, TAMs are often intrinsic cellular components of the essential tumor microenvironment (TME). In concert with lymphoid-lineage B and T cells at various developmental stages, TAMs can mediate enhanced tumor progression, often leading to poor clinical prognosis, at least partly through secretion of chemokines, cytokines, and various active proteases shown to stimulate tumor growth, angiogenesis, metastasis, and immunosuppression. Researchers recently showed that TAMs express certain key checkpoint-associated proteins [e.g., programmed cell death protein 1 (PD-1), programmed cell death-ligand 1 (PD-L1)] that appear to be involved in T-cell activation and that these proteins are targets of other specific checkpoint-blocking immunotherapies (anti-PD-1/PD-L1) currently part of new therapeutic paradigms for chemotherapy-resistant neoplasms. Although much is known about the wide spectrum and flexibility of MPs under many normal and neoplastic conditions, relatively little is known about the increasingly important interactions between MPs and B-lymphoid cells, particularly in the TME in patients with aggressive B-cell non-Hodgkin lymphoma (NHL-B). Normal and neoplastic lymphoid and myeloid cell/MP lineages appear to share many primitive cellular characteristics as well as transcriptional factor interactions in human and animal ontogenic studies. Such cells are capable of ectopic transcription factor-induced lineage reprogramming or transdifferentiation from early myeloid/monocytic lineages to later induce B-cell lymphomagenesis in experimental *in vivo* murine systems. Close cellular interactions between endogenous clonal neoplastic B cells and related aberrant myeloid precursor cells/MPs appear to be important interactive components of aggressive NHL-B that we discuss herein in the larger context of the putative role of B-cell/MP cellular lineage interactions involved in NHL-B pathophysiology during ensuing lymphoma development.

Keywords: B-cell lymphoma, lymphoma-associated macrophages, tumor microenvironment, immune suppression mechanism, macrophages

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INTRODUCTION

Macrophages (MPs) are intrinsic end-stage immune myeloid cells that contribute to homeostasis as well as the body's natural cellular immune and long-term inflammatory responses. As functional immune effector cells, they also serve as important links between the so-called intrinsic and adaptive immune systems that contribute to a biochemical milieu involving complex inflammatory events (1, 2).

Several subtypes of activated MPs exist, and they contribute differently to the immune microenvironment (3, 4). Specifically, MPs differ in their responses to signals from both the adaptive and innate immune systems, with various potential outcomes. Whereas the subtypes of MPs vary extensively, representing highly attenuated responses to different immune cellular events, the opposing forces of classic vs. alternative cellularly activated MPs are of particular interest. Induced by exposure to interferon-γ in the presence of various bacterial microbes, classically activated MPs (M1 MPs) produce various activated lytic enzymes, including interferon-α, and reactive oxygen species in addition to inflammation-promoting chemokines (5). In short, these MPs create a hostile microbicidal environment. In contrast, alternatively activated MPs (M2 MPs) repair tissues using molecules such as growth factors and transforming growth factor-β to reduce inflammation *via* molecules such as interleukin (IL)-10 and transforming growth factor-β. In the normal immune system, the different subtypes of MPs induce distinct types of immune responses to various antigens, specifically, viral and bacterial antigens (M1 MPs) and parasitic as well as fungal antigens (M2 MPs). The interplay between M1 and M2 MPs exists on a continuum. It can both resolve inflammation and, as in tumor microenvironments (TMEs), minimize inflammation and immune surveillance while increasing life expectancy (6).

Tumor-associated MPs (TAMs) are components of a highly complex and heterogeneous TME of productive host cells (7, 8). For example, specific TME signatures of lymphomas can aid in the maintenance of neoplastic cells experimentally *in vitro* and probably *in vivo*. The microenvironment's impact on cell growth and destruction varies greatly according to the inherent histotype of the lymphoma cell type. For example, the Hodgkin lymphoma (HL) tissue often consists of relatively few monoclonal cancer cells but at least 90% non-malignant cells (e.g., regulatory T cells), contributing to a fairly unique surrounding TME ecosystem (9), whereas Burkitt lymphoma seems to be largely devoid of a supportive cellular environment (6, 10). Even in cases of Burkitt lymphoma, though, immune signaling and attenuation (i.e., by IL-10) are crucial to intrinsic lymphoma cell proliferation (11). Clearly, TAMs play a distinct, specific, important role in neoplastic progression.

The presence of MPs in a tumor can be indicative of several characteristics of a lymphoma's clinical signature, including prognosis as well as efficacy of chemotherapy (12). Even before cells become cancerous, MPs can add to their surrounding inflammatory environment, producing mutagenic substances like reactive oxygen species that may support or augment oncogenesis (13). In addition, M2 MPs can express key immune checkpoint molecules, including programmed cell death protein 1 (PD-1) and programmed cell death-ligand 1 (PD-L1), generally inhibiting the overall inflammatory response, allowing the tumor cells to evade antitumor immunity (14).

Macrophages exist in complex TMEs and interact with other cells therein. In particular, interaction between lymphocytes and MPs may create a hostile tumorigenic intrinsic environment. For example, fibrosis in follicular lymphoma (FL) cases is correlated with the presence of both Th2 T cells and their related M2 MPs (15). In these cases, MPs and fibroblasts both contribute to fibrosis. In addition, CD8+ T-cell infiltrates and MPs are found at higher numbers in higher Ann Arbor-stage lymphomas, suggesting an association between not only MPs and cellular tumor extent but also between T cells and MPs in advanced B-cell lymphomas (16). Furthermore, communication between lymphocytes and MPs is diverse and variable. T and B cells both interact with TAMs in ways that can impact the progression of lymphoma and its clinical response to chemotherapy. Whether TAMs suppress an active immune response or impact the efficacy of immune therapy, they represent crucial junctions between innate and/or acquired immune systems that should not be overlooked or underappreciated in the pathological processes involved in hematologic malignancies.

B-CELL/MP INTERACTIONS IN B-CELL LYMPHOMAS

History

One of the first descriptions of the interactions between MPs and malignant B cells came in the 1960s with a number of studies in which researchers employed electron microscopy with ultrastructural pathological staining to identify MPs in lymphomas (17, 18). The most common description of lymphoma-associated MPs was the classic "starry sky" histological appearance in Burkitt lymphoma, in which the tumor cells have a very high turnover rate, so TAMs phagocytose and scavenge the tumor and stuff it with cytoplasmic cellular debris (at this point, the TAMs are called tingible body MPs). Upon fixation, the cytoplasm in TAM retracts, leaving round white spaces filled with debris resembling stars (19, 20). This pattern can be seen in both high-powered paraffin-embedded bone marrow and lymph node sections characteristic of Burkitt lymphoma (21, 22).

In the 1970s, investigators in several studies used a modified "skin window" (glass coverslip) technique to study MPs in normal subjects and patients with HLs or non-HLs (NHLs) (23, 24). They discovered that large MP-like cells in these patients were morphologically abnormal, exhibiting multinucleated patterns and, in some cases, aberrant multipolar mitotic figures. They concluded that the presence of abnormal MPs in lymphomas may be related to a significant and aggressive malignant process, but follow-up research of these findings was either lacking or inconclusive. In fact, several authors initially described these large MP-like cells both generically and descriptively as diffuse large cell lymphoma cells exhibiting considerable morphological plasticity and as dominant types of large cell in primitive lymphoreticular-type neoplasms (25). For many years, researchers generically described such large atypical (undifferentiated) lymphoma cells in terms of

their large size and irregular shape (atypia) as abnormal lymphoid tumor cells of obscure genetic cellular origin or as generic primitive lymphoid tumor cells, which were then referred to as reticulum cell sarcoma cells in the original pre-Rappaport early hematological fascicle of lymphoid classification terminology (26). These diffuse large cell lymphoma cells were later better characterized pathologically and subsequently designated as histiocytic lymphoma cells by Rappaport in his classic NHL/World Health Organization histopathological classification monograph. Primarily using light microscopy, clinical hematological researchers later showed that most of these cells were large, atypical polymorphous/polyploid lymphoid tumor cells categorized as various large lymphoid cell types, such as diffuse, undifferentiated, and/or immunoblastic histotypes, including occasional rare, possibly true histiocytic tumor cell types. However, at least 85% of these generically described diffuse large cell lymphomas are now shown to be derived from a clonally transformed neoplastic B-lymphoid lineage [diffuse large B-cell lymphoma (DLBCL)] without definitive evidence of a "true" or validated histiocytic cellular origin or lineage (27). In more recent and potentially more convincing follow-up studies originally conceptualized and performed by Lukes and Collins (28), they identified morphologically diffuse large cell lymphoma cells as actually being neoplastically transformed large, atypical B-lymphoid cells possibly of early B-cell lineage rather than precursor cells of the previously believed early myeloid/monocytic-derived or malignant histiocyte/MP origin originally conceived and favored by Rappaport and colleagues in their early World Health Organization classification.

In the early 1980s, several *in vitro* models of lymphoma-derived MPs were described (24, 29). In one study, pleural effusions from patients with diffuse "histiocytic" lymphoma (currently known as DLBCL) were cultured *in vitro*, giving rise to an MP population with a wide variety of cellular functions, including promotion of lymphoma cell growth and survival, as well as inhibition of immune responsiveness. These studies also demonstrated that lymphoma-derived MPs differed markedly from normal peripheral blood donor-derived MPs, as the former could, for instance, induce formation of lymphocytic rosettes around tumor MPs. However, the functional significance of lymphocytic rosette formation was difficult to explain at that time, requiring additional probing into mechanisms that could underlie this phenomenon. Such findings may have provided an initial clue or indication regarding how indigenous tumor-derived MPs interact and communicate with B-cell lymphoma cells and identified that such interactions could have important clinical as well as biological implications (30, 31).

Not until the early 2000s did authors begin to report on studies demonstrating that infiltrating MPs within lymphomas promote their biological and/or clinical progression. Researchers in those studies primarily used key phenotypic markers such as CD68 and CD163 to detect TAMs, correlating marker expression patterns with clinical outcome in patients with different types of lymphoma (32, 33).

TAMs in HL Patients

Investigators first described the clinically significant role of TAMs in HL cases in 1985 when they used peanut agglutinin biomarker staining of paraffin-embedded sections of HLs to identify MP histioctyes. They demonstrated that increased numbers of MP histioctyes in the HL sections correlated with unfavorable clinical and pathological parameters of the disease (34). In 2010, Steidl et al. (35, 36) used gene expression profiling to identify a TAM gene signature that was significantly associated with primary treatment failure. They further validated these findings using CD68 immunostaining, showing that an increased number of CD68+ TAMs in HL patients' lymph node biopsy samples was associated with adverse clinical prognosis. Since then, a large number of studies have further validated the important correlation between increased numbers of TAMs according to CD68 expression and a poor clinical course of HL (37–40). In fact, researchers identified CD68+ TAMs in relapsed/refractory HL samples, although additional clinical validation is probably required to confirm a role for these biomarkers as adverse prognostic markers of HL (37). However, several studies did not demonstrate a significant correlation between CD68+ TAMs and clinical prognosis for HL (41). Several reasons for these discrepancies are possible, including the background characteristics of the studied patient population, the antibodies and immunostaining reagents and technical methods used, and the numerical scoring methods used and analyzed.

Besides CD68, additional TAM biomarkers, such as the similarly expressed CD163 and colony-stimulating factor 1 receptor (CSF-1R) diagnostic antibody-based reagents, may be useful to stratify prognosis for HL (40, 42). Expression of CD163, a specific biomarker of M2 TAMs, seems to be a better, more efficient biomarker than CD68 in predicting clinical outcomes of HL (39). In addition, the lymphocyte/monocyte ratio was associated with the presence of TAMs and identified as a negative prognostic indicator for HL, suggesting another biologic marker that can be used to predict clinical outcome of HL (43).

Overall, these findings are encouraging, as they clearly demonstrated the clinical significance of TAMs regarding HL in various patient cohorts. However, the pathological and biological significance of TAMs in HL patients must be examined and validated. What are the biological functions of TAMs in the setting of HL, in which several cell types, including T cells, B cells, and Reed–Sternberg cells, are involved? Clearly, much more in-depth research in this field is needed to determine whether targeting TAMs in HL patients is feasible or effective in the clinic. Several novel strategies are used to target TAMs, which are described below, but currently, only one known clinical trial is testing treatment of relapsed or refractory HL patients with a CSF-1R inhibitor (44).

TAMs in FL Patients

Authors have described the clinical significance of TAMs in different subtypes of B-cell lymphomas, mostly as poor prognostic indicators (45–47). However, a few studies demonstrated that the presence of TAMs could indicate a favorable prognosis for these tumors (48, 49). Similar to those for HL described earlier, these discordant results may be attributable to differing methodological approaches as well as the cellular nature of the tissue samples being examined. In addition, the lymphoid cell type and differentiation stage of a B-cell lymphoma may determine the functional significance of TAMs. For instance, in FL patients, the tumor cells are admixed with heterogeneous lymphoid-like stromal

cells within infiltrated lymph nodes and bone marrow (50). These stromal cells are involved in the recruitment and polarization of mature monocytes into active M2 MPs. FLs are described as indolent low-grade B-cell lymphomas in which the tumor cells cannot survive on their own, requiring growth and/or survival stimulatory factors from their microenvironments, such as MPs, which appear to maintain the viability of the tumor cells. Various growth factors, such as IL-2, IL-4, IL-15, and CD40L, as well as the immune suppressor cytokine IL-4I1, are known to be secreted by TAMs and are involved in FL pathogenesis (51–53). Clearly, TAMs are biologically linked with FL, presumably providing the proper signals to not only maintain the viability of FL cells but also protect the tumor cells from the active immune system. Based on results of gene expression profiling analysis, investigators found that a defined MP-enriched gene expression pattern was associated with inferior clinical course of FL (54), indicating that the presence of TAMs in FL patients may predict clinical outcomes. In addition, researchers have used other macrophagic markers identified using immunohistochemistry, such as CD68 and CD163, to predict unfavorable outcomes of FL (45–47).

Our Laboratory Studies and Observations of TAMs in Mantle Cell Lymphoma (MCL) Cases

Mantle cell lymphoma is one of the most challenging human cancers to treat, particularly among hematopoietic neoplasms, and is often one of the most aggressive forms of B-cell non-Hodgkin lymphoma (NHL-B) (55–58). Only relatively recently recognized as a form of NHL-B (1992) (59), MCL was described in the older NHL (1964) literature and in the updated Kiel NHL classification systems as belonging to centrocytic "intermediate" B-cell lymphoma subtypes that are often aggressive, morphologically distinct, small B-cell histotypes of NHL-B (60, 61). MCL was morphologically defined as an aggressive lymphocytic (small cell) lymphoma with scattered epithelioid or "pink" histiocytes (MPs) and referred to as centrocytic intermediate-stage lymphocytic lymphoma. Later, MCL was immunophenotyped as "typical" MCL, the dominant form of the disease, with rarer aggressive blastoid and less aggressive "mantle-zone" variants. Recently, increasing numbers of MCL cases have emerged and been recognized to behave indolently and were associated with longer survival durations than is the classic form of MCL. However, closer scrutiny of these indolent MCL cases suggested that most were non-nodal, were less disseminated than the classic MCL, or tended to be leukemic, exhibiting certain immunophenotypic genotypic characteristics (56). These examples include the so-called *in situ* MCL cases, with or without SOX11 gene expression (62–65). Clearly, MCL is not the mostly monolithic pathological entity that it was previously assumed to be, and the initial indolence of the tumor and presence of pink histiocytes may be important pathophysiological clues, although their overall significance is still unclear. Only a few studies have linked monocyte count with the prognostic impact of MCL (66–69), and studies suggesting functional roles for MPs in MCL are limited. Clearly, active *in vitro* studies are needed for better characterization and biological functions of MPs in MCL biology and pathophysiology.

We recently demonstrated that certain microenvironmental interactions involving cellular subsets of monocyte/MP lineage are necessary for long-term *in vitro* cell culture and pathological characterization of primary MCL cells (70). Primary MCL tumor cells do not spontaneously grow after *in vitro* explanation; they need active cellular interactions with microenvironmental cellular components to stimulate and maintain expanded lymphoma cell growth and survival. Perhaps not surprisingly, monocytic and related cells of mostly myeloid accessory and precursor cell lineages make up a group of "nurse-like" cells from bone marrow and possibly other lymphoid tissues. These cells provide microenvironmental co-factors necessary for maintenance of lymphoma cells *in vitro* and, probably, *in vivo* (71–73). Our recent published studies of large numbers of mostly leukemic/effusion-selected MCL patients demonstrated that when adequate numbers of unstimulated and/or unseparated MCL cells from effusions (>90% morphological) or leukemic cell populations are cultured, the initial result is spontaneous formation of increased numbers of MPs after 7–14 days in cell culture. Furthermore, these MPs stain for CD68 biomarker (70). The MPs are presumably derived from cryptic CD68+ monocytes, as cultures of purified CD20+ lymphoma cells alone usually do not contain CD68+ cells. In addition, treating these cultures with the MP-depleting agent liposomal clodronate (74, 75) completely eliminated these MPs, suggesting that spontaneously formed MPs resemble endogenous TAMs. These TAMs are often bound and encircled by atypical lymphoma B cells (rosettes) *in vitro*, maintaining and nurturing the lymphoma cells in culture for variable durations, usually at least 2 months. The lymphoma cells then often transform into autonomously growing B-cell lymphoma cell lines or slowly die out owing to apparent spontaneous apoptosis. We recently discovered that culturing these primary MCL cells under hypoxic conditions enhances the activation of these TAMs, increasing lymphoma cells' viability and extending their survival. More importantly, we discovered that culturing primary MCL cells under hypoxic conditions causes them to progressively become adherent rosettes of lymphoma cell–TAM colony aggregates *in situ* (**Figure 1A**). These predictable clusters or aggregates of lymphoma cells and TAM cells reproducibly form in culture flasks, expanding in size and exhibiting protracted growth and survival (**Figure 1B**). In some cases, the TAMs frequently exhibit mitotic figures with morphological atypia, indicating that these TAMs are proliferating and may be abnormal (**Figure 1C**). Our *in vitro* data demonstrated a physical cellular (juxtacrine signaling) relationship between TAMs and lymphoma cells, mimicking the lymphoma cell/MP interactions seen in some bone marrow biopsies in lymphoma patients (76–78).

TAMs in DLBCL Patients

Diffuse large B-cell lymphoma, the most common human lymphoma, comprises a genetically and clinically diverse group of aggressive NHL-Bs among a small group of important human cancers that have increased in incidence in the United States over the past 4 decades (79–82). Current frontline DLBCL therapy, although fairly successful [~70–80% remission rates with the frontline chemotherapy regimen cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) combined with rituximab

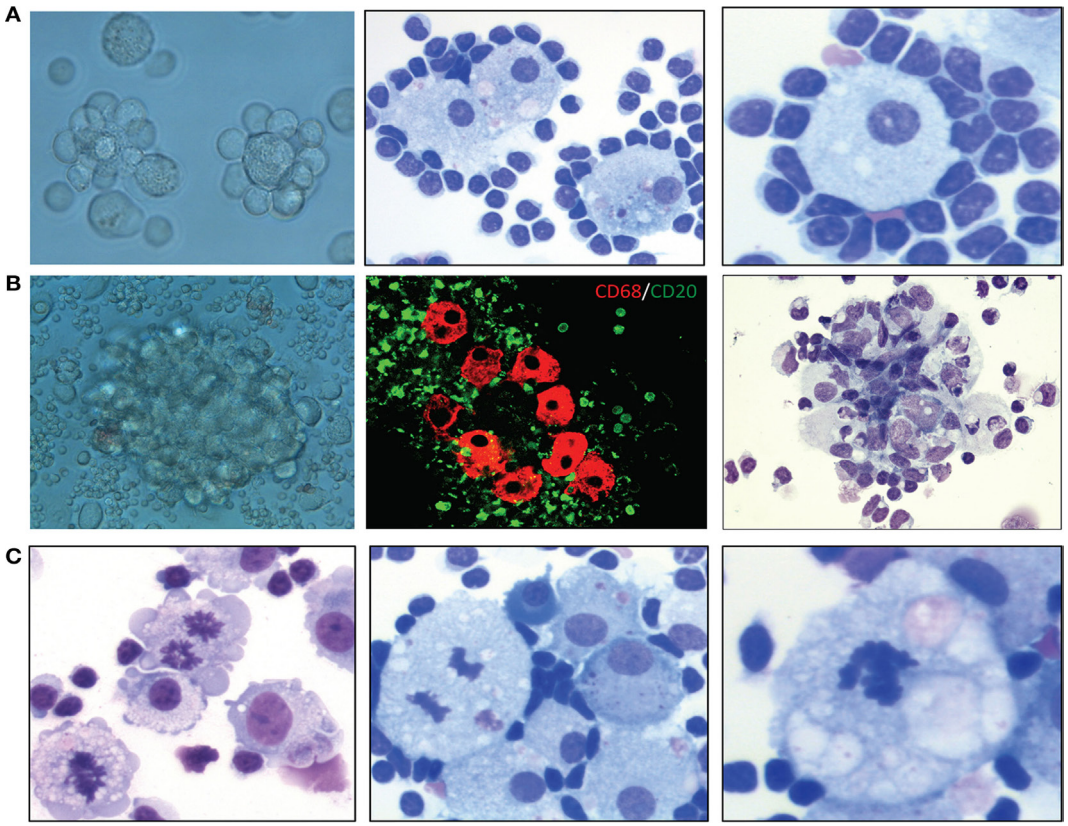

FIGURE 1 | Characterization of lymphoma-associated macrophages (MPs) in B-cell lymphoma cell cultures. **(A)** Examples of lymphoma cell–tumor-associated MP colony aggregation in culture after 2 weeks. Left, phase-contrast light microscopic image; middle, Wright–Giemsa stain (400×); right, Wright–Giemsa stain (400×). **(B)** Examples of MP clustering/aggregation in mantle cell lymphoma (MCL) cell culture under hypoxic conditions. Left, phase-contrast light microscopic image; middle, confocal microscopic analysis of CD68+ MPs (red) and CD5+ cells (green); right, hematoxylin and eosin stain (200×). **(C)** Hematoxylin and eosin and Wright–Giemsa stains showing mitotic figures in MPs in primary MCL culture after 8 weeks under hypoxic conditions (400×).

(R-CHOP)], is frequently followed by relapse (~40% of cases within 2–3 years), often as refractory DLBCL, resulting in only poor salvage therapy responses (<20% partial or complete responses) with short survival durations. Similar to FL, gene expression profiling has identified the TME and host inflammatory response signatures as defining features of DLBCL (83, 84). These studies demonstrated a strong correlation between TME signatures host cells and clinical prognosis for DLBCL. For instance, Lenz et al. (83) reported on a prediction model composed of two TME signatures, stromal-1 and stromal-2, that can predict clinical outcomes in DLBCL patients. Stromal-2 signature genes encoded for well-known markers of monocytic lineages that were predictive of unfavorable survival in DLBCL patients given CHOP alone or R-CHOP. Monocytic myeloid-derived suppressor cells and TAMs are presumably the important cellular types in the stromal-2 signature, as these cells also exhibited prognostic significance for DLBCL in other studies (85–88). Also, a number of studies have shown that a high absolute monocyte count at diagnosis is useful for prognostic stratification of patients with DLBCL (89–92). Khalifa et al. (93) demonstrated that increased CD14+ monocytes with loss of human leukocyte antigen-DR expression were seen in DLBCL patients with higher stage disease, more aggressive pathology, and in relapse or refractoriness to treatment. These studies clearly demonstrated that monocytes play an important role in the pathophysiology of DLBCL, possibly as precursors to TAMs, particularly those with the M2 phenotype. In addition, some studies demonstrated that high expression of CD68 in TAMs correlates with poor prognosis for DLBCL (76, 78, 94), whereas other studies did not demonstrate a specific or significant correlation (95, 96). This discrepancy is probably due to the diagnostic antibodies used in these studies as well as the scoring method used to analyze immunohistochemical CD68 staining. Therefore, double staining for CD68 and CD163 may be a better method of predicting outcomes of DLBCL, as their expression are associated with adverse outcomes in R-CHOP–treated patients (97).

Unlike FL and some MCLs, DLBCL is usually aggressive, with tumor cells that grow more or less autonomously and probably not needing external growth or survival stimuli, at least from TAMs. Given these characteristics, why do TAMs infiltrate or allow recruitment within DLBCL tumor tissues? A possible reason is that DLBCLs must be able to escape the immune surveillance of tumor-specific cytotoxic T cells by recruiting and polarizing M1 TAMs to M2 TAMs that highly express immune checkpoint molecules, such as PD-L1 and PD-L2, on their surfaces (98). These

ligands interact with the PD-1 receptor expressed on intratumoral T cells and provide inhibitory signals, thereby suppressing antitumor immune response (99). Immune checkpoint inhibitors, such as anti-PD-1 antibody, bind to the PD-1 expressed on activated cytotoxic T cells, thereby stimulating their proliferative capacity and enabling the immune system to resume recognizing, attacking, and destroying active cancer cells (100, 101). This may be one reason why anti-PD-1/PD-L1 therapy was effective against some cases of DLBCL in a clinical trial (102). Another possibility is that TAMs are recruited to a tumor site to protect the tumor cells from various effects of different chemotherapies. For example, Shen et al. (77) demonstrated that M2 TAMs secrete an enzyme called legumain that promotes the degradation of fibronectin and collagen I, resulting in tumor progression, at least in some murine DLBCL models.

ORIGIN OF TAMs IN B-CELL LYMPHOMAS

Derivation From Cells of Monocytic Lineage

A basic concept regarding the mononuclear phagocytic system is that in most cases, human MPs are derived from myeloid precursor-lineage monocytes (103). Cells of mononuclear phagocyte lineage progress through a series of specific morphologically distinct stages: they possess a myeloid progenitor in common with that of granulocytic cell types giving rise to monoblasts, promonocytes, and, later, monocytes that subsequently migrate into various hematopoietic tissues. In response to infection, monocytes move into focal tissue spaces around sites of infectious involvement and then differentiate into dendritic cells and typical MPs. Although hematological researchers have not formally proven it, many studies have suggested that lymphoma tissue-derived MPs can be derived from circulating monocytes. In fact, a number of studies demonstrated that a high monocyte count ratio at presentation in B-cell lymphoma patients was associated with increased numbers of CD163+ TAMs, which could predict poor clinical outcome (68, 69). The postulated mechanism of this relationship is that a high monocyte count can be a surrogate biomarker for the TME, reflecting the functions of recruited immunosuppressive peripheral blood monocytes recruited by lymphoma cells to differentiate targeted monocytes into polarized MPs (M2) that can in turn activate the tumor cells. Under stressful conditions, such as during *in vitro* cell culture, monocytes may be able to differentiate or be reprogrammed into MPs to provide tumor cells with growth and survival stimulatory factors in an autocrine or paracrine secretory fashion. This is probably the case in MCL patients, in whom pink histiocytes may develop to protect the surrounding tumor cells. A recent study demonstrated that B1 lymphocytes expressing IL-10 and other important chemokines play key roles in recruiting monocytes and promoting a protumoral M2 phenotype of MPs (104). The investigators indicated that *in vivo* polarization of MPs by B1 cells (normal counterparts of MCL cells) likely occurs only in specific anatomical compartments (the peritoneum and spleen) where B1 cells and MPs co-exist.

MP/Tumor Cell Fusion

Our preliminary data demonstrated that MPs that formed in culture (*in vitro*) co-existed with MCL cells for extended periods (>2 months), after which mitotic figures in the MPs began to appear (**Figure 2**). These results also suggested that these lymphoma-derived MPs are not normal, unlike non-mitotic normal MPs. How do these MCL-associated MPs differ from normal MPs? Our preliminary data also indicated that when we purified the MPs in the latter stages of tissue culture (3–4 months), some

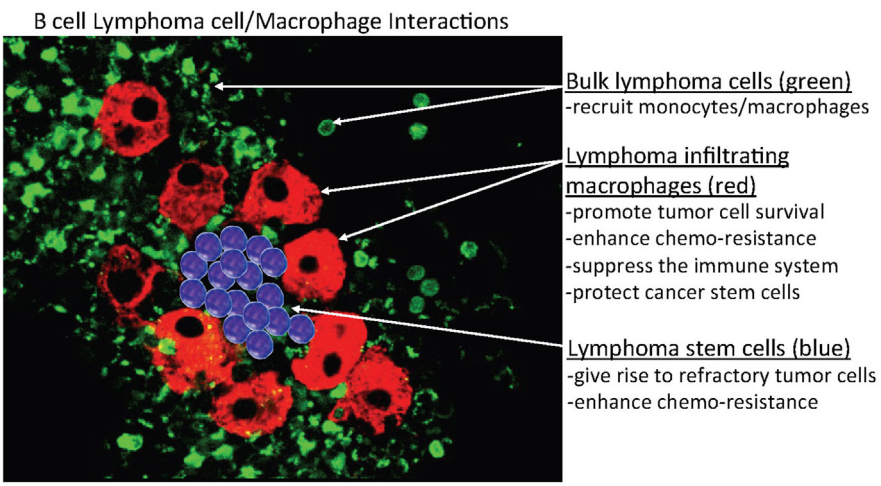

FIGURE 2 | Hypothetical model of B-cell/MP interactions in B-cell lymphomas. This model predicts that indolent and retransformed or non-transformed lymphoma cells do not initially exhibit autonomous or spontaneous-independent neoplastic cell growth but instead must undergo interactions with indigenous non-neoplastic cells, specifically, monocytes or macrophages (MP)-derived tumor-associated MPs (TAMs) that bind, stimulate, and activate targeted lymphoma cells, to adopt aggressive autonomous phenotypes. The postulated mechanism for this relationship is that a high monocyte count is a surrogate biomarker for the tumor microenvironment, reflecting the functions of immunosuppressive peripheral blood monocytes recruited by lymphoma cells to differentiate monocytes into polarized MPs (M2) that can in turn activate the tumor cells. In addition, the aggregated TAMs (red) can protect tumor cells and, potentially, cancer stem cells (blue) within their clusters from chemotherapy, enabling the tumor cells to survive (residual disease) and re-establish (relapse) at a later time.

of them phenotypically expressed PAX5 (a B-cell marker) and genotypically had the t(11;14) translocation (unpublished data). A possible explanation for this phenomenon is that at some point during tissue culture, polarized M2 MPs fuse with MCL cells, creating hybrid cells that may divide and/or proliferate. MP/cancer cell fusion is not uncommon, as recent studies demonstrated that these hybrid cells may spontaneously acquire cancer stem cell properties, including enhanced drug resistance, self-renewal, and increased metastatic activity (105–107).

B-Cell-to-MP Reprogramming

Another mechanism of the derivation of MPs in B-cell lymphomas may be lineage switching or reprogramming from B cells to myeloid cells, as certain subsets of B cells are endowed with self-renewal capacity and the ability to adopt gene expression patterns, morphology, and functional activities characteristic of MPs (108, 109). Studies performed by Xie et al. (110) provided strong evidence that committed mature B-cell-lineage cells can be reprogrammed to become MPs. Using a retroviral approach, they induced overexpression of the transcription factor C/EBPβ in CD19+ murine bone marrow B-cell progenitors. As a result, about 60% of the B cells had downregulated expression of B-cell-restricted genes (e.g., CD19, Rag1, B220, Mb-1, EBF, and Pax5) and upregulated expression of MP-restricted genes (e.g., MAC-1, Fc gamma RIII, Fc gamma R1, M-CSF-R, and PU.1). These reprogrammed B cells not only had MP phenotypes but also adopted the morphology of MPs and gained phagocytic abilities and/or functions. Apparently, introduction or switching of a single transcription factor can set into motion an entire series of events that can transform a B cell into a macrophagic cell. Whether this occurs naturally or only under certain pathological conditions, such as cancer, remains unclear, particularly in humans. In a recent article, McClellan and colleagues reported that when incubated *in vitro*, blasts from some precursor B-cell acute lymphoblastic leukemia cells could apparently be functionally reprogrammed into myeloid lineage-mimicking cells that morphologically resemble and can, in some cases, function similarly to normal MPs (111). Several other studies had similar findings, demonstrating that malignant B-lymphoid cells can be reprogrammed to become MPs with apparently lymphoid and myeloid characteristics under certain conditions (112–115). Interestingly, we observed a similar phenomenon in MCL cases, as *in vitro* culture of primary MCL cells led to the presence of MPs after several days (70). Whether such a reprogramming mechanism exists in MCLs must be confirmed to determine whether this mechanism can be used for therapeutic purposes.

FUNCTIONAL SIGNIFICANCE OF TAMs IN THE B-CELL LYMPHOMA MICROENVIRONMENT

Normal human MPs are typically ubiquitous, polymorphous, usually large, heterogeneous, multifunctional, myeloid-derived regulatory cells. These cells are active in most tissues and organs, mediating a wide array of biological regulatory activities to at least some extent in most normal eukaryotic tissues. Alternatively, putative neoplastic counterpart MPs (TAMs) are described as having both protumor and antitumor properties, but the majority of clinical lymphoid cancer studies have demonstrated that the presence of a high number of TAMs in the TME is related to poor prognosis, suggesting that TAMs predominantly exert protumoral activity *via* various mechanisms in many B-cell lymphomas.

Stimulation of Tumor Cell Growth and Survival

Normal MPs function mainly by engulfing foreign substances such as cellular debris and microbes as well as moribund and apoptotic cancer cells *via* phagocytosis. On the other hand, TAMs are usually large, polarized, multifunctional cells with great cellular plasticity that can play multiple key activating roles in the initiation and progression of many tumor types (116). TAMs are also prime candidates for creating the microenvironmental milieu, histologically represented by pathologists, particularly in MCL patients, as pink histiocytes, but they are present within tumor tissues in much greater numbers than once thought after immunohistochemical analysis identified them *via* staining of human tissue biopsy samples for anti-CD68 monoclonal antibodies. Many of these tumors frequently appear in tissue areas exhibiting chronic inflammation, likely aided by the mutagenic actions of MPs. Tumor growth and progression are often supported by MP-induced survival and stromal cell production *via* various MP-produced tumor-stimulating growth factors (117). Our *in vitro* investigations using primary MCL cell cultures demonstrated that MCL cells usually died within 2 weeks of *in vitro* culture if TAMs were not present, indicating or at least suggesting that TAMs are required for maintenance of the viability of primary MCL cells in long-term *in vitro* cell culture. The dependence of malignant B cells on some types of myeloid cells for continued survival is not unprecedented, as CD5+ B1-derived chronic lymphocytic leukemia (CLL) cells are highly dependent on nurse-like cells with M2 TAM phenotypes for mediation of growth and survival (118–120). However, whether the interaction between tumor cells and TAMs actually requires cell–cell interactions or can occur through a paracrine secretory mechanism has yet to be determined. In either case, coculture of TAMs with various types of lymphoma cells can activate various intrinsic pathways, such as signal transducer and activator of transcription 3, phosphoinositide 3-kinase/mammalian target of rapamycin, and nuclear factor-κB, providing key growth and survival signals in tumor cells (121–123).

Provision of Chemoresistance Mechanisms to Cancer Cells

Cancer cells at both primary and secondary metastatic tumor sites become resistant to various chemotherapeutic drugs through various molecular mechanisms mediating the activity of intrinsic and/or extrinsic cellular factors, although the latter can often remain largely overlooked. In most tumors, a high density and accumulations of TAMs predict poor outcomes, and TAMs are highly present in relapsed and refractory lymphomas, most likely playing an important role in multiple types of drug resistance. Mounting evidence suggests that the TME also play critical roles in multiple aspects of tumor progression, particularly in

therapeutic resistance (124). The lymphoma microenvironment can be composed of several different subsets of host cells, in particular, bone marrow stromal cells and TAMs, where they may play a key role in B-cell survival that to promote drug resistance (125). TAMs are often key components of the TME and often play an important role in the biology of different types of lymphoma, including FL, HL, DLBCL, and MCL (70, 126). The potential role of TAMs in B-cell lymphomagenesis and the emerging field of lymphoma B cell–TAM interaction should be further investigated to determine whether these two cellular subsets undergo bidirectional cross talk in the context of lymphoma drug resistance. TAM-mediated drug resistance can be a form of *de novo* drug resistance that can protect tumor cells from the initial effects of diverse therapies *via* both soluble factor-mediated and cell adhesion-mediated drug resistance. One hypothesis explaining this mechanism is that specific niches within the lymphoma microenvironment provide sanctuaries for subpopulations of tumor cells and a survival advantage through host-cell/tumor-cell interactions, activating key pathways that allow the target cells to survive the insult of toxic therapy, resulting in minimal residual disease (**Figure 2**). Over time, residual tumor cells are destined to expand and evolve through acquisition of additional genetic abnormalities (or selection of preexisting clones of tumor cells) that cause the gradual development of more complex, diverse, long-standing acquired resistance phenotypes. Persistence of residual tumor cells eventually cause relapsed disease, which is much less likely to respond to subsequent therapy after acquired resistance develops and the disease ultimately progresses. Therefore, understanding the relationship between host and tumor cells may help in the identification as well as design of more effective therapies to overcome dissemination or recurrence of cancer and improve the ultimate outcomes of future cancer therapies.

B lymphocytes are divided into two subpopulations—B1 and B2 cells—based mostly on expression of the definitive T-cell-associated protein CD5 (127). Natural B1 cells are further divided into B1a cells, which express CD5 on their membranes, and B1b cells, which do not express CD5 but share most other biological characteristics of B1a cells. CD5+ B1-cell origins and the predisposition of these cells toward giving rise to lymphoma and leukemia are long-standing issues that have yet to be successfully addressed experimentally (128). B1 (CD5+ B) cells appear early in ontogeny, produce mainly unmutated polyreactive antibodies, and are capable of self-renewal. B1 cells clonally expand as they age and are the primary malignant tumor cells in B-cell CLL and MCL cases. B1 cells are also immunogenic, capable of secreting inflammatory chemokines and cytokines. Our experimental focus in MCL studies has been on better characterizing the MCL microenvironment containing candidate endogenous MCL stem-like cell components. Several recent reports supported such a stem cell-like concept in human MCL cell populations with specific immunophenotypes (e.g., CD45+ CD19, CD133+ CD19−) (129, 130). However, whether individual clones of tumor-initiating stem cells within the MCL TME niche-expressing population of MCL cells remain stable over time and are relatively resistant to conventional MCL therapies remains unknown. TME niche-expressing MCL cells may be composed of cells that survive over the long term in tumors and give rise to daughter cells that can maintain the tumor's existence over time. Alternatively, they may be pluripotent and represent different clones, each having tumor-initiating cell characteristics at different points in time. Within the normal bone marrow microenvironment, MPs are crucial for the maintenance of normal hematopoiesis of stem cells (131, 132). TAMs may play a similar role in MCL cases, maintaining the stem-like cellular phenotype within the TME niche in the bone marrow as stable disease (indolent), with the MCL progressing to and/or transforming (blastoid) into more aggressive disease at a later time.

Immunosuppression

Tumor-associated MPs may also alter the behavior of the human cellular immune system, impacting the efficacy of recently developed immune checkpoint inhibitors (PD-1, PD-L1, etc.) and affecting T lymphocytes, leading to disease progression (133–136). Some malignant B cells acquire intrinsic mechanisms to escape from immune surveillance by tumor-specific cytotoxic T cells *via* overexpression of PD-L1 or PD-L2 on the cell surface or recruitment of TAMs expressing PD-L1. These ligands interact with the PD-1 receptor expressed on intratumoral T cells and provide an inhibitory signal, thereby suppressing the antitumor immune response. Checkpoint inhibitors, such as the anti-PD-1 antibody, bind to the checkpoint receptor PD-1 expressed on T cells, stimulating their proliferative capacity and enabling the immune system to reactivate its ability to recognize, attack, and destroy cancer cells. Several anti-PD-1/PD-L1 regimens have had encouraging therapeutic effects in patients with relapsed or refractory HL, FL, or DLBCL (137). Although immune checkpoint inhibitors are emerging as promising therapeutic options for patients suffering from different types of cancer, including aggressive B-cell lymphomas, the challenge is that not all treated cancer patients have had responses to these checkpoint inhibitors. Understanding the mechanisms that control PD-1/PD-L1 expression in various types of cancer cells and the key involved accessory cells may not only identify important predictive biomarkers for controlling the efficacy of anti-PD-1/PD-L1 antibody-based immunotherapy but also help in the development of novel targeted therapies that can be combined with checkpoint inhibitors for additional clinical efficacy. Although researchers have well established that PD-1 and/or PD-L1 blockade activates important immune T cells, little is known about the mechanistic role that the PD-1/PD-L1 pathway may play in TAMs. Several studies have focused on the expression of PD-L1 in TAMs and demonstrated that numerous well-known signaling pathways, such as the key oncogenic pathways MYC and signal transducer and activator of transcription 3, are responsible for PD-L1 regulation (98, 138, 139).

TARGETING TAMs IN B-CELL LYMPHOMA PATIENTS

Tumor-associated MPs, particularly those with the M2 phenotype, are among the major constituents of the tumor stroma in patients with different types of cancer, including B-cell lymphomas. Also, investigators have obtained compelling preclinical as well as clinical evidence that TAMs can promote neoplastic initiation, malignant progression, and further metastasis. TAMs are therefore potential targets for adjuvant therapies for aggressive

B-cell lymphoma, particularly relapsed or refractory lymphomas. Strategies designed to deplete TAMs or inhibit their recruitment into neoplastic lesions have been successful in experimental settings and are now considered promising therapeutic approaches in the clinic. To therapeutically target TAMs, researchers have proposed using various pharmacological as well as immunological strategies described below based on the results of previous preclinical studies.

Clodronate

Clodronate is a first-generation bisphosphonate-family compound that is now used in the clinic to prevent or actively inhibit the development of bone metastases and treat inflammatory diseases such as autoimmune rheumatoid arthritis and osteoarthritis (140). With experimental encapsulation of clodronate into liposomes, researchers developed an efficient reagent that selectively depleted MPs when successfully applied in several immunological studies (141). Recently, investigators found that the use of bisphosphonates as antiangiogenic agents suppressed tumor growth as well as metastasis in several lymphoma models, including DLBCL and T-cell lymphoma models, primarily through elimination of MPs (142).

The Bruton's Tyrosine Kinase Inhibitor Ibrutinib

Ibrutinib, a novel, first-in-class, orally bioavailable, irreversible Bruton's tyrosine kinase inhibitor, recently exhibited clinical effectiveness and tolerability in clinical trials in patients with various hematological malignancies, including refractory CLL and MCL (143, 144). However, whether ibrutinib actually inhibits the biological activity of MPs remains unclear. A recent study demonstrated that ibrutinib could actually target cells of monocyte/MP lineage in autoimmune disease models (145). In addition, clinical data indicated that ibrutinib-based treatment in MCL patients led to decreased secretion of MP inflammatory proteins and chemokines into the plasma (146). In the bone marrow microenvironment in CLL patients, ibrutinib disaggregated the interactions of MPs with leukemia cells by inhibiting secretion of the chemokine CXCL13, which decreased the chemoattraction of CLL cells (147). These findings support the concept that ibrutinib can target MPs in patients with B-cell lymphoma/leukemia and function *via* this chemokine/cytokine type of mechanism.

Trabectedin

Trabectedin is an antitumor chemotherapeutic drug originally isolated from a marine organism and approved by the U.S. Food and Drug Administration for the treatment of sarcoma and ovarian carcinoma (148, 149). Recently, researchers showed that trabectedin has selective cytotoxicity in cells of myeloid lineages, particularly TAMs, through the mechanism through induction of caspase-dependent apoptosis (150). Evidence in both mice and sarcoma patients suggests that selective MP depletion is a key mechanism of action mediating the antitumor activity of this agent (151). In biopsy samples obtained from sarcoma patients given trabectedin, authors noted a marked decrease in the number of TAMs and related stroma vessel networks (152). Trabectedin has undergone evaluation in several different *in vitro* and *in vivo* cancer models, exhibiting similar activities by targeting and selectively eliminating tumor-associated monocytes and MPs (153). Taking the acceptable toxicity profile of trabectedin and its unusual mode of action into account, this compound is an important therapeutic agent because it interferes with not only tumor cells but also myeloid cells in the TME. These results provide proof of principle for MP targeting in cancer patients and may have implications for the design of additional combination therapies, particularly for various relapsed and/or refractory B-cell lymphomas.

CSF-1/1R Inhibitors

CSF-1/1R signaling is a key survival signaling pathway in MPs, as blocking this signaling pathway preferentially depletes M2-like MPs while sparing M1-like MPs (154). CSF-1R signaling blockade can be achieved using antibodies against CSF-1/CSF-1R or other small-molecule inhibitors (155, 156). Anti-CSF-1R monoclonal antibodies are currently under preclinical and clinical evaluation for treatment of various solid tumors, indicating the possibility of using these antibodies against lymphoid malignancies (157, 158). Small-molecule CSF-1R kinase inhibitors such as pexidartinib (PLX3397) have effectively reduced the numbers of TAMs in several different cancer models (158–162). For example, deletion of TAMs by pexidartinib enhanced antitumor immunity and survival induced by immunotherapy in patients with solid tumors (160).

CONCLUDING REMARKS

B-lymphoid cells represent one of two essential components of the human cellular immune system that continue to be linked with MPs under normal, pathological, and neoplastic conditions. For more than 50 years, MPs have been recognized as relatively independent multifunctional hematological and immunological cells with only limited or tangential interrelationships with each other. Many studies have revealed that individualized biological and/or immunological functions of MPs represent wide functional flexibility, making them capable of recognition of and/or interaction with a wide variety of normal as well as neoplastic immune cells, although B-cell tumors at times have converted or transformed into monocytes/MPs or even have possibly been capable of actually becoming biphenotypic. In fact, researchers have shown that MPs and B cells have almost limitless cellular interactive potential, particularly in the immune and inflammatory systems, but are by no means limited to these essential areas. With the advent of increased realization and recognition of important aspects of modern molecular genetics along with the role and extended research capabilities, these multifunctional, seemingly limitlessly flexible cell types continue to play even larger and more complex roles in newly recognized forms of contemporary immunotherapy and cellular immunology. In fact, MPs and B cells, which are two of the most important primary mammalian immune/inflammatory cell types, are responsible for maintaining many elements of human immune system. These immune cell components are derived, at least in part, from the quite close relationships of components of myeloid/MP and B-lymphoid cell lineages that provide key functional cellular and humoral immune response components

with multiple intercellular linkages to the complex, intricate human immune system.

In the innate immune system, TAMs (M2, polarized, alternatively activated MPs), which can play multiple critical wide-ranging roles in basic biological activities, often mediate enhanced tumor progression in patients with poor clinical prognosis, demonstrating multiple functional pathophysiological capabilities. These include the ability to secrete key chemokines, cytokines, and various bioactive proteases that have been shown capable of stimulating tumor cell growth, angiogenesis, metastasis, chemoresistance, and immunosuppression. Of particular interest and possible importance, some recent studies demonstrated that M2-polarized MPs can express key immunotherapeutic targets, such as checkpoint proteins (e.g., PD-1) that appear to be involved in T-cell activation, as well as targets of other specific checkpoint-blocking immunotherapies (anti-PD-1/PDL-1) currently of interest as part of new therapeutic paradigms for understanding the conceptual basis for chemotherapy-resistant neoplasms.

Whereas much is known about the wide spectrum of plasticity and cellular flexibility of MPs in many normal and pathological cellular settings, relatively little is known about the increasingly important interactions between MPs and B-lymphoid cells, particularly in the TMEs of patients with aggressive forms of NHL-B. Our own studies have defined what we believe to be the necessary and sufficient conditions for establishing better *in vitro* models for defining the actual mechanisms driving the pathophysiology of B-cell lymphoma cells. Preliminary studies demonstrated that efficient *in vitro* NHL-B cell growth not only requires the presence of viable clonal NHL-B cells but also often requires the presence of large, autochthonous monocytes/MPs and probably other related myeloid-derived cells that are necessary to maintain lymphoma cell growth with adequate cellular viability to yield persistent, effective cell-line establishment. Such findings have proven to be essential for developing new effective therapeutic strategies, particularly for relapsed/refractory NHL-B, by targeting TAMs with monoclonal antibodies (anti-CSF-1R) or small-molecule inhibitors of CSF-1R kinase (PLX3397).

Cumulative studies over the past 50 years have brought to light the critical role of TAM/B-cell interactions in the pathophysiology of NHL-B. Now is the time to use this important and practical knowledge to further develop new novel strategies to better treat this deadly disease, as more than 30% (~20,000) of NHL-B patients die every year of disease processes that we should be able to reverse if not actually cure with a better understanding of the pathophysiology and capabilities demonstrated using improved model systems established in recent years.

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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Novel Immunotherapy Options for Extranodal NK/T-Cell Lymphoma

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Extranodal NK/T-cell lymphoma (ENKTCL) is a highly aggressive mature NK/T-cell neoplasm marked by NK-cell phenotypic expression of CD3ε and CD56. While the disease is reported worldwide, there is a significant geographic variation with its highest incidence in East Asian countries possibly related to the frequent early childhood exposure of Epstein–Barr virus (EBV) and specific ethnic–genetical background, which contributes to the tumorigenesis. Historically, anthracycline-based chemotherapy such as CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) was used, but resulted in poor outcomes. This is due in part to intrinsic ENKTCL resistance to anthracycline caused by high expression levels of P-glycoprotein. The recent application of combined modality therapy with concurrent or sequential radiation therapy for early stage disease, along with non-anthracycline-based chemotherapy regimens consisting of drugs independent of P-glycoprotein have significantly improved clinical outcomes. Particularly, this neoplasm shows high sensitivity to L-asparaginase as NK-cells lack asparagine synthase activity. Even still, outcomes of patients with advanced stage disease or those with relapsed/recurrent disease are dismal with overall survival of generally a few months. Thus, novel therapies are needed for this population. Clinical activity of targeted antibodies along with antibody–drug conjugates, such as daratumumab (naked anti-CD38 antibody) and brentuximab vedotin (anti-CD30 antibody conjugated with auristatin E), have been reported. Further promising data have been shown with checkpoint inhibitors as high levels of programmed death-ligand 1 expression are observed in ENKTCL due to EBV-driven overexpression of the latent membrane proteins [latent membrane protein 1 (LMP1) and LMP2] with activation of the NF-κB/MAPK pathways. Initial case series with programmed death 1 inhibitors showed an overall response rate of 100% in seven relapsed patients including five with a complete response (CR). Furthermore, cellular immunotherapy with engineered cytotoxic T lymphocytes targeted against LMP1 and LMP2 have shown encouraging results with durable CRs as either maintenance therapy after initial induction chemotherapy or in the relapsed/refractory setting. In this paper, we review this exciting field of novel immunotherapy options against ENKTCL that hopefully will change the treatment paradigm in this deadly disease.

Keywords: NK T cell lymphoma, CD30 ligand/CD30, CD38, programmed death 1, programmed death ligand 1, latent membrane protein 1, LMP2, EBV lymphoma

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BACKGROUND

Extranodal NK/T-cell lymphoma (ENKTCL) is a locally destructive and highly aggressive mature lymphoid neoplasm with a prevalence of <1% of all non-Hodgkin's lymphomas (NHLs) in the western world and up to 10% of NHLs in Asia and South America (1). The regional differences in prevalence is due in part to the Epstein–Barr virus (EBV) related pathogenesis of the disease and early childhood exposure to the virus (2). Furthermore, environmental and genetic factors may contribute to its etiology as recent SEER registry studies have shown higher incidence of the disease in Asian-Pacific Islanders and Hispanics as compared to non-Hispanic whites even in the United States (3, 4). Its classification and diagnosis is made by its immunophenotypic expression of CD2+, sCD3−, cytoplasmic CD3ε+, CD56+, and cytotoxic molecules, including perforin, granzyme B, and T-cell intracellular antigen 1 (1, 5, 6). EBV expression and *in situ* hybridization is imperative to the diagnosis as its presence is essential to its pathogenesis (7).

About two-thirds of the ENKTCL cases present as localized stage I and II disease mainly in the upper aerodigestive tract (UADT) (8–10). Therefore, treatment is usually a combination of chemotherapy with local radiotherapy occurring concurrently or sequentially, resulting in overall response rates of 80–90% (11–16). The 5-year progression-free survival (PFS) and overall survival (OS) rates range from 60 to 85 and 64 to 89%, respectively, which is still relatively poor for localized NHL. Over the last 20 years, the management of advanced stage ENKTCL has largely changed due to the discovery of high expression levels of P-glycoprotein on NK lymphoma cells, leading to intrinsic resistance of previously used adriamycin- and cyclophosphamide-based chemotherapy regimens (17). On the other hand, ENKTCL cells were found to lack expression of asparagine synthase and, therefore, rendered sensitive to L-asparaginase-containing chemotherapy regimens (18, 19). Even so, the complete response (CR) rates are around 50–60% with one long-term study reporting a 5-year OS of about 50% (20–23). Patients who relapse after having received L-asparaginase-containing regimens have a dismal outcome with OS of just a few months (24). Therefore, novel therapies are needed for this group of patients in the salvage setting and may even provide benefit when employed as part of a maintenance strategy in upfront therapy. The intrinsic pathogenesis of EBV-induced proliferation along with the innate expression of targetable CD markers make novel immunotherapy strategies an attractive option in L-asparaginase refractory cases. In this review, we focus on the currently available literature and case reports of immunotherapy approaches in both frontline and relapsed/refractory ENKTCL.

TARGETING CD30

Expression of CD30 has been widely reported in Hodgkin's lymphoma (HL) and various T cell lymphomas. It functions as a member of the tumor necrosis factor receptor pathway and is not usually expressed in normal human tissue, which makes it an attractive tumor target (25). CD30 expression in ENKTCL is variable around 50–70% in three separate studies but its clinical significance remains controversial (26–28). In one 22-patient study, CD30 expression of ≥50% was associated with worse event-free survival and OS (29). In another larger 72-patient study, CD30 expression ≥5% was associated with decreased risk of relapse, increased response, and improved OS when treated with non-anthracycline-based chemotherapy (27). The largest study included 317 ENKTCL patients and out of the 91 patients who had CD30 immunohistochemistry performed on cataloged tissue, they found no association between CD30 expression and survival outcomes (26). Similarly, the most recent study of 97 patients showed 56% of specimens had CD30 expression, but there was no association with OS or PFS at CD30 cutoffs of 1, 10, or 20% (28). These variable results are due to the retrospective nature of these studies, tumor variability with varying numbers of early versus late stage patients and different cutoffs for what constitutes positive CD30 tumor expression.

Brentuximab vedotin (BV) is a CD30-targeted antibody conjugated with auristatin E that has shown high efficacy in relapsed HL and multiple T cell lymphomas (30–33). Its efficacy stems not from mechanisms of direct immune activation through the CD30 antibody but rather through the internalization of the conjugated auristatin E (MMAE) leading to direct cytotoxicity. Initial studies of a "naked" CD30 monoclonal antibody (SGN-30) showed little to no efficacy in treating CD30-positive lymphomas (34). However, responses to BV were also apparent in T cell lymphomas that had low or absent CD30 expression, suggesting that the drug may also be dispersed to the tumor microenvironment and later released into the tumor cells as a bystander effect (35, 36). Similar results were also observed in diffuse large B cell lymphoma where even two patients with ≤1% tumor CD30 expression had CRs when treated with BV (37–39). Along with direct cytotoxicity and bystander effects as mechanisms of action for BV, mouse models have shown increased immune activation after treatment with BV with enhanced T cell activation and dendritic cell priming and migration toward tumor-draining lymph nodes (40, 41). Moreover, the antitumor effects of BV were much less pronounced in immunocompromised mice. Therefore, the rational combination BV with immune-activating agents such as anti-programmed death 1 (PD1) antibodies could potentiate increased efficacy. Unfortunately, almost all patients treated with BV do eventually relapse and the means of resistance are not entirely clear as the pathogenic role of CD30 has not been fully characterized. Chen et al. showed that BV-treated HL cell lines became increasingly more resistant to MMAE (the internalized chemotherapeutic component) with the mechanism possibly being increased expression of MDR1 protein, which is a known drug exporter (42).

Although there have been no clinical trials run specifically in relapsed/refractory ENKTCL, there have been two case reports of patients achieving CR after BV therapy. One patient had non-UADT ENKTCL and was heavily pretreated who then achieved a CR after just four cycles of BV (43). The patient quickly relapsed in 3 months after discontinuing therapy due to increasing dyspnea. The other patient was a 17-year-old female also with non-UADT ENKTCL who relapsed after two cycles of an L-asparaginase-containing regimen (44). After three cycles of

BV with bendamustine, she achieved a CR and was able to receive a haploidentical transplant with continued undetectable plasma EBV DNA levels posttransplant. With these encouraging results, multiple clinical trials of combining BV with both L-asparaginase and non-L-asparaginase-containing chemotherapy regimens either as sequential or combination therapy in the frontline setting have completed accrual (NCT01309789) and are being planned (NCT0324750).

Other CD30-specific therapies include engineered chimeric antigen receptor T-cells (CAR-T), which are antigen-specific T-cells with an antigen-recognizing extracellular single-chain variant fragment coupled with an activating intracellular domain that is then linked to one or more costimulatory molecules. CAR-T can be constructed toward specific targets such as CD30 and has already shown efficacy in clinical trials against various CD30-positive lymphomas. Ramos et al. treated seven HL patients and two patients with anaplastic large cell lymphoma (ALCL) with a CD28 co-stimulated anti-CD30 CAR-T with seven of these patients having previously received BV (45). Three patients achieved a CR (two HL and one ALCL) with two HL patients having stable disease. There were no reported cases of cytokine release syndrome. In another phase I trial, 18 patients (17 HL and 1 cutaneous ALCL) were treated with a 4-1BB co-stimulated anti-CD30 CAR-T construct, reporting 7 partial responses (46). Although these results are not quite as impressive as compared to their anti-CD19 CAR-T counterparts, CAR-T still remains a specific and viable treatment for relapsed/refractory ENKTCL in an area that does not have many proven options.

TARGETING CD38

CD38 is almost universally expressed within ENKTCL. In one study, investigators reported only 5% of NKTCL samples being completely negative for CD38 according to their proportion score with more than half of the samples being strongly positive (47). High CD38 expression within this disease is associated with worse PFS and OS independent of local tumor invasion. The naked anti-CD38 antibody daratumumab has high avidity toward CD38 and induces the greatest amounts of both complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (48). In heavily pretreated and relapsed/refractory multiple myeloma (MM) patients, daratumumab has already been approved in a variety of combinations due to high amounts of response and improvement in survival outcomes (49, 50). CD38 expression in MM has not correlated to responses to daratumumab as even patients with high proportion scores could be primary refractory, leaving the full mechanism of action of daratumumab up for further investigation (51). Response and resistance to the drug has been proposed as a combination of membrane CD38 expression, CD38/daratumumab binding, and endocytosis of the complex leading to clearance of daratumumab (51–53). More recently, upregulation of CD55 and CD59 was observed in MM patients progressing on daratumumab (54). Given the CDC mechanism of the drug, increased expression of complement inhibitory proteins, such as CD55 and CD59, suggests a method of resistance. Further supporting this, the investigators used all-trans retinoic acid to inhibit CD55/CD59 expression on relapsed MM cell lines, which then restored daratumumab CDC (54).

Activity of daratumumab in ENKTCL has been described in one case report in which a heavily pretreated patient was salvaged with the drug (55). This patient had initial stage IE disease treated with concurrent chemoradiation with a short response and widespread relapse including in the cerebrospinal fluid (CSF). She was then salvaged with an L-asparaginase-containing regimen followed by an allogeneic stem-cell transplant, but her disease relapsed 3 weeks after transplant. Daratumumab was initiated with initial rise in EBV titer levels within the first 4 weeks, but then eventual CR including clearance of her CSF at week 21. A multi-center phase II trial within multiple Asian countries is currently ongoing to assess the safety and efficacy of daratumumab within ENKTCL (NCT02927925). While most of the research on daratumumab has been in MM, ENKTCL-specific mechanisms of action and resistance will have to be investigated.

TARGETING PD1

Programmed death-ligand 1 (PD-L1) is an immunomodulatory cell-surface glycoprotein mostly expressed on antigen-presenting cells (APC) as part of natural T cell anergy and downregulation (56). Multiple tumor types upregulate PD-L1 to escape immune surveillance and enhance survival. As EBV contributes to the pathogenesis of ENKTCL, expression of the immunogenic latent membrane protein 1 (LMP1) by the virus acts to enhance PD-L1 expression through upregulation of the MAPK/NF-κB pathways (**Figure 1**) (57). In fact, almost all of the EBV-associated lymphomas, including B cell lymphomas, were associated with high expression levels of PD-L1 (58). Reported expression of PD-L1 in ENKTCL has been variable ranging from 39 to 100% with low PD1 expression within both the tumor and infiltrating immune cells (59–62). Nodal variants of NKTCL may have higher PD-L1 expression as compared to extranodal disease (61). Correlation of PD-L1 expression to clinical characteristics has shown that increased levels were associated with lower serum LDH and IPI stage (60). Contrastingly to the association of high PD-L1 expression with traditionally classified lower risk disease, Nagato et al. reported that increased PD-L1 expression within the tumor cells was correlated with increased serum PD-L1 levels and worse OS (59). Larger studies are needed to fully correlate PD-L1 with survival as other studies have shown no association (60).

The use of anti-PD1 antibodies such as pembrolizumab and nivolumab disrupt the PD-L1/PD1 interaction and can restore the antitumor activity of activated T cells (63). Kwong et al. reported a case series of patients with previously treated ENKTCL who received pembrolizumab (64). All seven patients had been previously treated with L-asparaginase containing regimens and two patients had received allogeneic stem-cell transplants. PD-L1 expression was considered "strong" in four patients with one patient having weaker expression at 20% and the other two patients not having PD-L1 testing performed on their tumor specimens. After a median follow up of 6 months, all patients experienced an objective response with five patients achieving CR.

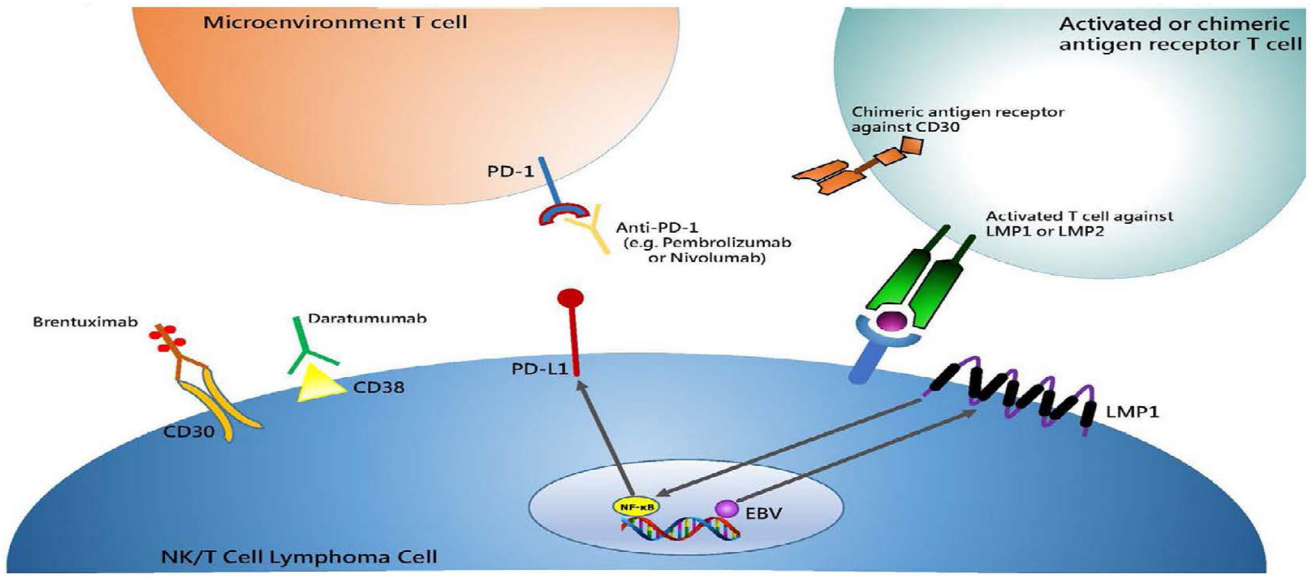

FIGURE 1 | Summary of immunotherapy drugs or treatment strategies in NK/T cell lymphoma and their respective cellular membrane targets. Antibody drugs target cellular membrane proteins, which include Brentuximab/CD30 and Daratumumab/CD38. Engineered chimeric antigen T-cells are targeted toward CD30 much like Brentuximab. Anti-PD1 antibodies, such as Pembrolizumab and Nivolumab, target microenvironment T-cells that become inactivated when bound with Programmed death-ligand 1 (PD-L1) expressed on tumor cells, inducing anergy. Latent membrane protein 1 (LMP1) is a transmembrane protein produced by Epstein–Barr virus (EBV), which subsequently activates the NF-κB pathway and leads to cell proliferation and lymphomagenesis. This in turn upregulates PD-L1, which makes immune checkpoint blockade an attractive target. Furthermore, LMP1 antigen is expressed within a MHC-complex on the cell surface to which activated T cells can then recognize and extinguish. *This was an originally produced image.*

TABLE 1 | Summary of immunotargets and drugs/therapies available against various intrinsic NK/T cell lymphoma markers or viral antigens. Best response rates are briefly summarized in the efficacy column.

Target	Drug/Therapy	Efficacy	Comment	Reference
CD30	Brentuximab	Two case reports both achieving CR		(43, 44)
CD30	Chimeric antigen receptor T cells	Mostly SD or PR with 3 patients achieving CR	Patients had either HL or ALCL, and currently remains untested in ENKTCL	(45, 46)
CD38	Daratumumab	One case report with CR		(55)
PD1	Pembrolizumab, Nivolumab	Case series with 7 patients treated. All patients achieved a response with 5 CRs		(64)
LMP1/ LMP2 (EBV antigens)	Activated/stimulated T cells	6 patients had active disease with 3 patients achieving durable remissions, but 2 with no response. Maintenance strategy after first-line treatment saw durable remissions in all patients		(65, 70)

PD1, programmed death 1; LMP, latent membrane protein; EBV, Epstein–Barr virus; CR, complete response; PR, partial response; SD, stable disease; HL, Hodgkin's lymphoma; ALCL, anaplastic large cell lymphoma; ENKTCL, extranodal NK/T cell lymphoma.

Given the universal EBV-induced pathogenesis of ENKTCL and LMP1 directed overexpression of PD-L1, checkpoint blockade remains a very attractive immunotherapy option for this disease. Currently, multiple clinical trials are ongoing to assess the efficacy of anti-PD1 therapies in relapsed/refractory ENKTCL (NCT03107962 and NCT03021057). As suggested previously, combining anti-PD1 agents with BV could further potentiate antitumor activity.

TARGETING EBV ANTIGENS

Further utilizing the EBV antigens present within ENKTCL, stimulated cytotoxic T lymphocytes (CTL) directed at LMP1 and LMP2 within the virus have shown efficacy in treating a multitude of EBV-derived lymphomas (65). Initial viral antigen targeting through autologous T cell activation was designed to treat viral reactivation after bone marrow transplants and was proven to be highly specific and efficacious (66, 67). Further expansion of this idea to treat EBV-associated post-transplant lymphoproliferative disorder showed sustained CRs of 68–84% (68, 69). Bollard et al. treated 52 EBV-associated lymphoma patients with a combination of LMP1/2 or LMP2-only targeted and stimulated CTLs of which 11 patients had ENKTCL (65). This study used adenoviral vector transduced and EBV-transformed APCs as stimulators for LMP-specific T cell expansion, which was later re-infused into the patients.

Of the 11 patients with ENKTCL treated in this study, 6 had active disease upon CTL infusion either as primary refractory or relapsed disease. Although two of these patients had no response to the CTLs, one patient achieved a CR that later relapsed within 9 months and the remaining three had durable remissions for more than 4 years. These results are impressive in light of known data for relapsed/refractory ENKTCL patients as half had durable CRs and undetectable plasma EBV levels. The other 5 out of 11 ENKTCL patients received CTLs as consolidative therapy after initial chemoradiation or after autologous stem-cell transplantation. All five of these patients remained in CR for 2–6 years. Even as a maintenance strategy for high-risk patients, this therapy may prove highly effective as one patient who had primary refractory disease but then achieved a CR after autologous stem-cell transplant remained in CR for 2 years following CTL infusion.

While these results may be remarkable for high-risk relapsed/refractory patients, the role of CTL therapy as maintenance therapy for localized disease after first-line therapy remains to be determined. Cho et al. treated eight localized disease and two advanced disease ENKTCL with LMP1/2-directed CTLs all of whom were in CR after initial induction chemotherapy with or without radiotherapy (70). Half of the patients also had consolidative autologous stem-cell transplants. The 4-year OS and PFS were 100 and 90% with only one patient who had initial stage IVE disease relapsing after 32 months. While these results seem impressive, it is unclear if the early stage patients truly benefited from maintenance CTL infusion as historical 5-year survival rates with chemoradiation can be upwards of 90%.

CONCLUSION

Extranodal NK/T-cell lymphoma remains an orphan disease with almost no phase III clinical trials to help guide therapy. Even less data are found in the relapsed/refractory cohort of patients in which most providers are using case reports and previous experience to choose treatments. With further understanding of the specific protein expression within ENKTCL, we are now able to target CD30, CD38, and PD1 as new drugs have become available (**Figure 1**). Combinations of these novel agents with conventional chemotherapy and each other are under investigation and may add more effective therapeutic choices. More specific to ENKTCL may be the use of EBV-antigen targeted CTLs that seem effective by themselves or as maintenance therapy for this disease. Although not yet tested in ENKTCL, CD30-targeted CAR-T may provide other T cell immunotherapy options for this disease (**Table 1**). Choosing one therapy over another is currently due to provider preference and patient-derived side effects from these drugs, but the overall goal would be to produce a deep response and move these relapsed/refractory patients onto an allogeneic bone marrow transplant. Future clinical trials with these novel immunotherapies will help to determine efficacy and whether to give these drugs upfront or in the salvage setting.

AUTHOR CONTRIBUTIONS

BH performed the literature search and wrote the manuscript for the review. He also generated the figures and tables for the manuscript. YO provided feedback, guidance and mentorship for the manuscript.

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Conflict of Interest Statement: 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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Restoration of Decreased T Helper 1 and CD8+ T Cell Subsets Is Associated With Regression of Lymphoproliferative Disorders Developed During Methotrexate Treatment

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Background: Lymphoproliferative disorder (LPD), including malignant lymphoma, is a relatively rare but life-threatening complication in RA patients under methotrexate (MTX) therapy. Spontaneous regression of LPD after MTX withdrawal is regarded as a distinct characteristic in part of such LPDs.

Objective: The present study aimed to investigate the immunological difference in regressive LPD and persistent LPD.

Methods: We studied RA patients who developed LPD during MTX administration ($n = 35$) and clinically matched controls ($n = 35$). The time of MTX cessation was defined as week 0, and LPD patients were divided into two groups according to LPD status at week 12: regressive group ($n = 22$) and persistent group ($n = 13$). Flow cytometric analysis of whole blood samples and serum cytokine assays were conducted for LPD ($n = 10$) and control patients ($n = 10$) at weeks 0, 4, and 12.

Results: There was a significant decrease in peripheral lymphocytes and the proportion of T helper 1 cells (Th1 cells), effector memory CD8+ T cells (EMCD8+ T) and Epstein–Barr virus (EBV)-specific CD8+ T cells at the time of LPD diagnosis, and a significant increase after MTX cessation was observed in the regressive group but not in the persistent group. The expansion of Th1 cells and EMCD8+ T cells significantly correlated with an increase in serum interferon (IFN)-γ concentration.

Conclusion: Changes in Th1 cells, EMCD8+ T cells and EBV-specific CD8+ T cells, which coincided with an increase in IFN-γ, were significantly different between regressive LPD and persistent LPD after MTX cessation.

Keywords: lymphoproliferative disorder, malignant lymphoma, regression, methotrexate, T cell subset

INTRODUCTION

Methotrexate (MTX) is an anti-rheumatic drug and the gold standard for treatment of rheumatoid arthritis (RA) worldwide (1, 2). However, MTX-associated side effects and certain adverse events can lead patients to abandon the treatment (1, 2). Among these, lymphoproliferative disorder (LPD), including malignant lymphoma, is a relatively rare but life-threatening complication (3, 4). Several studies have reported a high likelihood of developing LPD in MTX-treated patients (5, 6). On the other hand, previous reports revealed that chronic inflammation induced by RA itself is also a risk factor for LPD (7). It is difficult to distinguish between LPDs that develop associated with immunosuppression during MTX therapy and those induced by chronic inflammation or as an incidental complication; however, spontaneous regression of LPD following MTX cessation occurs in 30–70% of cases (3, 4, 7, 8). This had led to the suggestion that MTX may have the potential to cause lympho-proliferation. Our group and others previously reported the link between decreased lymphocyte counts at LPD diagnosis and subsequent restoration after MTX cessation and regression of LPD (8, 9), and suggested the association between activation of immune system and LPD regression.

Here, we investigated changes in lymphocyte subsets and serological factors during LPD regression, and assessed the difference in immune status between patients with regressive LPD and those whose LPD did not regress to investigate the pathogenesis of such LPDs.

MATERIALS AND METHODS

Patients and Data Collection

First, we retrospectively reviewed the medical records of RA patients in our institution from January 1995 to December 2013 and found 25 patients with pathologically defined LPD that developed during MTX treatment. We randomly selected 25 control RA patients without LPD who were treated with MTX in our institution matched for age, sex, MTX dose, and RA duration (LPD:control = 1:1). Second, we prospectively registered RA patients who were diagnosed with LPD while under MTX treatment ($n = 10$) from January 2014 to October 2015; clinically matched controls ($n = 10$) were randomly selected from RA patients without LPD who were administered MTX in the same period. We selected clinically matched patients based on categories of age, sex, MTX dose, and RA duration, and found several candidates for control per cases in our background cohort. And then we unintentionally selected one control RA patient based on patient linked-randomized number. The patients' blood samples were analyzed in the prospective cohort. In total, data from 35 LPD and 35 control RA patients were analyzed.

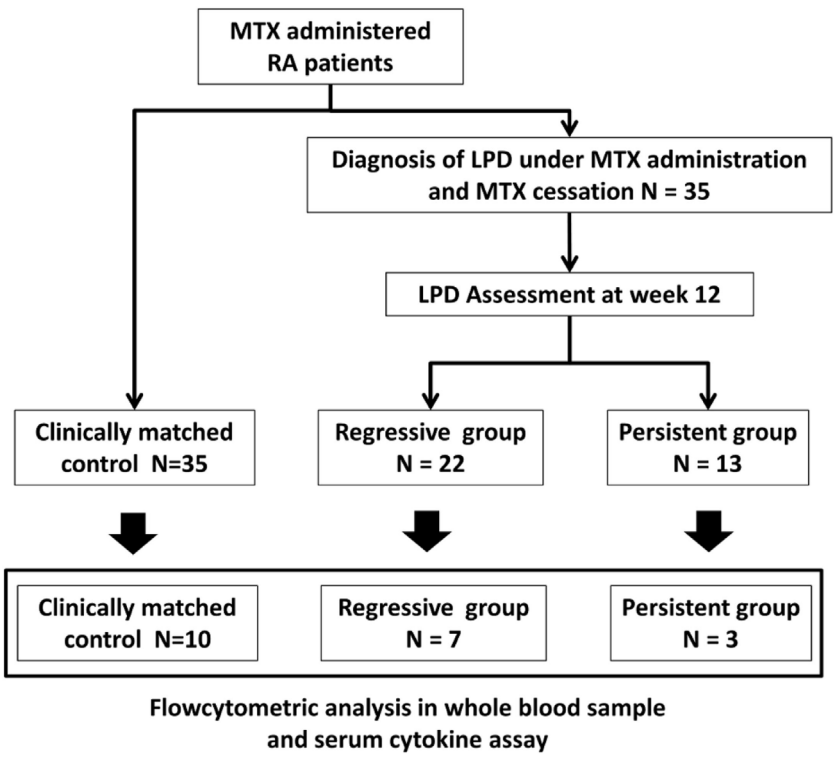

FIGURE 1 | Classification of LPD patients and patient flow. Twenty-two and 13 patients were divided into the regressive and persistent groups, respectively, according to LPD regression assessment at week 12. Control patients were age-, sex-, rheumatoid arthritis duration-, and MTX dose-matched with LPD patients ($N = 35$). Flow cytometric analysis of whole blood sample and cytokine analysis of serum sample were conducted for 10 patients from the control group, 7 patients from the regressive group, and 3 patients from the persistent group. MTX, methotrexate; LPD, lymphoproliferative disorder.

This study was approved by the ethics committee of Keio University School of Medicine (approval number: 20110136, 20130246, and 20130364) and the ethics committee of Saitama Medical Center, Saitama Medical University (approval number: 759). In accordance with the Declaration of Helsinki, written informed consent was obtained from the patients who had their blood samples analyzed, but consent from patients in the retrospective analysis of clinical features was waived in accordance with the regulations in Japan.

LPD Assessment

According to the WHO classification of tumors of hematopoietic and lymphoid tissues (4, 10), LPDs were classified into classical Hodgkin's lymphoma ($n = 8$), diffuse large B cell lymphoma (DLBCL) ($n = 18$), follicular lymphoma (FL) ($n = 2$), lymphomatoid granulomatosis (LYG) ($n = 2$), mucosa-associated lymphoid tissue (MALT) lymphoma ($n = 1$), NK/T cell lymphoma ($n = 1$), reactive hyperplasia ($n = 1$), and LPD with atypical cell proliferation ($n = 2$). The report of "LPD with atypical cell proliferation ($n = 2$)" was derived from other faculty and had no sufficient information of immunohistochemistry, and could not obtain additional pathological information. The presence of the Epstein–Barr virus (EBV) genome was assessed by *in situ* hybridization for EBV-encoded small RNAs (EBER), and clinical stage was determined using the Ann Arbor staging classification with Cotswolds modifications (11).

All patients stopped MTX treatment when LPD was suspected. The time of MTX cessation, which was simultaneous with diagnosis of LPD was defined as week 0, and the regression of LPD was assessed at week 12, in accordance with the revised response criteria of the International Working Group (12). Following the assessment of regression, patients were classified into two groups: the regressive group ($n = 22$), those who achieved complete ($n = 6$) or partial remission ($n = 16$), and the persistent group ($n = 13$), those with stable ($n = 5$) or progressive LPD ($n = 8$) (**Figure 1**). Complete remission was defined as the absence of all evidence of the disease; and partial remission as a LPD regression defined by ≥50% decrease in the sum of the product

TABLE 1 | Characteristics of LPD and control RA patients.

	Regressive group ($n = 22$)	Persistent group ($n = 13$)	Control group ($n = 35$)	Regressive vs. persistent, *P*	Regressive vs. control, *P*	Persistent vs. control, *P*
Demographics						
Age (years)	67 (58–73)	67 (64–71)	66 (57–72)	0.64	0.98	0.66
Female, *n* (%)	20/22 (91%)	11/13 (85%)	31/35 (89%)	0.57	0.78	0.71
RA features						
RA disease duration (months)	132 (57–227)	132 (115–298)	155 (88–201)	0.62	0.74	0.95
RF positive, *n* (%)	17/20 (85%)	9/13 (69%)	28/35 (80%)	0.28	0.64	0.43
ACPA positive, *n* (%)	14/18 (78%)	3/7 (43%)	17/23 (74%)	0.10	0.77	0.13
TJC	1 (0–3)	0 (0–2)	0 (0–1)	0.78	0.74	0.48
SJC	1 (0–3)	1 (0–2)	0 (0–2)	0.44	0.47	0.06
MTX duration (months)	65 (23–99)	98 (72–120)	87 (49–115)	0.08	0.17	0.41
MTX dose (mg/week)	10 (6–12)	8 (6–12)	10 (6–12)	0.82	0.61	0.56
MTX cumulative dose (mg)	2,080 (844–3,849)	3,064 (1,810–3,744)	2,760 (1,408–4,144)	0.29	0.46	0.99
Concomitant DMARDs	12/22 (55%)	6/13 (46%)	14/35 (40%)	0.63	0.28	0.70
Sjögren's syndrome	2/22 (9%)	4/13 (31%)	3/35 (9%)	0.11	0.95	0.07
LPD features						
Pathological phenotype (*n*, %)			–			
cHL	2 (9%)	6 (46%)	–			
DLBCL	14 (64%)	4 (31%)	–			
FL	0 (0%)	2 (15%)	–			
MALT lymphoma	1 (5%)	0 (0%)	–	0.07	–	–
NKT cell lymphoma	1 (5%)	0 (0%)	–			
LYG	2 (9%)	0 (0%)	–			
Reactive hyperplasia	1 (5%)	0 (0%)	–			
LPD with atypical cell proliferation	1 (5%)	1 (8%)				
EBER positive, *n* (%)	13/19 (68%)	7/11 (64%)	–	0.79	–	–
Clinical stage, I/II/III/IV, *n* (%)	10/4/4/4 (45/18/18/18, %)	3/3/5/2 (23/23/38/15, %)	–	0.47	–	–
White blood cell count (× 10^3/μL)	4.7 (3.0–7.0)	6.1 (4.4–8.3)	5.5 (4.4–6.4)	0.14	0.26	0.52
Lymphocyte count (/μL)	508 (286–825)	1,165 (517–2,035)	1,321 (1,045–1,691)	<0.01*	0.01*	0.55
LDH (IU/L)	226 (186–314)	257 (189–342)	194 (170–226)	0.82	0.02*	0.02*
CRP (mg/dL)	1.48 (0.26–2.88)	0.9 (0.11–2.20)	0.08 (0.03–0.47)	0.31	<0.01*	<0.01*
IgG (mg/dL)	1,218 (1,052–1,451)	1,511 (1,228–1,986)	1,518 (1,195–1,689)	0.05	0.12	0.63
sIL-2R (IU/L)	871 (447–1,436)	1,910 (894–2,600)	–	0.15	–	–

LPD, lymphoproliferative disorder; RA, rheumatoid arthritis; RF, rheumatoid factor; ACPA, anti-cyclic citrullinated peptides antibody; TJC, tender joint count; SJC, swollen joint count; MTX, methotrexate; DMARD, disease-modifying anti-rheumatic drug; cHL, classical Hodgkin's lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; LYG, lymphomatoid granulomatosis; MALT, mucosa-associated lymphoid tissue; EBER, Epstein–Barr virus-excreted RNA; LDH, lactate dehydrogenase; CRP, C-reactive protein; IgG, immunoglobulin G; sIL-2R, soluble IL-2 receptor.

**P < 0.05.*

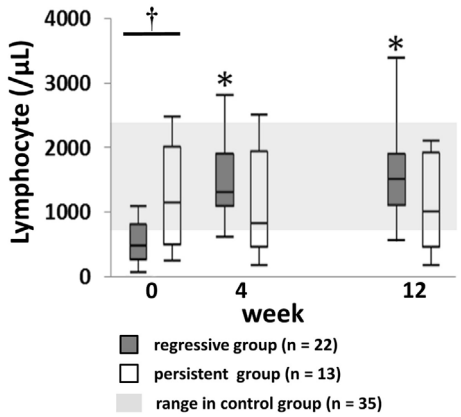

FIGURE 2 | Changes of lymphocyte numbers after MTX cessation in lymphoproliferative disorder (LPD) patients. Change in lymphocyte counts in regressive and persistent LPD compared with the range in the control group. Kruskal–Wallis test was used for comparing the three groups. †Regressive vs. control, $P < 0.05$; regressive vs. persistent, $P < 0.05$. *$P < 0.05$ for comparison with the value at week 0 in each group. MTX, methotrexate.

of the diameters of dominant masses, and no increase in size of other nodes and no new sites (12). Progressive disease was defined as any new lesion or increase by ≥50% of baseline, or cases that received chemotherapy ($n = 4$) or died of LPD ($n = 1$); and stable disease was defined as failure to attain complete or partial response that did not satisfy the definition of progressive disease (12).

We used the last observation carried forward method for the following conditions: intensification of RA treatment, initiation of chemotherapy, and patient death within 12 weeks post-MTX withdrawal. For matched control patients, we analyzed clinical data from the latest visit.

Flow Cytometric Analysis

Cell surface staining and flow cytometry analysis of fresh whole blood cells were performed at baseline (week 0), week 4, and week 12 for 10 LPD patients and 10 matched control RA patients. Peripheral blood cell subsets were defined by cell surface markers using a standardized method (13). Whole blood cells were stained for 30 min at room temperature in the dark with the following fluorophore-labeled mAbs:

TABLE 2 | Characteristics of LPD and control RA patients for whom flow cytometric analysis of whole blood sample and cytokine analysis of serum sample were conducted.

	Regressive group ($n = 7$)	Persistent group ($n = 3$)	Control group ($n = 10$)	Regressive vs. persistent, *P*	Regressive vs. control, *P*	Persistent vs. control, *P*
Demographics						
Age (years)	70 (44–71)	64 (63–66)	68 (59–73)	0.65	0.73	0.40
Female, *n* (%)	6/7 (86%)	2/3 (67%)	8/10 (80%)	0.49	0.76	0.63
RA features						
RA duration (months)	78 (20–175)	96 (49–124)	93 (44–137)	0.73	0.63	0.93
RF positive, *n* (%)	3/5 (60%)	2/3 (67%)	6/10 (60%)	0.85	1.00	0.84
ACPA positive, *n* (%)	4/5 (80%)	1/2 (50%)	5/10 (50%)	0.43	0.26	1.00
TJC	0 (0–1)	1 (0–6)	0 (0–1)	0.08	0.30	0.10
SJC	0 (0–1)	1 (0–2)	0 (0–2)	0.33	0.69	0.21
MTX duration (months)	32 (15–78)	96 (8–117)	49 (24–90)	0.43	0.46	0.61
MTX dose (mg/week)	12 (8–12)	12 (8–12)	10 (8–12)	0.88	0.52	0.58
MTX cumulative dose (mg)	988 (558–3,747)	3,712 (304–3,720)	1,700 (876–3,976)	0.80	0.33	0.87
Concomitant DMARDs	3/7 (43%)	3/3 (100%)	5/10 (50%)	0.09	0.77	0.12
Sjögren's syndrome	1/7 (14%)	1/3 (33%)	1/10 (10%)	0.49	0.79	0.33
LPD features						
Pathological phenotype, *n* (%)						
cHL	1 (14%)	2 (67%)	–	0.11	–	–
DLBCL	5 (71%)	0 (0%)				
LPD with atypical cell proliferation	1 (14%)	1 (33%)				
EBER positivity	4/7 (57%)	1/3 (33%)	–	0.49	–	–
Clinical stage, I/II/III/IV, *n* (%)	4/2/0/1 (57/29/0/14, %)	1/2/0/0 (33/67/0/0, %)		0.49	–	–
White blood cell count (× 10^3/μL)	4.5 (2.7–10.0)	5.2 (4.6–6.1)	5.4 (4.4–9.0)	0.57	0.73	0.87
Lymphocyte count (/μL)	480 (248–648)	2,001 (1,872–2,070)	1,522 (1,077–1,941)	0.01*	<0.01*	0.13
LDH (IU/L)	224 (186–350)	196 (177–238)	223 (176–240)	0.49	0.59	0.61
CRP (mg/dL)	1.19 (0.10–2.10)	0.22 (0.06–0.22)	0.10 (0.04–0.34)	0.14	0.03	0.80
IgG (mg/dL)	1,319 (781–1,532)	1,631(1,511–1,755)	1,191 (858–2,013)	0.10	0.88	0.51
sIL–2R (IU/L)	555 (283–1,210)	289 (206–956)	–	0.48	–	–
EBV-DNA (× 10^3 copies/mL)	1.0 (0.0–5.0)	0.8 (0.0–0.9)	–	0.36	–	–

LPD, lymphoproliferative disorder; RA, rheumatoid arthritis; RF, rheumatoid factor; ACPA, anti-cyclic citrullinated peptides antibody; TJC, tender joint count; SJC, swollen joint count; MTX, methotrexate; DMARD, disease-modifying anti-rheumatic drug; cHL, classical Hodgkin's lymphoma; DLBCL, Diffuse large B cell lymphoma; EBER, Epstein–Barr virus-excreted RNA; LDH, lactate dehydrogenase; CRP, C-reactive protein; IgG, immunoglobulin G; sIL-2R, soluble IL-2 receptor.
**P < 0.05.*

TABLE 3 | Proportion and absolute numbers of lymphocyte subsets.

	Regressive group (n = 7)	Persistent group (n = 3)	Control group (n = 10)	Regressive vs. persistent, P	Regressive vs. control, P	Persistent vs. control, P
Proportion (%)						
CD3+ T cells/lymph	68.1 (49.2–78.9)	78.2 (63.6–83.7)	62.2 (50.7–72.2)	0.59	0.36	0.08
CD4+/CD3+T cells	63.0 (44.6–82.2)	70.8 (56.0–77.6)	71.8 (55.3–80.8)	1.00	0.73	0.93
Naïve/CD4+ T	26.6 (24.0–45.7)	31.4 (28.5–36.2)	34.5 (19.1–38.2)	0.65	0.96	1.00
Central memory/CD4+ T	41.6 (29.3–46.1)	36.6 (33.9–44.0)	43.1 (37.4–49.1)	0.66	0.31	0.27
Effector/CD4+ T	1.1 (0.3–4.3)	0.4 (0.3–0.6)	1.4 (0.3–2.6)	0.27	0.81	0.49
Effector memory/CD4+ T	14.2 (6.1–31.6)	18.0 (13.7–25.6)	16.2 (11.9–20.9)	0.65	0.81	0.67
Th1/CD4+ T	14.8 (6.7–17.3)	19.8 (12.4–21.1)	18.1 (17.0–28.9)	0.17	0.02*	0.58
Th2/CD4+ T	65.2 (60.2–72.5)	55.8 (37.5–63.9)	64.0 (52.4–68.7)	0.17	0.46	0.36
Th17/CD4+ T	16.2 (11.7–18.8)	12.9 (8.0–15.8)	11.7 (7.6–13.5)	0.26	0.05	0.71
Th1/Th17/CD4+ T	4.1 (2.6–8.0)	8.4 (7.3–37.2)	6.2 (4.3–11.6)	0.07	0.34	0.14
Treg/CD4+ T	9.7 (3.6–11.5)	6.5 (5.8–7.5)	7.4 (5.6–9.2)	0.17	0.09	0.45
CD8+/CD3+ T cells	28.4 (14.0–49.2)	16.2 (12.9–25.3)	23.3 (14.9–30.7)	0.45	0.66	0.36
Naïve/CD8+ T	26.1 (14.9–40.4)	12.2 (5.1–31.2)	11.2 (9.3–16.5)	0.80	0.01*	0.26
Central memory/CD8+ T	18.3 (10.9–23.9)	16.7 (9.2–20.4)	10.9 (3.6–18.5)	0.82	0.05	0.27
Effector/CD8+ T	19.8 (6.7–30.4)	15.0 (8.2–30.6)	19.1 (11.8–27.5)	1.00	0.73	0.67
Effector memory/CD8+ T	22.5 (15.7–25.4)	34.3 (26.6–40.9)	45.7 (37.0–49.1)	0.02*	<0.01*	0.08
EBV–specific CD8+/CD8+ T	0.1 (0.1–0.2)	0.4 (0.3–0.6)	0.6 (0.4–1.2)	0.11	0.02*	0.56
B cells/lymph	8.8 (1.6–12.1)	3.3 (3.0–17.3)	11.9 (6.8–15.2)	1.00	0.19	0.55
NK cells/lymph	9.6 (5.8–33.1)	7.1 (5.8–9.55)	16.0 (12.5–23.4)	0.49	0.59	0.06
Absolute numbers[a]						
Lymphocytes	480 (248–648)	2,001 (1872–2070)	1,522 (1,077–1,941)	0.02*	<0.01*	0.15
CD3+ T cells	288 (153–534)	1,565 (1,317–1,567)	837 (680–1,037)	0.02*	0.01*	0.05
CD4+ T cells	257 (54–439)	1,021 (876–1,109)	639 (447–693)	0.01*	<0.01*	0.02*
Naïve CD4+ T	82 (11–185)	318 (316–321)	185 (121–235)	0.02*	0.05	0.05
Central memory CD4+ T	107 (24–148)	375 (320–449)	271 (237–313)	0.04*	<0.01*	0.02*
Effector CD4+ T	2 (1–19)	4 (3–6)	8 (2–16)	0.82	0.31	0.45
Effector memory CD4+ T	20 (15–45)	157 (140–284)	92 (61–195)	0.02*	<0.01*	0.08
Th1	26 (8–47)	206 (168–209)	114 (82–220)	0.02*	<0.01*	0.27
Th2	189 (38–320)	552 (510–666)	374 (269–487)	0.02*	0.01*	0.06
Th17	36 (7–84)	156 (83–174)	60 (50–85)	0.07	0.20	0.05
Th1/17	9 (2–26)	88 (73–506)	34 (23–79)	0.02*	0.02*	0.10
Tregs	16 (6–37)	78 (64–79)	36 (25–52)	0.02*	0.04*	0.02*
CD8+ T cells	75 (51–97)	212 (201–395)	192 (118–281)	0.07	0.02	0.55
Naïve CD8+ T	19 (9–83)	25 (20–66)	20 (16–32)	0.36	0.46	0.27
Central memory CD8+ T	11 (9–28)	36 (24–43)	20 (10–26)	0.17	0.66	0.08
Effector CD8+	20 (4–22)	30 (17–122)	27 (18–79)	0.17	0.05	1.00
Effector memory CD8+ T	16 (8–28)	69 (49–162)	82 (49–132)	0.02*	<0.01*	0.93
EBV-specific CD8+ T	0 (0–1)	2 (1–3)	2 (1–7)	0.11	0.02*	0.85
B cells	43 (4–110)	62 (60–358)	143 (98–454)	0.37	0.01*	0.55
NK cells	64 (60–99)	142 (109–198)	272 (147–394)	0.07	0.01*	0.27

Th, T helper; EBV, Epstein–Barr virus; NK, natural killer.

[a]*Number per 1 mm³ whole blood are indicated as absolute numbers.*

Values indicate median (Q1–Q3).

**P < 0.05.*

anti-CD3-Pacific Blue/fluorescein isothiocyanate (FITC)/PerCP-Cy5.5, anti-CD4-VioGreen (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD8- phycoerythrin (PE)-Cy5/PE-Cy7, anti-CD20 allophycocyanin-cyanine 7 (APC-Cy7), anti-CD25-PE-Cy5, anti-CD45RA-FITC, anti-CD56-PE-Cy7/APC, anti-CD127-FITC, anti-chemokine (C-X-C motif) receptor 3 (CXCR3)-PE, anti-chemokine (C-C motif) receptor 6 (CCR6)-PerCP-Cy5.5, anti-CCR7-PerCP-Cy5.5, HLA-A*2402 -restricted EBV-Tetramer-PE, anti-HLA-DR-APC/APC-Cy7 (all from BD Biosciences, Franklin Lakes, NJ, USA), and anti-mouse immunoglobulin G isotype-matched controls (VioGreen from Miltenyi Biotec, the others from BD Biosciences). Stained cells were washed twice with phosphate buffered saline and analyzed on a MACSQuant analyzer (Miltenyi Biotec). The lymphocyte subsets analyzed were CD4+ and CD8+ T cells (including naïve; CD45RA+CCR7+, effector; CD45RA+CCR7−, central memory; CD45RA−CCR7+, effector memory cells; CD45RA+CCR7−). CD4 T cells were classified into Th1 cells (CXCR3+CCR6−), Th2 cells (CXCR3−CCR6−), Th17 cells (CXCR3−CCR6+), and Treg cells (CD25+CD127low). Since detection of EBV-specific CD8+ T cells by the EBV-tetramer was restricted to HLA-A*2402 (14), HLA-A typing was also performed in LPD patients and control RA patients. Data on EBV-specific CD8+ T cells in patients who did not have HLA-A*2402 were excluded from the sub-analysis. Other cell subsets were defined as follows: B cells (CD3−CD20+) and natural killer cells (CD3−CD56+).

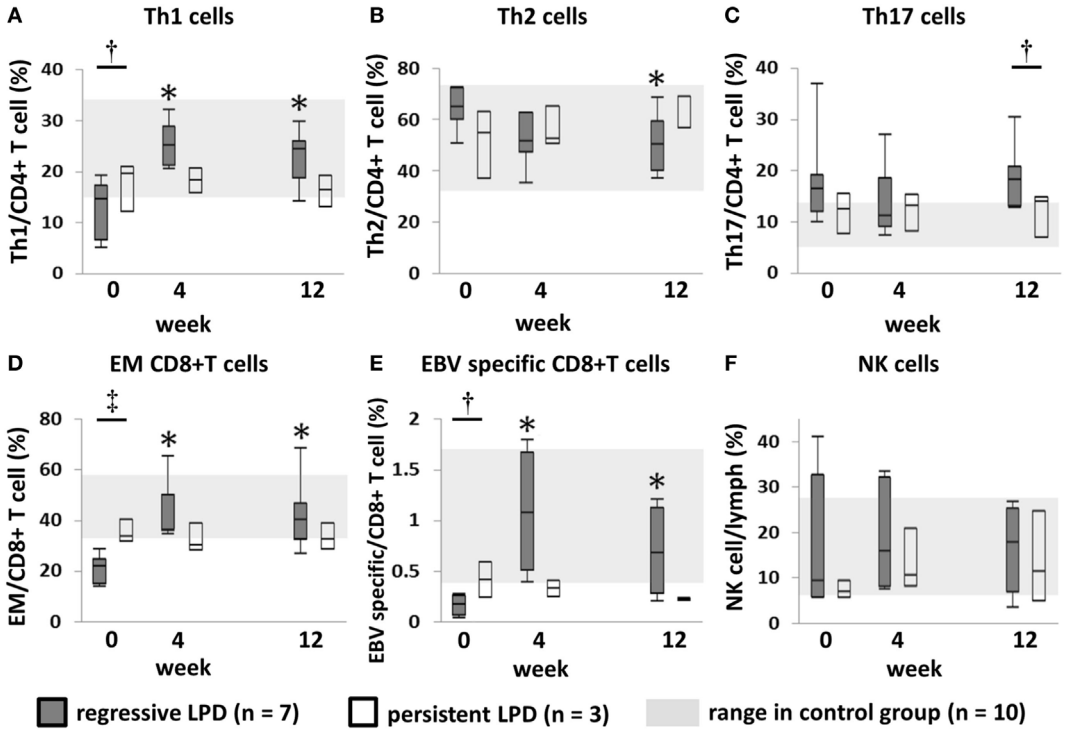

FIGURE 3 | Changes in the proportion of each lymphocyte subset after methotrexate (MTX) cessation. Transition of the proportion of **(A)** Th1, **(B)** Th2, and **(C)** Th17 cells among CD4+ T cells; **(D)** EMCD8+ T cells and **(E)** EBV-specific CD8+ T cells among CD8+ T cells; and **(F)** NK cells among lymphocytes, after MTX cessation. Comparison between the three groups was conducted using the Kruskal–Wallis test. †Regressive vs. control, $P < 0.05$. ‡Regressive vs. control, $P < 0.05$; regressive vs. persistent, $P < 0.05$. *$P < 0.05$ for comparison with the value at week 0 in each group. Th1/2/17, T helper 1/2/17; EM, effector memory; EBV, Epstein–Barr virus; NK, natural killer.

Serum Cytokine Assay and Quantification of EBV Viral Load

The concentration of serum interferon (IFN)-γ and interleukin (IL)-2, IL-7, IL-10, IL-12p70, IL-15, and TNF-α were measured by enzyme-linked immunosorbent assay kits (MSD, Gaithersburg, MD, USA), according to the manufacturer's protocol. Quantitative EBV-PCR level in whole blood sample were measured by a clinical laboratory testing company (BML Inc., Tokyo, Japan).

Statistical Analysis

Descriptive values are expressed as medians (Q1–Q3) or range. Comparisons of values and percentages of clinical parameters between the three groups were conducted using the Kruskal–Wallis test and Chi-squared test. Comparisons between two groups were conducted using paired or unpaired Wilcoxon test and Fisher's exact test. Correlations were analyzed by the Spearman's correlation coefficient. *P*-values less than 0.05 were regarded as statistically significant. All statistical analyses were performed with JMP software 11.2.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Baseline Characteristics of Regressive LPD, Persistent LPD, and Control Groups

The characteristics of patients at the time of LPD diagnosis and grouped into regressive ($n = 22$) and persistent groups ($n = 13$), and the control group ($n = 35$) are shown in **Table 1**. There were no significant differences in the demographic characteristics and RA features among the groups. Regarding the histological subtype of LPD, the proportion of patients with classical Hodgkin's lymphoma was lower and DLBCL was higher in the regressive group than the persistent group. The prevalence of EBER-positive LPD was similar between the regressive and persistent groups. Laboratory tests showed significantly higher levels of lactate dehydrogenase (LDH) and C-reactive protein (CRP) in the regressive and progressive groups compared with the control group, but there was no difference between the regressive and progressive groups. Absolute lymphocyte number was significantly lower in the regressive group compared with the progressive and control groups (508, 1,165, and 1,321/μL, respectively, $P < 0.01$). The decreased count of lymphocytes in the regressive group rapidly recovered to a level equivalent with that of the control group at week 4 (1,358/μL) after MTX withdrawal. By contrast, the lymphocyte number in the persistent group did not significantly change after MTX withdrawal (**Figure 2**).

The characteristics of the prospectively registered LPD patients ($n = 7$ with regressive LPD, $n = 3$ with persistent LPD) and controls ($n = 10$) are summarized in **Table 2**.

Lymphocyte Subsets at LPD Diagnosis

The proportion and absolute number of specified lymphocyte subsets at LPD diagnosis in the regressive and persistent LPD

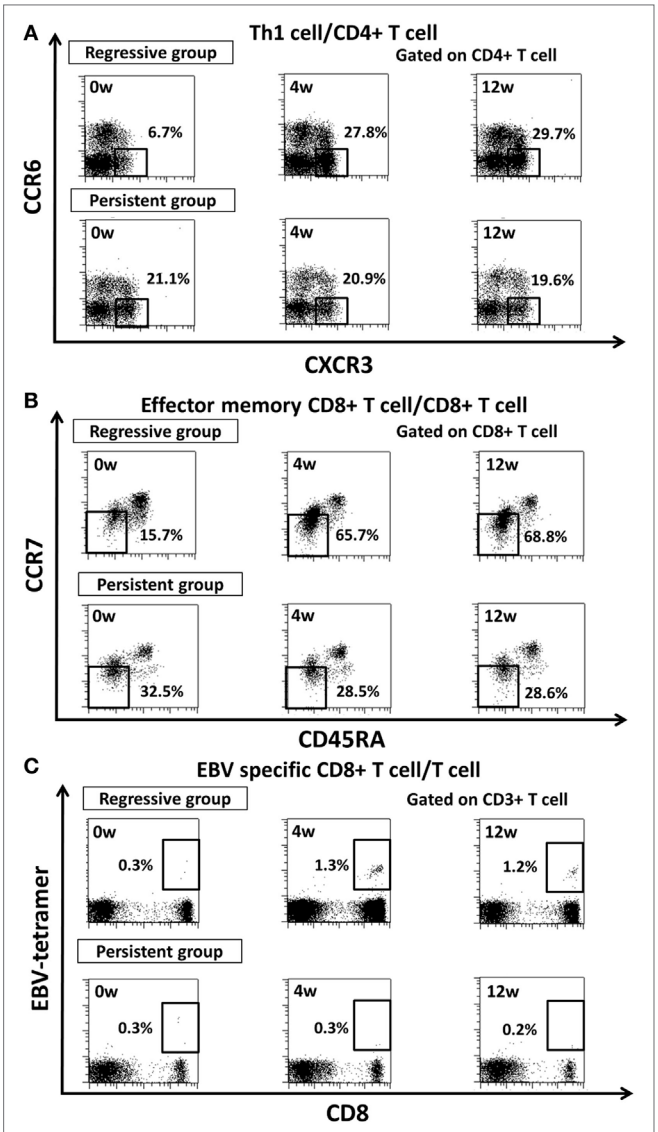

FIGURE 4 | Representative figure for transition of proportion of **(A)** Th1 cell, **(B)** effector memory CD8+ T cell, **(C)** EBV-specific CD8+ T cells after methotrexate cessation in regressive group and persistent group. Th1, T helper 1; EM, effector memory; EBV, Epstein–Barr Virus.

groups are summarized in **Table 3**. We analyzed EBV-specific CD8+ T cells in those with the HLA-A*2402 allele (regressive LPD: $n = 4$, persistent LPD: $n = 2$, control group: $n = 5$). The incidence of HLA-A*2402 was similar to that reported in a previous study in Japan (15).

The proportions of three subsets of cells among CD4+ T cells or CD8+ T cells were significantly lower in the regressive group compared to the control group (T helper 1 cells in CD4+ T cells:Th1 cell/CD4+ T (Th1 cell/CD4+ T), 14.8 vs. 19.8%; effector memory CD8+ T cells within CD8+ T cells:EMCD8+ T cell/CD8+ T (EMCD8+ T cells/CD8+ T), 22.5 vs. 45.7%; EBV-specific CD8+ T cells within CD8+ T cells: EBV-specific CD8+/CD8+T (EBV-specific CD8/CD8+ T), 0.2 vs. 0.6%, $P < 0.05$ for all comparisons of between regressive group vs. control group). The absolute numbers of these three subsets were also significantly lower in the regressive group compared to the persistent and control groups ($P < 0.05$ for all comparisons between regressive vs. persistent, and regressive vs. control). By contrast, the proportion of specific subsets of cells was not significantly different between the persistent and control groups. Therefore, we focused on the transition of the three cell subsets after MTX withdrawal.

Changes in Th1, EMCD8+ T Cells, and EBV-Specific CD8+ T Cells in Regressive LPD and Persistent LPD

In the regressive group, the proportions of Th1 cells, EMCD8+ T cells and EBV-specific CD8+ T cells increased significantly to reach levels equivalent to those of the control group at week 4, and this was maintained through to week 12 after MTX cessation (**Figures 3A,D,E**). In the persistent group, the proportion of these subsets of cells was equivalent to the level in the control group at week 0, and did not significantly change after MTX cessation until week 12 (**Figures 3A,D,E**). The transition of the three subsets is shown in a representative case in **Figure 4**. In addition, the proportions of Th2 cells, Th17 cells, and NK cells did not significantly change from week 0 to 4 in both the regressive and persistent groups, compared to the control group (**Figures 3B,C,F**). The changes in the absolute number of these cell subsets are shown in **Figure 5**. In the regressive group, the absolute numbers of Th1 cells, Th2 cells, EMCD8+ T cells, EBV-specific CD8+ T cells, and NK cells were significantly lower than those of the control group at week 0, but increased to levels that were equivalent with those of the control group at week 4 and were maintained to week 12 after MTX cessation (**Figures 5A–F**). The absolute numbers of each cell subset in the persistent group were equivalent to the numbers in the control group from week 0 to week 12.

All patients in the regressive group with the HLA-A*2402 allele ($n = 4$) had EBER-positive LPD. Therefore, we could not assess the transition of EBV-specific CD8+ T cells in EBER-negative regressive LPD cases. In addition, the transition of Th1 cells and EMCD8+ T cells was not significantly different between the pathological classifications of LPDs (Figure S1 in Supplementary Material).

Activation Markers on Effector Memory CD8+ T Cells

Since we expected EMCD8+ T cells to be the key of the anti-LPD effector cells (16), we measured the proportion and absolute numbers of HLA-DR+EMCD8+ T cells to assess the activation status of EMCD8+ T cells. Both the proportion and absolute numbers of HLA-DR+EMCD8+ T cells in the regressive group were significantly lower than those of the persistent and control groups at week 0 ($P < 0.05$ in the comparison between regressive vs. control and regressive vs. persistent), but were significantly increased to higher level than that of control group at week 4, and were at equivalent level with control group at week 12 (**Figure 6**). On the other hand, while mean fluorescence intensity (MFI) of HLA-DR did not significantly differ among groups at week 0, it significantly increased to a higher level in the regressive group compared with the control group at week 4 (**Figure 6C**). The proportion and absolute numbers of HLA-DR+EMCD8+ T cells, and MFI of

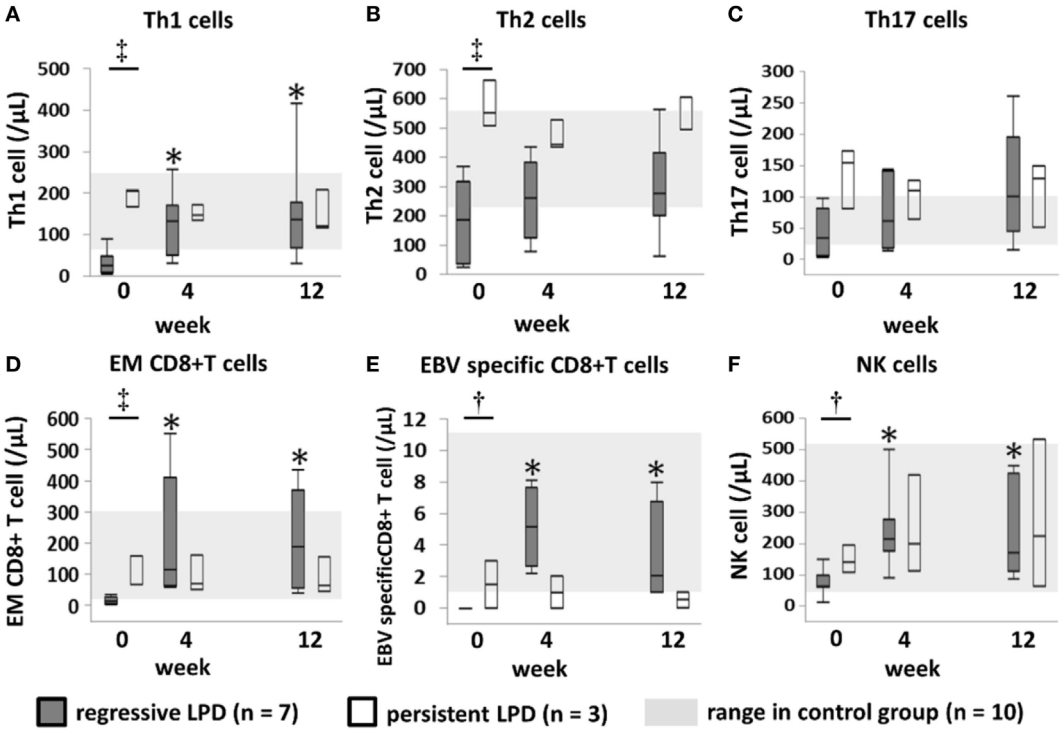

FIGURE 5 | Transition of absolute number of lymphocyte subsets. Transition of the absolute number of **(A)** Th1, **(B)** Th2, and **(C)** Th17 cells among CD4+ T cells; **(D)** EMCD8+ T cells and **(E)** EBV-specific CD8+ T cells among CD8+ T cells; and **(F)** NK cells among lymphocytes, after MTX cessation. Comparison between the three groups was conducted by Kruskal–Wallis test, and comparison between two groups was conducted by Wilcoxon test. †Regressive vs. Control, $P < 0.05$. ‡Regressive vs. Control, $P < 0.05$; Regressive vs. Persistent, $P < 0.05$. *$P < 0.05$ for the comparison with the value at week 0 in each group. Th1/2/17, T helper 1/2/17; EM, effector memory; EBV, Epstein–Barr Virus; NK cell, natural killer cell.

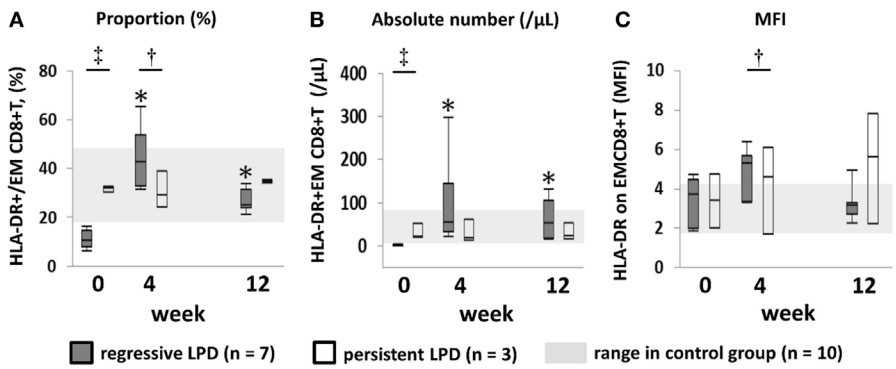

FIGURE 6 | Transition of proportion and absolute number of activated EMCD8+ T cell, mean fluorescence intensity (MFI) of HLA-DR on EMCD8+ T cell after methotrexate (MTX) cessation. Transition of **(A)** proportion and **(B)** absolute number of activated EMCD8+T cell, **(C)** MFI of HLA-DR onEMCD8+ T cell after MTX cessation. Comparison between the three groups was conducted by Kruskal–Wallis test, and comparison between two groups was conducted by Wilcoxon test. †Regressive vs. Control, $P < 0.05$. ‡Regressive vs. Control, $P < 0.05$; Regressive vs. Persistent, $P < 0.05$. *$P < 0.05$ for the comparison with the value at week 0 in each group. EM, effector memory.

HLA-DR on EMCD8+ T cells, did not show significant changes from weeks 0 to 12 in the persistent group (**Figures 6A–C**).

Correlation Between Restoration of Th1 Cells and EMCD8+ T Cells and Change in Serum Cytokine Levels

To identify the key cytokine in this process, we measured serum IFN-γ, IL-2, IL-7, IL-10, IL-12p70, IL-15, and TNF-α levels after MTX cessation (**Figure 7**). Serum IFN-γ levels were not significantly different among groups at week 0, but it increased to significantly higher level than that of persistent and control group at week 4. However, no such increase was observed in the persistent group. IL-2, IL-7, IL-10, IL-12p70, IL-15, and TNF-α levels in both the regressive and persistent groups did not significant change from weeks 0 to 12 and were equivalent to those of the control group. However, IL-15 levels in the regressive group seemed to decrease from weeks 0 to 4 in some cases.

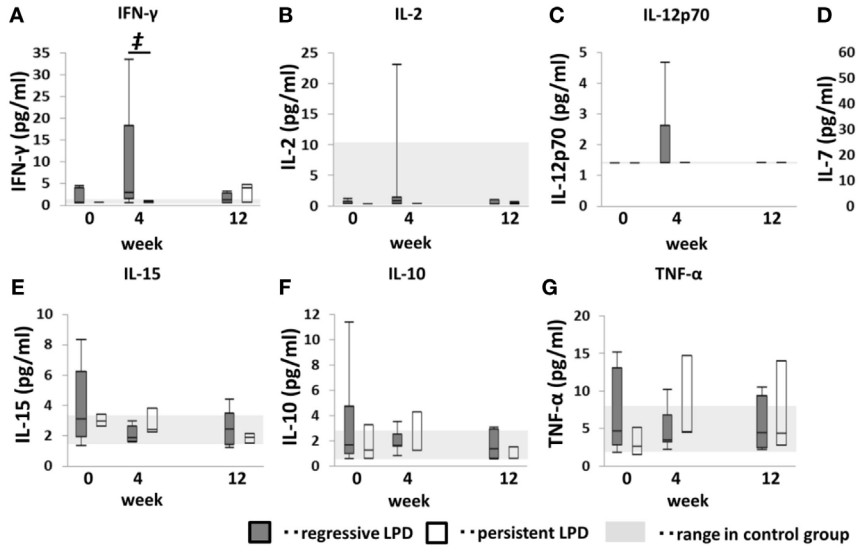

FIGURE 7 | Changes in serum cytokines after methotrexate (MTX) cessation in lymphoproliferative disorder (LPD) patients. Transition of **(A)** interferon (IFN)-γ, **(B)** IL-2, **(C)** IL-12p70, **(D)** IL-7, **(E)** IL-15, **(F)** IL-10, and **(G)** TNF-α after MTX cessation. Comparison between the three groups was conducted using the Kruskal–Wallis test. †Regressive vs. control, $P < 0.05$. ‡Regressive vs. control, $P < 0.05$; regressive vs. persistent, $P < 0.05$. *$P < 0.05$ for comparison with the value at week 0 in each group.

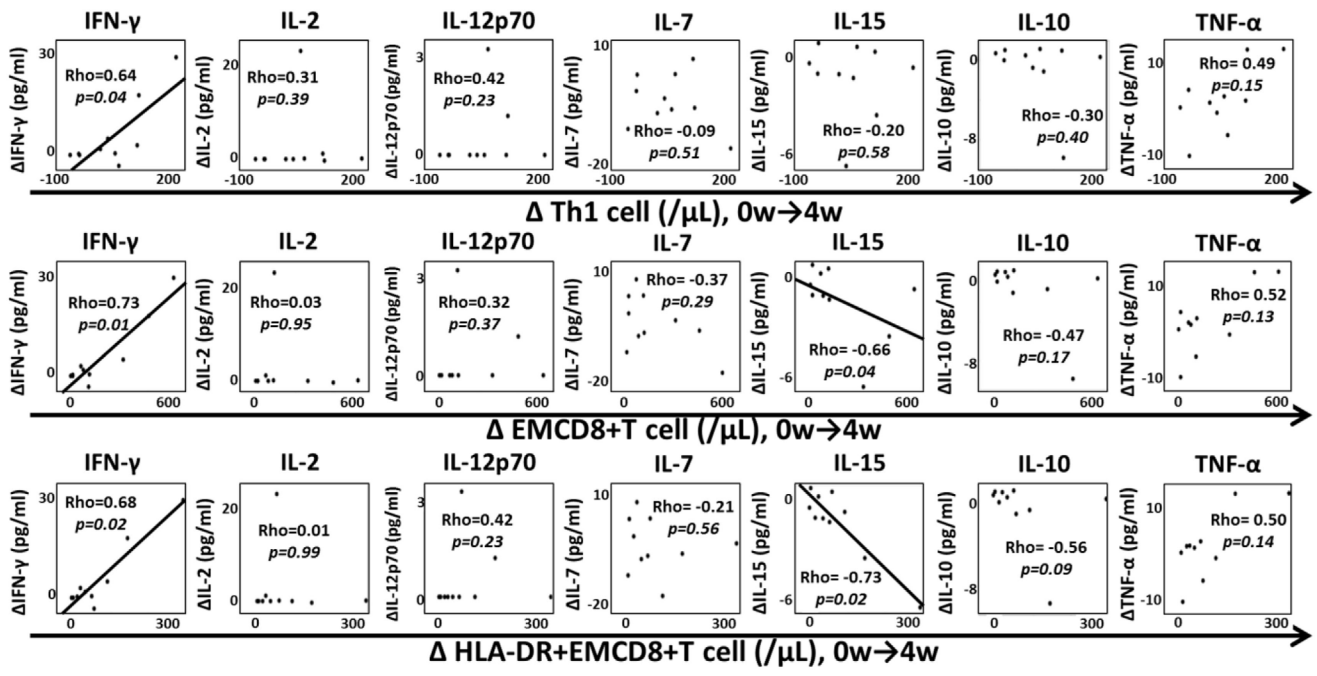

FIGURE 8 | Correlation between increased lymphocyte subsets and cytokine change. Correlation between increase in Th1 cells, EMCD8+ T cells, HLA-DR+EMCD8+ T cells, and changes in interferon (IFN)-γ, IL-2, IL-12p70, IL-7, IL-15, IL-10, and TNF-α from weeks 0 to 4. Correlations were analyzed by Spearman's correlation coefficient. Th1, T helper 1; EM, effector memory.

We assessed the correlation between changes in Th1 cells, EMCD8+ T cells, HLA-DR+EMCD8+ T cells and each cytokine from weeks 0 to 4 (**Figure 8**) to investigate the role of cytokines in the expansion of T cell subsets. The increase of IFN-γ (ΔIFN-γ) was significantly correlated with ΔTh1 cells, ΔEMCD8+ T cells and ΔDR+EMCD8+ T cells ($P < 0.05$). In contrast, ΔIL-15 was inversely correlated with ΔEMCD8+ T cells and ΔDR+EMCD8+ T cells ($P < 0.05$). There was no correlation between the other

cytokines and the increase in these cell subsets. In addition, there was no significant correlation between ΔIFN-γ, ΔIL-15, and ΔTh2 cells, ΔNK cells, or ΔTh17 cells (data not shown). Transition of quantitative EBV-PCR level after MTX cessation and correlation between change of EBV-PCR and EBV-specific CD8+ T cell were demonstrated in Figure S2 in Supplementary Material.

DISCUSSION

Previous studies by our group and others (8, 9) have suggested that the decrease in lymphocytes at the time of LPD diagnosis and their restoration following MTX withdrawal may associate with the pathogenesis and regression of LPD developed during MTX administration. Here, we focused on the changes of lymphocyte subsets that were associated with the regression of LPD. Immunophenotyping of peripheral blood cells revealed a restoration of the proportion and absolute numbers of Th1 cells, EMCD8+ T cells and EBV-specific CD8+ T cells during the regression of LPD developed during MTX administration.

Our data also showed an association between the increase in Th1 cells and EMCD8+ T cells and that of IFN-γ after MTX cessation. The lack of such changes in persistent LPD indicates that the pathogenesis of regressive and persistent LPD is discriminable. We also showed that EBV-specific CD8+ T cells decrease at the time of LPD diagnosis and are restored after MTX cessation in regressive EBER-positive LPD patients. A previous study of post-transplant lymphoproliferative disease reported that EBV-specific T cells show an anti-LPD effect even in small numbers (17). Interestingly, the transition of lymphocyte subsets was not significantly different between the pathological phenotypes of LPDs, suggesting a common regression mechanism among each phenotype; however, further studies are needed to confirm this hypothesis.

Currently, all LPDs that develop during MTX administration are classified as "other iatrogenic immunodeficiency-associated LPD" according to the latest WHO classification of lymphoid neoplasms (4), regardless of the status of LPD following MTX cessation. This is the first study to report differences in immune status between regressive and persistent LPDs developed during MTX administration in RA patients. Excessive inhibition of Th1 cells, EMCD8+ T cells and EBV-specific CD8+ T cells by MTX at the time of LPD development, and their restoration after MTX cessation appear to be features specific to the pathogenic and regression mechanism of "regressive LPD" (**Figure 9**), since a decrease in the proportion and absolute number of these cell subsets was not observed in persistent LPD. This suggests that persistent LPD is not caused by inhibition of the "LPD surveillance system". Therefore, our findings could represent the first evidence of distinct types of MTX-associated LPD: "regressive LPD" and those caused by inhibition of the "LPD surveillance system".

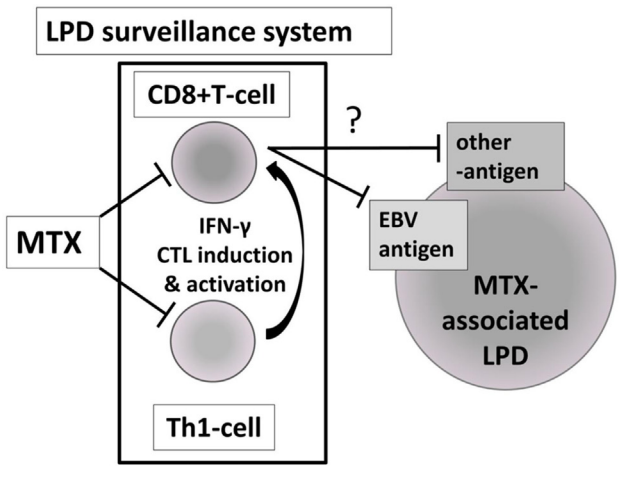

FIGURE 9 | Hypothesis of the pathological and regression mechanism of "regressive LPD" as a narrow sense of "MTX-associated LPD". MTX, methotrexate; LPD, lymphoproliferative disorder; Th1, T helper 1; EBV, Epstein–Barr virus.

Th1 cells are one of the major sources of IFN-γ (18); it is assumed that the Th1 response was initiated before the increase of IFN-γ in regressive LPDs. MTX inhibits Th1 response and cytokine production, including IFN-γ (19–21), suggesting that excessive inhibition of Th1 cells by MTX may be involved in the pathogenesis of MTX-associated LPD. Furthermore, the important role of CD4+ T cells in promoting CD8+ T cell proliferation and their response to antigens is well established (22, 23). IL-15 is required for the basal proliferation of memory CD8+ T cells (24); the decrease in IL-15 from baseline after MTX withdrawal, which was correlated with the increase in EMCD8+ T cells, might suggest the IL-15 secretion induced by the decrease of EMCD8+ T cells.

Some limitations of our study warrant mention. First, the number of LPD cases for which peripheral blood was analyzed by flow cytometry was limited because of the low incidence of LPD during MTX administration, even though the number of patients was rather large for such a rare disease. We also could not analyze the difference in cell subsets between each pathological phenotype of LPD because of the small number of patients. Second, as we only focused on the cell subsets that were significantly different in proportion between control and regressive groups, we cannot rule out potential associations between regression of LPD and cell subsets that showed a difference in absolute numbers, such as NK cells, and B cells. Third, we did not examine the anti-LPD function of the cell subsets within the peripheral blood. Previous studies indicate that circulating lymphocyte subsets including cytotoxic CD8+ T cells and EBV-specific CD8+ T cells in a cancer-bearing situation have specific anti-tumor function (16, 25, 26).

In conclusion, assessment of the immunological status of LPD patients treated with MTX enabled to close on the pathogenesis of regressive LPD after MTX cessation. Studies that examine the difference in immunological status between pathological phenotypes of LPDs are warranted in the future.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "ethics committee of Keio University School of Medicine"; and the "ethics committee of Saitama Medical Center, Saitama Medical University"; with written informed consent from all subjects. All subjects gave written informed consent in

accordance with the Declaration of Helsinki. The protocol was approved by the "ethics committee of Keio University School of Medicine" and the "ethics committee of Saitama Medical Center, Saitama Medical University."

AUTHOR CONTRIBUTIONS

SS performed most of the experiments. SS, KS, MT, and TT participated in the study conception and design of the work. SS, KS, KYo, YK, KYa, TS, TM, SO, KK, KA, JT, MT, and TT participated in the acquisition of data. SS, KS, YK, KYa, MT, and TT participated in the analysis and interpretation of data. SS, KS, Kya, and TT were involved in drafting the manuscript. All authors were involved in revising it critically for important intellectual content and approved the final version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | Transition of Th1 cells and EMCD8+ T cells between different pathological classifications of lymphoproliferative disorders (LPDs). Transition of the proportion of Th1 cells among CD4+ T cells in **(A)** regressive LPD and **(B)** persistent LPDs, and transition of the proportion of EMCD8+ T cells among CD8+ T cells in **(C)** regressive LPD and **(D)** persistent LPDs. Th1, T helper 1; EM, effector memory.

FIGURE S2 | Transition of quantitative Epstein–Barr virus (EBV)-PCR level after methotrexate (MTX) cessation and correlation between change of EBV-PCR and EBV-specific CD8+ T cells. **(A)** Transition of quantitative EBV-PCR level after MTX cessation and **(B)** correlation between change of EBV-PCR and EBV-specific CD8+ T cells.

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REVIEW
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Mechanisms of Immune Evasion and Immune Modulation by Lymphoma Cells

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Purpose: Targeting cancer cells by modulating the immune system has become an important new therapeutic option in many different malignancies. Inhibition of CTLA4/B7 and PD1/PDL1 signaling is now also being investigated and already successfully applied to various hematologic malignancies.

Methods: A literature review of PubMed and results of our own studies were compiled in order to give a comprehensive overview on this topic.

Results: We elucidate the pathophysiological role of immunosuppressive networks in lymphomas, ranging from changes in the cellular microenvironment composition to distinct signaling pathways such as PD1/PDL1 or CTLA4/B7/CD28. The prototypical example of a lymphoma manipulating and thereby silencing the immune system is Hodgkin lymphoma. Also other lymphomas, e.g., primary mediastinal B-cell lymphoma and some Epstein–Barr virus (EBV)-driven malignancies, use analogous survival strategies, while diffuse large B-cell lymphoma of the activated B-cell type, follicular lymphoma and angioimmunoblastic T-cell lymphoma to name a few, exert further immune escape strategies each. These insights have already led to new treatment opportunities and results of the most important clinical trials based on this concept are briefly summarized. Immune checkpoint inhibition might also have severe side effects; the mechanisms of the rather un(der)recognized hematological side effects of this treatment approach are discussed.

Conclusion: Silencing the host's immune system is an important feature of various lymphomas. Achieving a better understanding of distinct pathways of interactions between lymphomas and different immunological microenvironment compounds yields substantial potential for new treatment concepts.

Keywords: CD58, CD70, Epstein–Barr virus, HLA-G, lymphoma, microenvironment, PDL1, PD1

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INTRODUCTION

Next to surgery, chemotherapy and radiotherapy, immunotherapy has become a new effective strategy to treat human cancer (1). This field spans from cytokine therapy, tumor vaccines, and infusions of primed T-cells to drugs specifically targeting immune checkpoint signaling such as programmed cell death 1 (PD1/CD279) and its ligand PDL1 and the cytotoxic T-lymphocyte-associated protein 4 (CTLA4/CD152) and its ligand B7, both ligands being expressed on target- or antigen-presenting cells to inhibit T-cell activation. Though initially these treatments were designed for solid cancers,

this concept is now readily applied in a variety of hematolymphoid neoplasms. In addition, in hematolymphoid neoplasms, another form of "immunotherapy," allogenic hematopoietic stem cell transplantation has been used for a long time already showing a tremendous improvement of patients' prognosis (2).

The history of immunotherapy reaches back more than 100 years to studies of Paul Ehrlich, and, despite obvious efficacy, its application regarding the type of treatment and its targets is still controversially discussed (3). It has been studied in various animal models with inconclusive results. While immune-deficient nude mice, which display a markedly reduced amount of T-cells, do not show an increased rate of tumors (4), specifically genetically modified mice with knock-outs of *recombination activating gene 2*, *signal transducer and activator of transcription 1 (STAT1);* or the *gamma-interferon receptor* show increased cancer rates even if not treated with carcinogens or crossed with animals with a cancer development stimulating mutation (5, 6). The reason for the lower tumor rates in nude mice is explained by a reduced, yet sustained amount of non-thymic T-cells as well as an upregulation of innate immunity. Looking at humans, patients with iatrogenic, viral or genetically caused immunodeficiency are known to have higher rates of both, virus-related cancers, such as lymphomas, squamous cell skin cancer or Kaposi sarcomas, and of non-virus-related cancers, such as colon and lung cancer. Mechanistically, immunosurveillance of tumors, especially those, which have escaped cellular senescence (7), is mainly exerted *via* control of antigens presented by the cells *via* the major histocompatibility complex 1 (MHC1) allowing T-cells to discriminate altered, i.e., tumor cells from normal cells; CD4- and CD8-positive T-cells are the key players in controlling outgrowth of tumors (5). This mechanism puts tumor cells under pressure and leads to a selection of subclones, which have achieved the capability to evade the immune response.

In many types of tumors, cancer cells undertake considerable efforts to keep the host's immune system at bay; this involves both the tumor cells themselves, which express immunosuppressive surface proteins such as PDL1, B7, or human leukocyte antigen (HLA) G, less MHC1 or its compound β-2 microglobulin (B2M), as well the microenvironment of the tumors, which is influenced and manipulated by the tumor cells (8). Here, upregulation of regulatory T-cell subsets and subsequent anergy of cytotoxic T-cells, crosstalk with tumor growth-promoting M2 macrophages and overexpression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) play all an important role (9–11); since the role of IDO and respective therapeutic inhibition has several times been addressed and extensively reviewed, we kindly refer to some excellent publications covering this topic (12, 13). Furthermore, both compartments secrete various factors such as interleukins and interferons as well as tumor necrosis factor alpha or transforming growth factor beta. These factors can promote tumor cell survival on the one hand and prime the microenvironment, particularly the immune system in a pro-tumorigenic manner on the other (14).

Importantly, with the broad introduction of immunotherapy it has become obvious that not all patients respond in the same way, which is both due to tumor heterogeneity (15) as well as to individual (immuno-)genetic polymorphisms (16). In order to tackle this issue, specific biomarkers are needed to allow stratification of patients to ensure tailored treatment approaches, which might increase tumor response rates.

In this review, we mainly focus on the role of lymphoma tumor cells in the immunological crosstalk and not that of the microenvironment, as this topic will be covered by the review of Dr. Xu in this journal issue.

HODGKIN LYMPHOMA—THE CLASSICAL PARADIGM FOR IMMUNOMODULATIVE CANCER

Classical Hodgkin lymphoma (cHL) comprises about 20% of lymphoid malignancies. Before the development of effective chemo- and radiotherapy regimens, it was a fatal disease (17) with patients dying—apart from mechanical problems due lymphoma burden—mainly due to infections because of severe immunosuppression caused by the cHL, exemplifying the importance of the interaction between tumor cells and the immune system. Another peculiar feature of cHL is the fact that the tumor cells [Hodgkin- and Reed–Sternberg cells (HRS cells)] comprise less than 1% of the lymphoma mass, and the majority of the tumor bulk is constituted by reactive or inflammatory cells in varying compositions, which depends on the cHL subtype. HRS cells both rely on their microenvironment on the one hand and need to specifically silence it on the other in order to prevent being attacked by it. This has been shown for T-cells as well as for tumor-associated macrophages (TAM). Regarding the latter, it has been shown that HRS cells induce PDL1 expression in macrophages (**Figure 1A**) in order to boost the immunosuppressive environment (18). Additionally, TAM and tumor-infiltrating lymphocytes express PD1, thus PD1/PDL1 blockade can both stop their immunosuppressive abilities and turn on tumor-surveilling attributes (19). It has been shown that the HRS cells are derived from germinal center B-cells as they carry clonally rearranged and somatically mutated immunoglobulin heavy- and light-chain genes (20, 21). HRS cells show a global downregulation of B-cell-related gene expression (22), which explains their specific immunoprofile. Genetic drivers of HRS cells are mutations in the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) pathway, of compounds of the JAK–STAT signaling and genes involved in MHC composition and expression, and communication with T-cells (23). Deciphering the mutational landscape of HRS cells has helped to get new insights into tumorigenesis of cHL as well as elucidating mechanisms how this tumor interacts with and, thus, manipulates the immune system (24, 25).

An important feature of cHL [and primary mediastinal B-cell lymphoma (PMBCL)] is gain of chromosome *9p24* (**Figure 2A**), which leads to an overexpression of PDL1 (25, 26) that can also be shown *in situ* (27, 28), and seems to be of probable prognostic importance in patients treated with standard treatment regimens (25) and offers the opportunity to be specifically targeted, resulting in unprecedented response rates in otherwise hopeless cases of multiple-relapsing cHL (29). Other genes in this region encompass *JAK2*, *PDL2*, and *JMJD2C*, the upregulation of all of which seems to be vital for HRS cells (30), explaining why blocking PD1

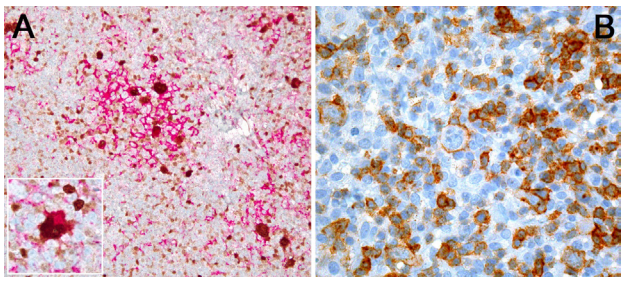

FIGURE 1 | **(A)** PDL1 expression study of classical Hodgkin lymphoma with PDL1 (red chromogen)-MUM1p (brown chromogen) double-staining; note that a lot of PDL1$^+$ cells, corresponding to tumor-infiltrating macrophages, do not express MUM1p while yielding dendroid cytoplasmic projections and form "immunosuppressive microniches," in which PDL1 and MUM1p co-expressing Hodgkin- and Reed–Sternberg cells (see also inset) are scattered. **(B)** PD1 expression by single tumor cells (large ones) and plenty of tumor-infiltrating lymphocytes in T-cell- and histiocyte-rich B-cell lymphoma.

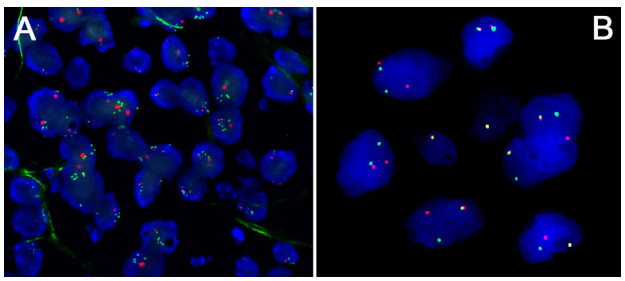

FIGURE 2 | **(A)** Amplification of the *PDL1/JAK2* locus at *9p24* in a primary mediastinal B-cell lymphoma (PMBCL); note multiple green FISH signals corresponding to the locus of interest compared to only 2 red centromere 9 signals/cell. **(B)** Rearrangement of the *CIITA* locus at *16p13* a PMBCL; note fused green and red signals corresponding to the non-rearranged wild-type allele and free green and red signals corresponding to the rearranged allele.

might be more effective than blocking PDL1 in cHL as the first might prevent the tumor cells also from relying on PDL2 as a substitute of blocked PDL1 (31).

Other immune escape mechanisms in cHL (and PMBCL) are deactivating translocations of *CIITA* (**Figure 2B**), the transactivator gene of MHC class II, that can be found in a subset of cHL (32), and downregulation of MHC class II that is reported to be also an adverse prognostic factor in affected individuals (33). The same applies to MHC class I (34), although this study could not confirm the impact of MHC class II as a prognostic factor. Regarding MHC class I, mutations in the *B2M* gene, which is important for MHC class I composition and function, are among the commonest in cHL and have been shown to be a predictor of inferior outcome independently of the *9p24* status (34).

CD58, also known as lymphocyte function-associated antigen 3, is a glycosylated surface molecule on both B- and T-cells, which provides a stimulatory signal for T cells *via* the CD2 receptor. The function of CD58 in cHL is two-faced: on the one hand, it is necessary for HRS cells to communicate with CD4-positive T-cells (35), on the other hand, loss of CD58 expression due to mutations can facilitate immune escape, especially in advanced disease, when HRS cells become less dependent on the surrounding microenvironment (36, 37). HLA-G, a non-classical HLA molecule, plays a similar role in cHL and modulates the microenvironment to foster immunotolerance. HLA-G expression has been demonstrated both on HRS cells and the microenvironment, with high HLA-G expression on HRS cells and, conversely, low expression in the microenvironment correlating with a better outcome in one study (38).

Epstein–Barr virus (EBV) infection of HRS cells is a common feature in 30% of cHL in the Western world and >90%—especially in pediatric cases—in Central America (39). EBV infection is clonal and, thus, an early event in cHL. It immortalizes B-cells by rescuing them from apoptosis (40). EBV shows latency II state in HRS cells, with expression of the EBV-encoded genes EBV nuclear antigen 1 (EBNA1), latent membrane protein 1 (LMP1), and LMP2a. In EBV-negative cHL, the oncogenic impact of EBV seems to be substituted by mutations of genes related to the NF-κB pathway (e.g., *C-REL*) as well as several receptor tyrosine kinases (41). EBV can also upregulate PDL1 expression (42). This is primarily mediated by LMP1. LMP1 activates both the JAK/STAT pathway directly *via* JAK3 as well as activated protein 1 (AP1) *via* the microtubule-associated protein kinase (MAPK) pathway, both of which promote *PDL1* gene expression (42). Interestingly, while frequencies of *9p24* gains and amplifications are similar in EBV-positive and EBV-negative cHL, PDL1 expression is mostly and more selectively upregulated in EBV-positive cHL (25). EBNA1 and LMPs also directly interact with immune cells helping to create an immunosuppressive environment with enhanced amounts of regulatory T-cells (43).

Finally, HRS cells secrete a plethora of immunosuppressive soluble mediators, which is beyond the scope of this review (44, 45).

VARIOUS MECHANISMS OF IMMUNOMODULATION IN LYMPHOMAS—A CLOSER LOOK

In the second part of this review and after having focused on one specific lymphoma subtype, which is the prototype for immunomodulative cancer, we will have a closer look at the various mechanisms touched in the previous sections, namely, PD1/PDL1, CTLA4/B7, HLA-G, CD58 and B2M, CD70, and CD27 as well as EBV. Beside a discussion on how these pathways exert their function and by which types of lymphomas they are used, we will also focus on interactions between them and show their synergistic and/or complementary mode of action.

PD1/PDL1—The Best Studied and Most Frequently Therapeutically Used Pathway of Immune Evasion

PD1 and its ligand PDL1 have already been discovered in the early and late 90s, respectively (46, 47). A second ligand of PD1, PDL2, the expression of which is more restricted than that of PDL1, has been identified as well (48). These molecules are important tools to control T-cell activity and proliferation, and

can both inhibit T-cells as well as stimulate immunosuppressive regulatory T-cells (49, 50). Another recently discovered ability is the effect of PDL1 on TAM briefly touched in the section on cHL. Gordon et al. recently showed that PDL1 blockade increases the phagocytic capability of TAM in rodent models and leads to increased survival and tumor control (19). This is an interesting and potentially also clinically relevant finding considering the bad prognostic effect of high numbers of TAM in cHL (51, 52), which might thus be counterbalanced by PDL1 inhibition. In contrast to CTLA4, which is discussed in the next paragraph, PD1 and its ligands exert their function in the peripheral tissue and thus do not lead to a systemic affection of the immune system, which has been nicely shown in several animal models (53, 54). The cytoplasmic tail of PD1 contains an immunoreceptor tyrosine-based switch motif (ITSM) and an immunoreceptor tyrosine-based inhibitory motif (ITIM), of which the ITSM is essential for the transmission of inhibitory signals [reviewed in Ref. (55)]. Upon T-cell receptor (TCR) stimulation and ligation with either PDL1 or PDL2, the ITSM and ITIM undergo phosphorylation, leading to the recruitment of the phosphatases SHP-1 and SHP-2, which in turn lead to dephosphorylation (inactivation) of the crucial T-cell signaling molecules ZAP70 and CD3ζ, and, in addition, of the phosphatidylinositol 3-kinase, which interrupts AKT and ERK signaling; even more, upon PD1 engagement by PDL1, protein tyrosine kinase-θ, which is necessary for the activation of the transcription factors NFκB and AP1, is attenuated and the negative regulator of T-cell activation, the E3 ubiquitin ligase CBL-b is upregulated (56–58). As a net effect, TCR-mediated activation and T-cell proliferation are impeded.

PD1/PDL1 expression in lymphomas (**Figures 1A,B**) has been investigated by a variety of studies with mostly consistent results (27, 28); it can be demonstrated in up to a third of DLBCL, mainly of the activated B-cell type (59), and in PMBCL, in other lymphoma entities it is expressed in only a low percentage of cases (27). Interestingly, in chronic lymphocytic B-cell leukemia (CLL), PDL1 expression has been described in the proliferation centers (60). PDL1 expression is observed both in the tumor microenvironment (particularly in tumor-infiltrating macrophages) and in lymphoma cells, while PD1 is primarily expressed in T-cells of the microenvironment. In T-cell- and histiocyte-rich B-cell lymphomas, PDL1 expression is seen in both T-cells and histiocytes, while the tumor cells themselves are negative for PDL1 (27). Importantly, in extranodal natural killer (NK)- and T-cell lymphoma of the nasal type, which is known to have an aggressive and mostly fatal course, PDL1 is substantially upregulated due to EBV infection of the tumor cells, and PD1 blockade has been shown to be very effective in otherwise hopeless relapse cases in a small case series (61).

As mentioned above, the genetic mechanism of PDL1 overexpression has been first elucidated in cHL consisting of alterations in chromosome *9p24.1*. Similar alterations have been found in PMBCL (62) and DLBCL (63) as well as lymphomas of immune-privileged sites such as the central nervous system and the testis (64). In addition to gene gains, PDL1 expression is inducible by LMP1 of EBV *via* activation of STAT- and AP1-mediated pathways. As to be expected, other causes of STAT activation also enhance PDL1 expression as seen in anaplastic lymphoma kinase-positive anaplastic large cell lymphomas (65) or in instances with active cytokine signaling (66). Another mechanism of enhancing PDL1 expression was just recently reported by Kataoka et al., who demonstrated the presence of disruption of the 3′-untranslated region (UTR) of the *PDL1* gene leading to a marked increase of PDL1 that is stabilized by truncation of the 3′-UTR (67). Finally, at least in DLBCL, translocations of *IGH*, *PIM1*, and *TP63* with the *PDL1* locus that lead to latter's overexpression have been described, too (63).

As in solid tumors, the direct applicability of PD1/PDL1 expression to predict therapy responsiveness and prognosis remains to be fully elucidated. Xing et al. could show that PDL1 expression in DLBCL treated with standard R-CHOP treatment is associated with a better overall survival rate, yet not with remission after first therapy, relapse- or progression-free survival (68). Several studies with small patient cohorts suggest that best responses are seen in lymphomas harboring *9p24* alterations such as lymphomas of immunoprivileged sites (69). In PMBCL, high PDL1 expression and low MUM1p expression is correlated with a better outcome than *vice versa* expression of these two proteins (70). A study on refractory lymphomas revealed that there is a discrepancy between PDL1 expression and amplification of the *PDL1* locus, supporting the hypothesis that other mechanisms—next to gene amplifications—are involved in upregulation of PDL1 expression (71). It has also become evident that in several lymphoma types such as follicular lymphoma and CLL, adding PDL1 blockers to conventional therapy regimens shows a benefit in comparison to only very limited treatment response if given as single agents (72). For comprehensive overviews of ongoing and already finished clinical trials, we refer to several recent excellent clinically centered reviews as well as the contributions of Proff. Renner and Stenner in this issue.

CTLA4—A Key Player Seemingly Not Only in T-Cell Lymphomas

CTLA4 belongs to the superfamily of immunoglobulins (73). It is generally expressed in T-cells, and regulatory T-cells are constitutively positive (74). It shares its ligands B7-1 (CD80) and B7-2 (CD86) together with CD28, which has a function opposite to CTLA4 as it is a stimulator of TCR signaling (75). CTLA4's affinity and avidity to these ligands is greater than that of CD28 due to its bivalent binding to the B7 molecules (76). The main function of CTLA4 is T-cell inactivation, which is exerted by two different mechanisms: it competitively binds its ligands B7-1 and B7-2 leading to a reduced stimulatory signaling of CD28; furthermore, *via* its cytoplasmic tail, CTLA4 can inhibit various intracellular signaling pathways in T-cells such as NF-κB, AP1, and nuclear factor of activated T-cells (77), it can impede the cell cycle (78) and inactivate MAPK, extracellular signal-regulated kinase-1 (ERK) and c-Jun NH2 terminal kinase signaling, and thus impair interleukin 2 production (79). In contrast to PD1/PDL1, which exert their function in the periphery, CTLA4 is acting rather early in the time course of the immune response as it is involved in priming T-cells in primary lymphoid organs (80).

CTLA4 expression is noted in a variety of T-cell lymphomas, namely, peripheral T-cell lymphomas and mycosis fungoides/

Sézary syndrome. Besides inducing T-cell anergy and, thus, fostering immune escape, CTLA4 has also a direct oncogenic effect: a fusion of the two opponents CTLA4 and CD28 has recently been described in a variety of T-cell lymphomas and proposed to be a major driver of lymphoma development (81). The fusion protein consisting of the extracellular and transmembrane domains of CTLA4 and the cytosolic signaling domain of CD28 showed increased activation of intracellular MAPK and ERK signaling in cell culture experiments, confirming observations of earlier studies (82). Herrmann et al. reported CTLA4 expression in B-cell lymphomas, primarily in DLBCL (83). These lymphomas were shown to be able to exert their immunosuppressive function by binding of B7.1 and thus reducing CD28 activation on tumor-infiltrating/immunosurveillance T-cells; furthermore—as in T-cell lymphomas—CTLA4 can enhance proliferation *via* the STAT3 pathway, which is an important driver also in B-cell lymphomas (84). So far, CTLA4 inhibition is not commonly used in lymphoma therapy. In cHL, CTLA4 blockade has been tested in transplanted patients (85) and in combination with brentuximab, the latter still being an ongoing trial (86).

HLA-G—The Unknown Member of the HLA Family

HLA-G is a non-classical MHC class I molecule and besides the classical function of HLA proteins—presenting protein fragments on the cell surface—it exerts its function mainly by immunomodulation (87). In contrast to the classical HLA molecules, the non-classical HLA are highly conserved molecules with only few alleles. Immunomodulation by HLA-G occurs *via* a plethora of ways as it can interact with different receptors found on T-cells, B-cells, macrophages, dendritic cells, and NK cells (88). It interferes with proliferation and cytotoxicity as well as promotes apoptosis. Furthermore, it also inhibits chemotaxis by downregulating several chemokine surface receptors (89), hampers the function of neutrophils (90), and reduces neoangiogenesis (91). HLA-G expression has been investigated in a variety of cancers and is correlated with worse overall survival or increased risk of tumor progression and metastases in most studies (88). In lymphomas, HLA-G has been explored in only few studies so far and the results regarding the predictive role of HLA-G expression are still equivocal (92). As alluded to above, HLA-G expression has been demonstrated in cHL (**Figures 3A,B**) and its high expression in the tumor microenvironment has been correlated with an inferior response rate (38). Bielska et al. demonstrated that *HLA-G* polymorphisms, which have a direct impact on the expression of HLA-G RNA, differ between different prognostic groups of DLBCL (93), and similar findings were reported in CLL patients (94). Both studies showed independently that especially the 14 base pair deletion polymorphism (rs66554220) in the 3′ UTR of *HLA-G* has an adverse prognostic impact.

CD58 and B2M—Important Prerequisites for Immunosurveillance

Both CD58 and B2M are important for the correct assembly of MHC class I molecules (95) and alterations thereof are another

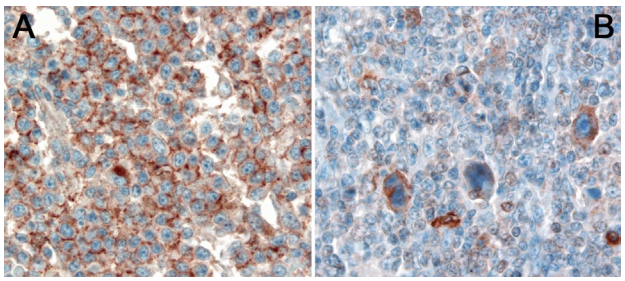

FIGURE 3 | **(A)** Expression of HLA-G in a diffuse large B-cell lymphoma. **(B)** Expression of HLA-G by Hodgkin- and Reed–Sternberg cells of classical Hodgkin lymphoma.

immune escape mechanism of tumors (96). Inactivating mutations of *CD58* have been initially described in approximately one sixth of DLBCL with no preference for either cell of origin subtype (97). They are as frequent as mutations of *B2M*; in our study on 76 DLBCL in immunocompetent patients, the mutational frequency of *B2M* was 16% (98). Interestingly, loss of CD58 cell surface expression is more commonly observed than assumed from its mutational frequency and many DLBCL show a concomitant loss of HLA class I and CD58. As loss of HLA class I alone might increase susceptibility to lysis by NK cells (99), the concomitant loss of CD58, which is a CD2 ligand, might act in a counterbalancing way. The reduced cytolysis of DLBCL cells lacking CD58 expression has been confirmed in cell culture experiments (97). *CD58* mutations have also been described in a small percentage of peripheral T-cell lymphomas along with *B2M* mutations (100). Mutations of *CD58* and *B2M* are thought to be a main reason for non-responsiveness to immune checkpoint inhibition (101). Cao et al. showed that both mutations and copy number losses of *CD58* and *TP53* genes are independent unfavorable prognostic factors in DLBCL (102). This is the first study attributing such a high impact to *CD58* mutations.

B2M mutational rates vary in specific subtypes of DLBCL: in DLBCL of the testis and the central nervous system, i.e., DLBCL arising in immunoprivileged sites, *B2M* mutations have been reported to be frequent (103), while in our study on posttransplant DLBCL, no *B2M* mutations were detected (104). From this finding, we concluded that *B2M* mutations do not provide an additional advantage in the state of immunosuppression as there is, for obvious reasons, no genetic pressure for immune escape on the tumor cells.

The CD70–CD27 Axis: Another Key to T-Cell Control

CD27 belongs to the tumor necrosis factor family; it is involved in the activation of both innate and adaptive immunity. It is expressed in thymocytes and naïve T-cells as well as activated T-cells (105), memory B-cells (106), and NK cells in the bone marrow but not in circulating NK cells (107). CD27 has a unique ligand, CD70, which has become a focus of potential therapeutic interaction. A plethora of different tumor entities including many lymphomas (**Figure 4**) have been shown to express CD70

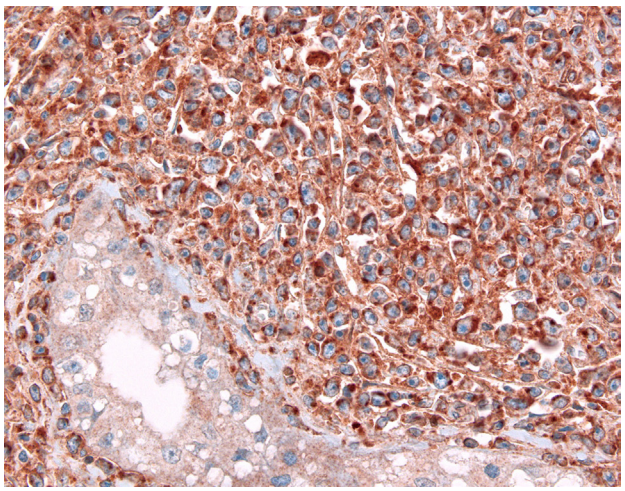

FIGURE 4 | Expression of CD70 in a testicular diffuse large B-cell lymphoma; note a negative seminiferous canaliculus.

(108, 109), whereas CD27 expression is primarily restricted to hematopoietic tumors (108). Tumors use the CD70–CD27 axis in order to manipulate T-cells in an immunosuppressive manner by increasing the proportion of inhibitory regulatory FoxP3$^+$ T-cells (110), induction of T-cell apoptosis (111), and skewing T-cells toward anergy and exhaustion (112).

First studies using monoclonal antibodies directed against CD70 have been tested with rather low response rates [complete remission in 1/19 lymphoma patients (113)]. Currently, several trials of combining anti-CD70 therapy and chemotherapy and radiotherapy are ongoing. The rationale behind this approach is that by activating the immunosurveillance of the microenvironment by CD70 blockade, the effect of conventional chemotherapy and radiotherapy is increased (114).

EBV—The Classical Model of Oncogenicity and Immune Escape

Epstein–Barr virus's role in lymphomagenesis was first discovered in Burkitt lymphoma (BL). While the *MYC* translocation is important for upholding the proliferative activity of BL, the main effect of EBV is thought to be effectively preventing c-myc-induced apoptosis (115). EBV-infected non-neoplastic memory B-cells express only one EBV-specific protein (EBNA1)—known as "latency type 1"—in order to avoid recognition by the immune system, and these cells provide the life-long reservoir of EBV in humans. This latency type 1 is sustained in many B-cell lymphomas including BL, DLBCL and terminally differentiated B-cell lymphomas, while in cHL and many NK- and T-cell lymphomas, virus-infected tumor cells express to a certain extent LMP1 and LMP2A&B (without EBNA2), known as latency type 2, and in lymphomas of immunosuppressed individuals EBNA2-3C are expressed along with LMPs, referred to as latency type 3 (116). Importantly, latency type 2 is an intriguing therapeutic target for PD1/PDL1-blocking agents as exemplified in cHL and NK/T-cell lymphomas (29, 61), while the latter latency type 3 would be recognizable by a functional immune system and is tolerated due to the concomitant immunosuppression in affected individuals as exemplified by recurrent tumor control in seldom instances, in which the respective immunosuppression can be restored (117, 118) (see also: expansion of decreased T helper 1 and CD8$^+$ T cell subsets associates with regression of lymphoproliferative disorders developed during methotrexate treatment. Saito et al., published in the same journal issue). Even more, EBV relatedness in several of the above listed instances may even stand for distinct diseases, as it has been shown for DLBCL and PTLD (104) and recently also for plasmablastic lymphoma (119) that EBV-positive and EBV-negative tumors have both different pathogenesis as well as different prognosis. EBV exerts effects on the tumor cells related to proliferation and preventing apoptosis, and on the microenvironment, particularly on the host's immune system. In the setting of human immunodeficiency virus (HIV) infection, a marked increase of EBV-related lymphomas has been initially observed (120). With the introduction of highly active antiretroviral therapy (HAART), the incidence of HIV-related lymphomas has considerably changed: while there was a steep decline of EBV-associated lymphomas of the CNS and DLBCL, cHL incidence has risen, and the incidence of BL has remained stable (121). This shows that the risk to develop certain types of lymphoma is related to the function of the immune system. While several subtypes thrive in severe immune suppression (EBV-related DLBCL in general), cHL is dependent on an at least partially functioning immune system due to HRS cell interaction with the microenvironment, particularly their dependence on CD4$^+$ T-cell signaling (122), and thus their restoration by HAART "paradoxically" promotes cHL development. In BL, it is postulated that the expansion of the germinal center reaction and the pronounced activation of polyclonal B-cells seen in the early stages of HIV—induced by several viral proteins (123)—increases the amount of EBV-infected B-cells with *MYC* translocations (115). This reservoir of translocated and virus-infected B-cells, already "replenished" at the very beginning of HIV infection, increases the risk of BL outgrowths, which is independent of future control over HIV.

Apart from improving T-cell function and numbers, a key to treatment of EBV-related lymphoma is modulation of the ubiquitin–proteasome system. This vital cell component is used by EBV in several ways: it is inhibited by the virus to foster immune evasion (124); furthermore, it is used for modulation of cell cycle checkpoint proteins such as proto-oncogene serine/threonine protein kinase 1 (PIM1) (125) or tumor suppressors such as p16 and retinoblastoma protein (pRb) (126); finally, it is involved in inhibition of apoptosis by fostering degradation of p53 and BCL6. The proof of concept of inhibiting the ubiquitin-proteasome system has been delivered in several EBV-associated malignancies (both carcinomas and lymphomas); however, larger clinical trials for testing this approach in the clinical setting are still required (127). In plasmablastic lymphoma, which is EBV-associated in the vast majority of cases (128), bortezomib treatment has already shown considerable improvement of treatment response and survival rates in small cohorts (129).

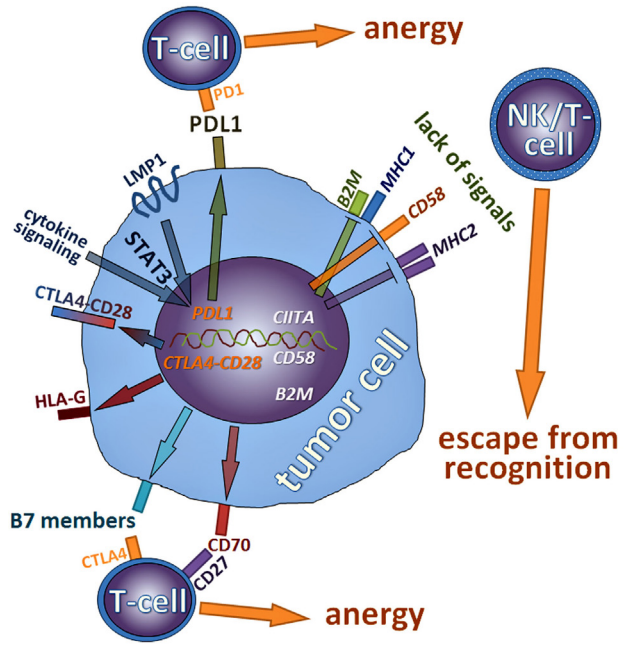

FIGURE 5 | Schematic summary of mechanisms discussed in this review lymphoma cells employ to influence or circumvent the immune system; molecules that are rather repressed are in italics; genes inactivated by mutations are in white color, while those activated are in orange.

Hematological Side Effects of Immunomodulative Therapies

Adverse events (AE)/side effects of immune checkpoint inhibition drugs are reported to be rarer than those of classical chemotherapy agents (130). In contrast to the well understood genesis of pathologic changes in peripheral organs, which can mainly be explained by a graft-versus-host-like pathophysiology, the underlying mechanisms for hematological side effects of checkpoint inhibitors are not yet fully understood. Hematological AE in general seem to be more common in lymphoma patients than in patients treated for solid tumors (131). They manifest as isolated neutropenia, thrombocytopenia, or anemia, in some cases as pancytopenia, which may all have in common decreased auto-tolerance mechanisms under immunomodulation (132). Furthermore, development of hemophilia A in patients treated with anti-CTLA4 antibodies has been described (133, 134). In one study on DLBCL patients, a condition referred to as myelodysplastic syndrome (MDS) occurred in a single patient and was listed among the AE (131). However, in our point of view, it is difficult to attribute a MDS to immune checkpoint inhibition as several potential other causes should be considered (e.g., therapy-associated myeloid neoplasm after several previous chemotherapy courses!) and the mechanism how immune checkpoint inhibitors entice MDS-related mutations remains completely unclear. Though the pathophysiology of hematological AE seen in the context of immunomodulative therapies is not fully elucidated yet, it is highly likely that they develop in an autoimmune disease-like manner. In AE suspect instances, it is vital to rule out other potential causes of cytopenias such as lymphomatous bone marrow involvement, substrate deficiencies or toxicities of former (chemo-)therapies including evolving therapy-associated myeloid neoplasms, concomitant treatment with myelotoxic medications, e.g., certain NSAR, mycophenolate, or mTOR inhibitors (135). Interestingly, occurrence or worsening of graft-versus-host disease (GvHD) in previously transplanted individuals, whom immune checkpoint inhibition was given, has been reported as an AE in some studies, while others reported a reduced incidence (132). Importantly, in an experimental setting, PDL1 inhibition reduced GvHD without hampering the graft versus lymphoma effect in mice (136).

CONCLUSION

In this review, we have summarized mechanisms lymphoma cells employ to influence or circumvent the immune system (**Figure 5**). We have shown that many mutations and pathway alterations discovered in cHL—the pathognomonic example for a lymphoma interfering with the immune system—can also be found in other types of lymphomas and that these alterations, to which many lymphomas are oncogenically addicted, can be specifically targeted. Indeed, it has become evident that manipulating the immune system taints to be an advantageous management strategy for many tumors including lymphomas. Thorough research has elucidated several mechanisms how this is achieved, it has also become clear that both tumor cells and microenvironmental compounds should be considered and modulated in a proper manner. These findings have led to a plethora of new potential treatment options, which have already proven to be beneficiary for patients.

However, it has also become evident that there is no uniform treatment response, highlighting the need for individualized analysis of patients' tumors and the corresponding individual immunological/immunogenetic background in order to decipher on the one hand the specific pathways used by the tumor to hamper the hosts' immune system and the potential responsiveness of the latter. It has also become evident that immunotherapy can and probably should be synthetically combined with the other pillars of cancer therapy—surgery, chemotherapy, and radiotherapy—as this can markedly improve the impact of each therapy approach.

AUTHOR CONTRIBUTIONS

Both AT and TM conceived and wrote the manuscript.

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