EPSTEIN-BARR VIRUS-ASSOCIATED T/NK-CELL LYMPHOPROLIFERATIVE DISEASES

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EPSTEIN-BARR VIRUS-ASSOCIATED T/NK-CELL LYMPHOPROLIFERATIVE DISEASES

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Epstein-Barr virus (EBV)-associated T/NK-cell lymphoproliferative diseases (EBV-T/NK-LPDs) are a group of mostly neoplastic diseases of unknown etiology. These diseases are characterized by ectopic infection of EBV in T or NK cells, that are unusual cellular targets for the B-lymphotropic virus. Multiple entities of EBV-T/NK-LPDs can be diagnosed in a single patient and one entity of EBV-T/NK-LPDs may evolve into another during the clinical course, suggesting a common mechanism in the pathogenesis, including a genetic predisposition. Treatment of these diseases is difficult and often requires hematopoietic stem cell transplantation. The research topic "Epstein-Barr virus-associated T/NK-cell lymphoproliferative diseases" was intended to highlight recent progresses in the research on these intractable diseases and to provide a platform for discussion on their enigmatic pathogenesis and the development of novel therapeutic strategies. This eBook contains review articles and case reports contributed to the research topic that overall indicate that the recent technical innovation in genomic research has begun to provide critical novel findings in both the host and virus genetics of EBV-T/NK-LPDs, giving new insights into the etiology and pathogenesis of the diseases.

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EDITORIAL
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Editorial: Epstein-Barr Virus-Associated T/NK-Cell Lymphoproliferative Diseases

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Keywords: EBV-associated T/NK-cell lymphoproliferative diseases, chronic active EBV infection (CAEBV), hydroa vacciniforme-like lymphoproliferative disorder, severe mosquito bite allergy, extranodal NK/T-cell lymphoma-nasal type (ENKTL), aggressive NK-cell leukemia (ANKL), EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH), systemic EBV-positive T-cell lymphoma of childhood

Editorial on the Research Topic

Epstein-Barr Virus-Associated T/NK-Cell Lymphoproliferative Diseases

In the current 2016 version of the WHO classification of tumors of hematopoietic and lymphoid tissues, four disease entities are included as EBV-associated T/NK-cell lymphoproliferative diseases (EBV-T/NK-LPDs), namely systemic EBV-positive T-cell lymphoma of childhood, chronic active EBV infection (CAEBV) of T- and NK-cell type (including the systemic form and the two cutaneous forms, hydroa vacciniforme-like LPD and severe mosquito bite allergy), aggressive NK-cell leukemia (ANKL), and extranodal NK/T-cell lymphoma, nasal type (ENKTL). In addition, EBV-positive nodal peripheral T-cell lymphoma is included as a provisional entity. Although EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH) is not included, this syndrome is also characterized by non-neoplastic proliferation of EBV-infected T or NK cells.

EBV-T/NK-LPDs share some epidemiological, clinical, and pathophysiological properties, including geographical distribution almost restricted to East Asia and Central/South America. Multiple entities of EBV-T/NK-LPDs can be diagnosed in a single patient and one entity of EBV-T/NK-LPDs may evolve into another during the clinical course, suggesting a common mechanism in the pathogenesis, including genetic predisposition. However, the pathogenesis of EBV-T/NK-LPDs is largely unknown and therapy for most of these diseases is difficult and tends to depend on hematopoietic stem cell transplantation (HSCT). This Research Topic is intended to summarize recent progresses in the field of EBV-T/NK-LPDs and to provide a platform for discussion on their enigmatic pathogenesis and the development of novel therapeutic strategies.

Kimura and Fujiwara provide an overview of EBV-T/NK-LPDs, describing the definition, characteristics, and current therapies of these diseases. They also list unsolved questions concerning EBV-T/NK-LPDs and highlight the area where more investigations are required.

As Kim et al. note, the diagnosis of EBV-T/NK-LPDs is difficult because of their unusual clinical presentation and discrepancies between clinical and pathological findings (e.g., aggressive clinical course without apparent morphological atypia in neoplastic cells). In a comprehensive review of each entity of EBV-T/NK-LPDs, these authors describe characteristic clinical features, histology, immunophenotype, and molecular findings of the diseases that are essential for their correct diagnosis and proper management.

In a comprehensive review on CAEBV, Arai points out that the pathophysiology of CAEBV has two facets, inflammation and neoplasm. Although the molecular mechanism of EBV-induced

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T/NK-cell proliferation and survival has not been elucidated, she describes recent evidence suggesting pivotal roles for the NF-κB and JAK/STAT pathways. She suggests that therapies targeting these pathways may be effective against both the inflammatory and neoplastic features of CAEBV and may improve the result of HSCT via resolving disease activity before transplantation.

Ai and Xie review the literature on EBV-T/NK-LPDs reported from Chinese mainland, one of the few areas where these diseases are endemic. Although the general clinical picture of CAEBV appears similar between China and Japan, including poor prognosis without HSCT, they imply some differences, including higher incidence of lymphadenopathy and interstitial pneumonitis and lower incidence of hypersensitivity to mosquito bite in Chinese mainland as compared to Japan. However, they note that EBV-infected cell type has been determined only in a small percentage of patients in China and therefore the statistics of EBV-T/NK-LPDs must be interpreted carefully.

ANKL is a rare malignant lymphoproliferative disease of mature NK cells typically with the large granular lymphocyte morphology. It occurs mainly in younger adults and has very poor prognosis. Ishida contributes a minireview discussing recent molecular and clinical issues associated with ANKL. Next-generation sequencing of ANKL cells reveals frequent somatic mutation in genes involved in the JAK/STAT pathway and those associated with epigenetic gene regulation, suggesting the possibility of identifying a novel molecular target for therapy.

Harabuchi et al. contribute a comprehensive review on ENKTL. Especially, they summarize basic findings on ENKTL, starting from their own discovery of EBV DNA and proteins in tumor cells and including recent characterization of various cytokines, chemokines, and microRNAs that are expressed in association with EBV infection of T/NK cells and could be therapeutic targets.

De Mel et al. summarize recent progresses in genome-wide gene expression profiling (GEP) on EBV-T/NK-LPDs. GEP reveals upregulation of the JAK/STAT and NF-κB pathways that promote cell survival and proliferation in ENKTL. It also shows that upregulation of survivin and deregulation of p53 inhibit apoptosis in ENKTL and CAEBV. Furthermore, GEP identifies a number of possible therapeutic targets, including immune checkpoint molecules, CD38, and elements of the JAK/STAT pathway.

Tanita et al. describe a 21-year-old male patient with EBV-positive γδT-cell LPD who exhibits low T and NK-cell numbers, low T/NK-cell proliferative response, and deficiency in STAT3/5/6 phosphorylation following stimulation with cytokines. Whole exome sequencing identifies a hemizygous hypomorphic mutation of the *IL2RG* gene (c.C982T, p.R328*) that may be responsible for his immunodeficiency. This result suggests the possibility that CAEBV can develop on the basis of unidentified primary immunodeficiency.

Ishimura et al. describe a male case of CAEBV with a hypomorphic mutation of the *SH2D1A* gene (c.G7T, p.A3S). They also describe two male cases of CAEBV/EBV-HLH with a hypomorphic variant of the *XIAP* gene (c.1045_1047delGAG, p.E349del) and a female case of CAEBV with the same *XIAP* variant in a heterozygous state. These cases suggest that the two genes responsible for X-linked lymphoproliferative disease may be involved in the pathogenesis of EBV-T/NK-LPDs in a fraction of patients.

In a perspective article, Yachie highlights the value of flow-cytometric analysis of circulating lymphocytes in the diagnosis of EBV-T/NK-LPDs. He indicates that the expansion of HLA-$DR^+TCR\gamma\delta^+$ cells and that of HLA-DR^+CD56^+ cells are a diagnostic marker suggestive of hydroa vacciniforme-like LPD and severe mosquito bite allergy, respectively. Expansion of $CD8^+CD5^{dim/negative}$HLA-DR^{high} cells is a specific marker for the diagnosis of EBV-HLH. Flow-cytometry can also demonstrate monoclonality of EBV-infected T cells by revealing the expansion of a cell population expressing a particular TCR Vβ gene.

Sawada and Inoue describe a unified treatment strategy for EBV-T/NK-LPDs that is composed of the step 1 (immunochemotherapy), step 2 (multi-drug chemotherapy) and step 3 (allogeneic HSCT). They analyze a single-institution experience of planned (i.e., not emergent) transplantation ($n = 63$) for the treatment of CAEBV and show that reduced-intensity conditioning (RIC) is superior to myeloablative conditioning (MAC) with the 3-year overall survival of $90.7 \pm 4.0\%$ ($n = 54$) for the former and $66.7 \pm 15.7\%$ ($n = 9$) for the latter. Thy also use the unified strategy for the treatment of EBV-HLH; majority of cases require only the step 1 or the steps 1 and 2.

Sato contributes an opinion article that mainly summarizes recent preclinical studies on molecular target drugs for the treatment of EBV-T/NK-LPDs, including the anti-CC chemokine receptor 4 (CCR4) antibody mogamulizumab, the proteasome inhibitor bortezomib, HDAC inhibitors, mTOR inhibitors, and JAK inhibitors. He also mentions on the recent progress in organizing a nationwide registry of EBV-T/NK-LPD in Japan.

To conclude, the recent technical innovation in genomic research has begun to provide critical novel findings in both the host and virus genetics of EBV-T/NK-LPDs, providing new insights into the etiology and pathogenesis of the diseases. This trend will accelerate the development of improved therapeutic strategies for EBV-T/NK-LPDs including novel chemotherapy.

AUTHOR CONTRIBUTIONS

SF and HK participated in the editorial process of the Research Topic and co-wrote the editorial.

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MINI REVIEW
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Overview of EBV-Associated T/NK-Cell Lymphoproliferative Diseases

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Epstein-Barr virus-associated T/natural killer-cell lymphoproliferative diseases (EBV-T/NK-LPDs) are a group of rare diseases resulting from ectopic infection of T or natural killer (NK) lymphocytes with Epstein-Barr virus (EBV). EBV-T/NK-LPDs include chronic active EBV infection, EBV-associated hemophagocytic lymphohistiocytosis, hydroa vacciniforme-like lymphoproliferative disease, and severe mosquito bite allergy. Extra-nodal NK/T-cell lymphoma-nasal type and aggressive NK-cell leukemia can also be included in this broad spectrum. Currently, the etiology of EBV-T/NK-LPDs is unknown and no curative therapy has been established, except for hematopoietic stem cell transplantation. While most cases of EBV-T/NK-LPDs have been documented in specific areas of the world, they have also been documented more broadly across East Asia and Latin America. Consequently, active research and discussion of EBV-T/NK-LPDs are both necessary and important within the extensive international community of scientists and clinicians, to elucidate their etiology and develop a standard therapy.

Keywords: EBV-T/NK-LPDs, chronic active EBV infection, hydroa vacciniforme-like LPD, severe mosquito bite allergy, extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia

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INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that persistently infects more than 90% of the world's adult population. EBV infection primarily targets B cells and epithelial cells. Although infection with EBV is usually asymptomatic, the development of symptomatic disease has been associated with a delayed primary infection leading to infectious mononucleosis in adolescents and young adults, and with various EBV-associated malignancies. The virus also causes various opportunistic diseases in immunocompromised hosts.

In apparent immunocompetent hosts, EBV can also induce chronic disease with prolonged infectious mononucleosis-like symptoms and a sustained EBV DNA load in the peripheral blood. Although rare, this disease has been called chronic active EBV disease or chronic active EBV infection; both are abbreviated as CAEBV (1, 2). Interestingly, while EBV-infected B-cell proliferation has been observed in most patients with CAEBV in Western countries, the virus is found mainly in T or NK cells proliferating clonally in East Asian countries, including Japan (3, 4).

In addition to CAEBV, there is a group of rare diseases that develop in the apparent absence of immunodeficiency and are characterized by ectopic infection of EBV in T or NK lymphocytes. They are EBV-associated hemophagocytic lymphohistiocytosis (HLH), hydroa vacciniforme (HV)-like lymphoproliferative disease, and severe mosquito bite allergy (SMBA). These diseases have

a particularly high incidence in East Asia and Latin America and are collectively called EBV-associated T/NK-cell lymphoproliferative diseases (EBV-T/NK-LPDs) (5, 6). However, the EBV-T/NK-LPDs concept has not been fully established. In fact, there are several unanswered questions about EBV-T/NK-LPDs. For instance, it is not known how EBV infects T and NK cells, or how they proliferate. It is also not known why EBV-infected T/NK cells are not removed by host immune responses, or whether they are immunodeficient or malignant diseases. Moreover, the scientific community cannot yet explain why the incidence rates of EBV-T/NK-LPDs are uneven across geographic territories, or whether there are any genetic predisposing factors. Finally, the ideal timing and protocol for hematopoietic stem cell transplantation for CAEBV remain to be determined.

This review investigates both original works and secondary sources on EBV-T/NK-LPDs. It is intended to stimulate discussion on the enigmatic pathogenesis and disease concept of EBV-T/NK-LPDs, to facilitate, eventually, the development of a standard therapy.

DEFINITIONS AND BRIEF DESCRIPTION OF REPRESENTATIVE EBV DISEASES

EBV preferentially infects B cells via the CD21 cell surface protein, and is associated with a variety of diseases of B-cell origin. These include infectious mononucleosis, Burkitt lymphoma, diffuse large B-cell lymphoma, and Hodgkin lymphoma (7). EBV nucleic acids are detected in 25∼50% of Hodgkin lymphoma in the USA and Europe (8, 9). EBV causes immunodeficiency-associated lymphoproliferative diseases, such as post-transplant lymphoproliferative disorders and lymphomas associated with HIV infection (7). EBV can infect epithelial cells and is associated with nasopharyngeal carcinoma and gastric cancer (7). EBV is also associated with diseases of T- and NK-cell origin, such as extranodal NK/T-cell lymphoma-nasal type (ENKTL), aggressive NK-cell leukemia (ANKL), and EBV-T/NK-LPDs as already mentioned. However, the specific T or NK cell receptors that contribute to the disease state are not known.

According to the 2017 World Health Organization Classification of Tumors of Haematopoietic and Lymphoid Tissues, four diseases are classified as EBV-positive T-cell and NK-cell LPDs of childhood: systemic EBV-positive T-cell lymphoma of childhood, the systemic form of CAEBV of the T/NK-cell type, HV-like LPD, and SMBA (10). ENKTL and ANKL are also described in the text and are classified under the umbrella of EBV-positive T/NK-cell proliferation. Since EBV-associated HLH is non-neoplastic, it is not listed as an EBV-positive T-cell and NK-cell lymphoproliferative disease of childhood in this classification. It is, however, important to note that EBV-associated HLH is also characterized by EBV-positive T/NK-cell lymphoproliferation. Furthermore, EBV-associated HLH is an intractable, potentially fatal disease (11). EBV-T/NK-LPD that are described and classified in the 2017 WHO lymphoma classification are shown in **Table 1**.

CAEBV of the T/NK-Cell Type, Systemic Form

CAEBV is defined as an EBV-related illness characterized by symptoms such as fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, and interstitial pneumonia lasting for more than 3 months (3, 12). Some patients also exhibit skin-related symptoms, such as HV-like eruptions or hypersensitivity to mosquito bites. In these patients, increased amounts of EBV in the affected tissues or peripheral blood were also found. The incidence of the T/NK cell type of this disease varies markedly by race, with most cases occurring in East Asians. Most of the patients are children or young adults who exhibit clonality of EBV-infected cells. CAEBV is a potentially life-threatening illness, but the prognosis of CAEBV is variable. Some patients rapidly develop severe complications, such as multi-organ dysfunction and malignant lymphomas, whereas others remain stable without therapeutic intervention (12). Initially, CAEBV was thought to mainly be a disease of childhood, but, recently, increasing numbers of adult cases have been identified with slightly different clinical features compared to those of pediatric cases (13, 14).

HV-Like LPD

In the 2008 WHO lymphoma classification, HV-like LPD was termed HV-like lymphoma. However, given the broad spectrum of the disease, and the lack of reliable morphological or molecular criteria, the term HV-like LPD was proposed in the 2017 classification (10). Sub-classifications of HV-like LPD include classic HV, severe (or systemic) HV, and HV-like lymphoma (15).

HV-like LPD consists of a recurrent vesiculopapular lesion with central umbilication and crust formation, which mimics herpetic vesicles usually occurring on sun-exposed areas. HV-like eruptions result from infiltration of T cells into the superficial dermis and subcutaneous tissue. In classic HV, gamma-delta T cells comprise the majority of the T cell population in the skin and mucosal epithelium (16, 17). Classic HV exhibits only skin involvement and has favorable outcomes (12, 15). The prognosis of HV-like LPD varies. Classic HV eventually resolves in adulthood. However, other types of HV-like LPD develop into a progressive disease, with worsening cutaneous symptoms and systemic dissemination (10). Patients who have systemic symptoms like fever, wastage, lymphadenopathy, and hepatosplenomegaly are categorized as severe or systemic HV.

SMBA

SMBA is an EBV-positive NK-cell lymphoproliferative disorder defined by a hypersensitivity to mosquito bites. The disease is characterized by a high fever after mosquito bites and has various skin manifestations, including ulcers, necrosis, and scarring (18). Similar to HV-like LPD, some patients may have systemic symptoms. Most SMBA cases have been reported in Japan, with other cases reported in Korea, Taiwan, and China. Patients with SMBA have a long clinical course, with an increased risk of developing hemophagocytic syndrome and ANKL. Half of the SMBA patients were reported to have died of hemophagocytic syndrome or leukemia/lymphomas (19).

TABLE 1 | 2017 WHO lymphoma classification of EBV-associated T-cell and NK-cell proliferation.

Disease entity	Association with EBV (%)	Infected cells	Age group	Population at high risk
Systemic EBV$^+$ T cell lymphoma of childhood	100	T	Pediatric, adolescent	East Asians
Chronic active EBV infection of T/NK type	100	T, NK	Pediatric, adolescent	East Asians
Hydroa vacciniforme-like lymphoproliferative disorder	100	γδT, NK	Pediatric, adolescent	Asians, native Americans
Severe mosquito bite allergy	100	NK (T)	Pediatric, adolescent	East Asians
Aggressive NK cell leukemia	>90	NK	Adult	Asians
Extra nodal NK/T cell lymphoma, nasal type	100	NK, T	Adult	East Asians

ENKTL

ENKTL, also called nasal NK/T-cell lymphoma, is a predominantly extranodal lymphoma of NK-cell or T-cell lineage characterized by necrosis, vascular damage and destruction, cytotoxic phenotypes, and an association with EBV (20). ENKTL is more prevalent in East Asians and Native Americans in Mexico and Central/South America.

In ENKTL, the upper aerodigestive tract, including the nasal cavity and paranasal sinuses, is most commonly involved. It often progresses, exhibiting extensive midfacial destructive lesions that sometimes spread to other sites, including the skin and gastrointestinal tract. ENKTL is highly aggressive and poorly responds to therapy, resulting in low survival rates.

ANKL

ANKL is a rare disease, but is considerably more prevalent among Asians. It involves systemic neoplastic proliferation of NK cells, frequently associated with EBV infection, and has an aggressive course (21). Patients with ANKL usually present with fever, constitutional symptoms, and a leukemic blood picture. Most cases have a fulminant clinical course, frequently complicated by multiple organ failure, coagulopathy, and hemophagocytic syndrome. The prognosis of this EBV-T/NK-LPDs is dismal.

ETIOLOGY

The pathogenesis of EBV-T/NK-LPDs is unclear. Some evidence indicates that EBV infects T/NK cells during the primary infection (22), although this does not necessarily lead to EBV-T/NK-LPDs. EBV-infected T or NK cells may proliferate and evade apoptosis with the help of viral oncogenes and may eventually cause EBV-T/NK-LPDs in rare cases (23–25). There are however several unknown mechanisms underlying this process, as summarized in **Table 2**.

First, the mechanism of T/NK cell infection by EBV remains to be determined. T/NK cells lack CD21 and HLA-DR, both of which are EBV receptors in B cells. Therefore, the exact mechanism of EBV attachment and entry into T/NK cells remains largely unknown (29). Tabiasco et al. reported that NK cells activated by EBV-infected B cells acquire CD21 molecules by synaptic transfer, and these ectopic receptors allow binding of EBV to NK cells (28). EBV may attach to T cells via CD21, which is expressed in immature T cells and common progenitor cells (26, 27, 35). Recently, dual infections of both T-cell and NK-cell lineages with a single clone of EBV have been increasingly reported in patients with CAEBV. EBV may infect lymphoid progenitor cells that express CD21 and have the capacity to differentiate into both T and NK cells, at least in some patients (22).

TABLE 2 | Critical questions about the pathogenesis of EBV-associated T/NK-cell lymphoproliferative diseases.

Question	Possible answer or supportive evidence	References
What are the EBV receptors on T/NK cells?	1. CD21 expressed on lymphocyte progenitors 2. Transfer of CD21 from B cells to T/NK cells via immunological synapse 3. Non-CD21 receptor	1. (26, 27) 2. (28) 3. (29)
How does EBV induce T/NK-cell proliferation?	1. Involvement of the co-activating receptor CD137 2. Involvement of the cellular transcription factor NF-κB 3. Involvement of the cellular transcription factor STAT3	1. (23) 2. (24) 3. (25)
Why are EBV-infected T/NK cells not removed by cytotoxic T cells?	1. EBV-infected T/NK cells exhibit latency 2 type viral gene expression and do not express immunodominant proteins such as EBNA2 and EBNA3s	1. (3)
What explains the biased geographical distribution of EBV-T/NK-LPDs?	1. Genetic predisposition 2. Local environmental factors 3. Specific EBV strains	1. (30) 2. (31) 3. (32)
Are EBV-T/NK-LPDs immunodeficient?	1. Deficiency of EBV-specific CTLs was reported in CAEBV 2. Clinical manifestations resembling CAEBV have been documented in a patient with common variable immunodeficiency	1. (33) 2. (34)
Are EBV-T/NK-LPDs malignant?	1. Driver gene mutations identified in CAEBV	1. (2)

Second, it is not known whether EBV-T/NK-LPDs are malignant neoplastic diseases like overt leukemia or lymphoma. Based on current knowledge, ENKTL and ANKL appear to be malignant neoplastic diseases. EBV-T/NK-LPDs can be considered as a pre-leukemic or lymphomatous stage in which genetic mutations accumulate and drive the process of lymphomagenesis. Recent comprehensive genetic analyses indicate that DDX3X, TP53, STAT3, and BCOR1 are driver genes that are frequently mutated in patients with ENKTL (36, 37). Interestingly, somatic driver mutations that are seen in ENKTL were found in EBV-infected cells from CAEBV patients. This suggests that EBV-infected T/NK cells evolutionally expand

with driver gene mutations, and that mutations in DDX3X are important for the development of overt lymphoma and leukemia in patients with CAEBV (2). Activation-induced cytidine deaminase (AID), which belongs to the APOBEC3 protein family and induces somatic hypermutations and class switch recombination, is a candidate cause of such host gene mutations. Upregulation of AID driven by EBV infection plays an important role in c-myc translocation and tumorigenesis of Burkitt lymphoma and other lymphomas of B-cell origin (38). High expression of AID is seen in EBV-infected T/NK cells from patients with EBV-T/NK-LPDs, and may be associated with driver gene mutations (39).

Finally, although EBV is ubiquitous, it is unknown why only some people develop EBV-T/NK-LPDs in specific areas of the world. While the existence of immunodeficiencies in certain areas has been postulated, mutations in known immune-associated genes are not seen in the majority of cases (33, 34). An association with a specific HLA (A26) was reported, but it did not account for all cases (22, 30). Other explanations suggest that environmental factors are associated with the progression or development of T/NK-cell tumors. In a case-control study, exposure to pesticides and chemical solvents was correlated with ENTKL development (31). Specific EBV strains or variants that have a greater tendency to develop T/NK-cell tumors may exist (32), but there is no direct proof. Thus, the etiologies of EBV-T/NK-LPDs may be multifactorial and they are likely to consist of heterogeneous entities.

TREATMENT

The prognosis of EBV-T/NK-LPDs is highly variable. Some patients rapidly develop severe complications, whereas others remain stable without therapeutic interventions. So far, no standard treatment for EBV-associated T/NK cell lymphoma has been established. Additionally, there are no effective antivirals against EBV and no molecular-targeted therapies, unlike rituximab against B cell neoplasms. The outcomes of EBV+ diffuse large B-cell lymphoma or post-transplant lymphoproliferative disorders have improved with the addition of rituximab to conventional chemotherapies (40, 41). EBV-infected T/NK cells are also generally resistant to chemotherapy, due to the expression of the multidrug resistance protein p-glycoprotein (42). To combat difficulties in treatment, hematopoietic stem cell transplantation has been introduced as a curative therapy. Although recent efforts at introducing reduced-intensity conditioning resulted in excellent transplantation outcome (43), the rates of complications associated with transplantation are high (12). It is therefore necessary to develop novel approaches to treat EBV-associated T/NK cell lymphoma.

Proteasome inhibitors, histone deacetylase (HDAC) inhibitors, and Janus kinase (JAK) inhibitors have been tested using *in vitro* or *in vivo* xenograft models as possible therapeutic approaches (25, 44–46). Additionally. immune checkpoint inhibitor therapy may be beneficial for patients with EBV-T/NK-LPDs, since programmed death-1 (PD-1) blockade with pembrolizumab has proven effective in relapsed or refractory ENKTL (47). To establish a standard therapy against EBV-T/NK-LPDs, new strategies and extensive further research are needed.

AUTHOR CONTRIBUTIONS

HK and SF contributed to concept development and the writing and review of this manuscript, and gave final approval of the version to be published.

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REVIEW
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Epstein-Barr Virus-Associated T and NK-Cell Lymphoproliferative Diseases

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EBV-associated T and NK-cell lymphoproliferative diseases (EBV-T/NK LPDs) are characterized by the transformation and proliferation of EBV-infected T or NK cells. The 2016 revised World Health Organization classification recognizes the following EBV-positive lymphoproliferative disorders (LPD): chronic active EBV infection (CAEBV) of T- and NK-cell type (cutaneous and systemic forms), systemic EBV-positive T-cell lymphoma of childhood, aggressive NK-cell leukemia, extranodal NK/T-cell lymphoma, nasal type, and the new provisional entity primary EBV-positive nodal T/NK-cell lymphoma. EBV-associated hemophagocytic lymphohistiocytosis (HLH), although not included in the WHO classification because it is a reactive, inflammatory disease, is included in this review because it can be life-threatening and may have overlapping features with other EBV+ T/NK LPDs. EBV+ T/NK LPDs are rare diseases difficult to diagnose and manage properly, because some LPDs have unusual presentations, and discrepancies between clinical and histological findings might be encountered. Furthermore, EBV+ T/NK disorders share some clinico-pathological features, and may evolve into other categories during the clinical course, including malignant transformation of CAEBV. Here, we review the EBV+ T/NK LPDs in terms of their definitions, clinical features, histology, immunophenotype, molecular findings, and pathogenesis. This review aims to increase our understanding and awareness of the differential diagnosis among the different EBV+ T/NK LPDs. New insights into the genetic characteristics of these disorders will also be discussed.

Keywords: EBV, lymphoproliferations, hemophagocytic lymphohistiocytosis, chronic active EBV infection, systemic EBV positive T-cell lymphoma, aggressive NK-cell leukemia, extranodal NK/T-cell lymphoma, primary EBV nodal T and NK-cell lymphoma

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INTRODUCTION

Epstein-Barr virus (EBV) is a gamma herpesvirus with a double-stranded DNA genome in core. EBV has a tropism for B cells but can infect various types of human cells including T cells, NK cells and even epithelial cells (1). EBV causes chronic latent infection with lifelong persistence in about 95% of the world population (2). Memory B cells are important as the main reservoir of EBV during the latency period. The impairment of balance between host immune response and EBV virus can lead to various EBV-associated lymphoproliferative disorders (LPDs) of B, T, or NK cells.

EBV-associated LPDs can be categorized into B or T/NK-cell types based on which cells are infected by the virus.

EBV-associated T and NK-cell LPDs are characterized by the transformation and proliferation of EBV-infected T and NK-lymphocytes that usually carry an EBV-latency type 2. These disorders occur commonly in Asians and Native Americans from Central and South America (3). EBV-associated T and NK-cell LPDs represent a broad spectrum of diseases encompassing various reactive and malignant disorders (4). They are classified into 6 categories, consisting of EBV-associated hemophagocytic lymphohistiocytosis (HLH), chronic active EBV infection (CAEBV) of T- and NK-cell type, systemic EBV-positive T-cell lymphoma of childhood, aggressive NK-cell leukemia, extranodal NK/T-cell lymphoma, nasal type, and primary EBV-positive nodal T/ NK-cell lymphoma, the latter incorporated as a new provisional subgroup within peripheral T-cell lymphoma, not otherwise specified (**Table 1**). The first three disorders are prevalent in the pediatric and adolescent population, whereas the last three groups usually affect adults (3). Systemic EBV-positive T-cell lymphoma of childhood (previously LPD), aggressive NK-cell leukemia, extranodal NK/T-cell lymphoma, nasal type, and primary EBV-positive nodal T/NK-cell lymphoma are considered malignant proliferations in the 2016 revised WHO classification (3). CAEBV of T- and NK-cell type represents a reactive process of EBV-associated T and NK-cell LPDs with potential to progress into a malignant disorder. CAEBV is divided into one systemic and two cutaneous forms; hydroa vacciniforme (HV)-like LPD (previously lymphoma) and severe mosquito bite allergy. EBV-associated HLH is a clinicopathological syndrome complicated by abnormal hyperinflammatory immune response and is also considered a reactive disorder.

This review aims to describe the clinicopathological features of various EBV-associated T and NK-cell LPDs including reactive lymphoproliferations as well as overt lymphomas. The main features are summarized in **Table 2**.

TABLE 1 | EBV-associated T and NK- cell lymphoproliferative diseases.

Disease entities
EBV-positive hemophagocytic lymphohistocytosis
Chronic active EBV infection of T- and NK-cell type
Systemic form
Cutaneous form
Hydroa vacciniforme-like lymphoproliferative disease
Severe mosquito bite allergy
Systemic EBV-positive T-cell lymphoma of childhood
Aggressive NK-cell leukemia
Extranodal NK/T-cell lymphoma, nasal type
Primary EBV-positive nodal T and NK-cell lymphoma*

Considered a provisional entity within peripheral T-cell lymphoma, not otherwise specified.

EBV-ASSOCIATED HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

HLH is a life-threatening inflammatory disease characterized by uncontrolled and overwhelming activation of the immune system. HLH is a clinical syndrome that can be diagnosed when the patient meets diagnostic criteria based on the following findings: (1) clinical features such as fever and splenomegaly; (2) laboratory abnormalities including cytopenias, hyperferritinemia and liver dysfunction; and (3) pathological findings showing hemophagocytosis in bone marrow (BM), spleen, or lymph nodes (LN) (4, 5). HLH can be divided into primary and secondary forms according to underlying causes. The primary or familial type is an inherited disorder with an autosomal recessive inheritance of mutations affecting the cytotoxic function of T and NK cells, and the secondary type corresponds to an acquired type associated with various conditions including infections, autoimmune disorders, and malignancy (6, 7). However, the clinical distinction between primary and secondary HLH may be ambiguous, because patients with HLH-associated gene defects frequently have an infectious event triggering the clinical symptoms (8). Furthermore, advanced molecular techniques including whole-exom sequencing can identify underlying genetic abnormalities that have not been detected by conventional clinical testing in patients with presumed secondary HLH (9). Recent whole-genome sequencing analysis identified several types of genetic defects related with immune dysfunction, including potentially novel ones, in 58% of patients with HLH (9). Therefore, systematic and comprehensive investigations are essential for all patients with newly diagnosed HLH.

Viral infections are the most common cause of secondary HLH, and EBV is the most frequently HLH-associated virus (5). In a nationwide survey performed in Japan, EBV-associated HLH accounted for about 30% of HLH, followed by other infection- or lymphoma-associated HLH (5). EBV infection was reported to be about 75% in patients with HLH in a retrospective study done in China (10). HLH can also occur in the clinical course of other EBV-associated T and NK-cell LPDs including CAEBV of T/NK cell type or systemic EBV-positive T-cell lymphoma of childhood. An independent diagnosis of EBV-associated HLH, as a separate entity, can be made when other EBV-associated LPDs are excluded in the differential diagnosis. EBV-associated HLH is typically associated with EBV infection of T or NK cells rather than B cells.

Among several predisposing genetic conditions, X-linked lymphoproliferative disease (XLP) is often associated with EBV-associated HLH. Especially, HLH arising in patients with XLP type 1 is nearly exclusively linked to EBV (11). In patients with XLP type 2, HLH is commonly associated with EBV, but also arises in response to other infectious agents besides EBV or without an identifiable infectious cause (11). EBV-associated HLH occurs predominantly in children and adolescents. The majority of cases have been reported in East Asians (5, 12). Differences in geographic distribution indicates that there may be some genetic predisposition in the pathogenesis of EBV-associated HLH (13).

Clinical Features

EBV-associated HLH typically presents with continuous high-grade fever and splenomegaly. Other findings including

TABLE 2 | Summary of pathological features of EBV-associated T and NK- cell lymphoproliferative diseases.

	Clinical features	Histological features	Immunophenotypic features	Lineage and Clonality
EBV-associated HLH	High fever and splenomegaly Cytopenia and liver dysfunction Serological test or the detection of EBV DNA or RNA from the tissues Exclusion of other EBV-associated T/NK-LPDs	Hemophagocytosis by activated histiocytes in BM, spleen, or LNs Presence of relatively small numbers of EBV+ T cells	Predominantly cytotoxic CD8+ T cells	T cell (80%) NK cell (20%) Monoclonal TCR (50%)
CAEBV, systemic	Persistent IM-like illness >3 months in duration High fever, hepatosplenomegaly, HV-like eruptions, hypersensitivity to mosquito bite, uveitis, diarrhea, and lymphadenopathies Cytopenia and liver dysfunction Increased EBV DNA (>102.5 copies/mg) in peripheral blood or demonstration of EBV RNA or viral protein in affected tissues	Nonspecific inflammatory changes with no histological evidence of malignant lymphoproliferations	CD4>>CD8> γδ T cells CD56+ (41%)	T cell (59%) NK cell (41%) Monoclonal TCR (50%) Monoclonal EBV (84%)
Hydroa vacciniforme-like LPD	Cutaneous form of CAEBV Recurrent vesiculopapular eruptions usually in sun-exposed skin area Indolent, self-limited clinical course with a risk to progress to other EBV-associated T/NK-LPDs	Intraepidermal spongiotic vesicles Lymphoid infiltrates with angiocentric and periadnexal involvement Small lymphocytes with no or mild atypia	Predominantly cytotoxic CD8+ T cells CD56+ (30%)	T cell (70%) NK cell (30%) Monoclonal TCR Monoclonal EBV
Severe mosquito bite allergy	Cutaneous form of CAEBV Exaggerated hypersensitivity reaction to mosquito bites (erythema, bullae, ulcers, scarring, high fever, lymphadenopathy, liver abnormalities, and hepatosplenomegaly) Prolonged clinical course with a risk to progress to other EBV-associated T/NK-LPDs	Epidermal necrosis, ulcer and bullae Polymorphous infiltration of small lymphocytes, large atypical cells, and other reactive inflammatory cells including histiocytes and eosinophils	CD3ε+, CD56+ NK cells	NK cell Polyclonal TCR Monoclonal EBV
Systemic EBV+ T-cell lymphoma of childhood	High fever, hepatosplenomegaly, pancytopenia, and coagulopathy, and abnormal liver function Monoclonal proliferation of EBV-positive T cells in tissues or peripheral blood Occurs shortly after acute primary EBV infection in previously healthy children or in the setting of CAEBV Fulminant clinical course that resulted in death within days to weeks	Increased infiltration of small lymphoid cells with histiocytic hyperplasia and striking hemophagocytosis in BM, spleen, and liver Small lymphocytes with no or minimal atypia	Predominantly CD8+ cytotoxic T cells CD2+, CD3+	T cell Monoclonal TCR
Aggressive NK-cell leukemia	High fever, general malaise, hepatosplenomegaly, hepatic failure, and pancytopenia Systemic neoplastic proliferations of NK cells in peripheral blood and bone marrow Fulminant clinical course Presence of EBV-negative subset (<15%)	Varying degrees of leukemic cell infiltration in BM, LN, liver and spleen (sometimes focal or subtle) A broad cytological spectrum ranging from normal large granular lymphocytes to atypical pleomorphic lymphocytes	CD3ε+, CD56+ NK cells CD2+, FASL+ surface CD3-, CD5- CD16+ (75%)	NK cell Polyclonal TCR
Extranodal NK/T-cell lymphoma, nasal type	EBV-positive aggressive lymphoma Nasal type (70–80%): occurs in nasal and nasopharyngeal area, relatively less aggressive disease Extranasal type (20–30%): in skin, GI tract, and testis, aggressive disease Extensive ulceration and necrosis in mucosal sites	Diffuse infiltration of atypical lymphoid cells with angiocentricity and angiodestruction Frequent coagulative necrosis Broad cytological spectrum Variable amounts of inflammatory cells	Mostly CD3ε+, CD56+ NK cells (CD25+, FAS+, FASL+, HLA-DR+, surface CD3-, CD4-CD5-) Occasionally CD3ε+, CD56- cytotoxic T cells (CD8+, CD5+,TCR γδ or αβ+)	NK cell (80–85%) T cell (15–20%) Monoclonal TCR (10–40%)
Primary EBV+ nodal T/NK-cell lymphoma	A rare type of EBV+ PTCL with primary nodal presentation Generalized lymphadenopathy Limited extranodal lesions without nasal involvement	Relatively monomorphic proliferation of large atypical cells with centroblastic feature or diffuse proliferation of pleomorphic cells composed of small, medium, to large atypical cells	Predominantly CD8+ cytotoxic T cells, γδ T cells CD56+ (7.5–15%), CD4+ (15–20%)	Mostly T cell, rarely NK cell Monoclonal TCR

EBV, Epstein barr virus; HLH, hemophagocytic lymphohistiocytosis; CAEBV, chronic active EBV infection; NK, natural killer; BM, bone marrow; LN, lymph nodes. TCR, T-cell receptors; GI tract, gastrointestinal tract; PTCL, peripheral T-cell lymphoma; LPDs, lymphoproliferative disorders; IM, infectious mononucleosis.

lymphadenopathy, jaundice, edema, and skin rash may also be present. Laboratory tests show cytopenias affecting more than two lineages in the peripheral blood (PB), abnormalities in liver function tests (e.g., hypertriglyceridemia, hypofibrinogenemia, elevated serum transaminases, hyperbilirubinemia, prolonged prothrombin time, and prolonged partial thromboplastin time), hyperferritinemia, cerebral spinal fluid (CSF) pleocytosis, hyponatremia, and hypoproteinemia (6). Among these, cytopenia, hypertriglyceridemia, hypofibrinogenemia, and hyperferritinemia are included in the diagnostic criteria of HLH (**Table 3**) (14). EBV-HLH tends to show these clinical findings in a more rapid and severe fashion, compared to other HLHs (12). However, clinical manifestations may be highly variable and some patients can have nonspecific presentations by unusual organ involvement, including intestinal perforation, cutaneous lesions, pulmonary infiltrates, and central nervous system (CNS) disease (12, 13, 15). Therefore, clinical suspicion is important for the appropriate diagnosis and treatment of EBV-associated HLH.

EBV-associated HLH induces hypercytokinemia resulting from abnormal hyperactivation of the immune system. Various cytokines including IFN-γ, TNF, sIL-2R, IL-6, IL-10, and IL-18 are secreted by activated macrophages and T-lymphocytes (12). They have been used as biological indicators reflecting the severity of HLH. The level of sIL-2 receptor (sCD25) is used as a diagnostic marker. It is crucial to detect EBV infection in the patient for diagnosing EBV-associated HLH. The presence of EBV can be determined by serological tests or by detection of EBV DNA or RNA from PB or any tissue. Viral load can be estimated by measuring EBV copy numbers using the real time PCR assay, which correlates better with clinical severity than serology (12). The clinical course of EBV-associated HLH varies from mild to severe or fatal.

Morphology and Immunophenotypical Findings

Histologically, activated macrophages engulfing RBCs, leukocytes, platelets, and their precursor cells are scattered in the sinusoids of BM, spleen, liver, and LN, but are not required for diagnosis. EBV-infected T cells are also found with hemophagocytic histiocytes and show a cytotoxic phenotype with expression of CD8 and granzyme B in the majority of cases (4). The characteristics of infiltrating T cells are different between EBV-associated HLH and other EBV-associated T and NK-cell LPDs. Generally, EBV+ T cells frequently show a cytotoxic phenotype with CD8 expression in EBV-associated HLH and systemic EBV-positive T-cell lymphoma, whereas CD4+ cells or NK cells are predominantly infected by EBV in other EBV-associated T and NK-cell LPDs. In addition, the EBV+ cells are present in relatively small amounts in EBV-associated HLH, compared to other LPDs. *In situ* hybridization (ISH) with the EBV-encoded small RNA (EBER) is used to detect EBV-infected cells. Double staining with EBER ISH and CD20, CD3, or CD56 can be done to identify which cells are infected by EBV. HLH induced by EBV-infected NK cells has been reported to occur uncommonly, accounting for 20% in a previous report (4, 16).

TABLE 3 | Diagnostic guidelines for HLH used in the HLH-2004 trial.

The diagnosis of HLH can be established if one of either 1 or 2 below is fulfilled.

1. A molecular diagnosis consistent with HLH.
2. Diagnostic criteria for HLH fulfilled (at least 5 out of the 8 criteria below)

1) Fever ≥38.5°C
2) Splenomegaly
3) Cytopenia involving ≥2 cell lines
   - Hemoglobin <90 g/L (in infants <4 weeks: hemoglobin <100 g/L)
   - Platelets <100 × 109/L
   - Neutrophils <1.0 × 109/L
4) Hypertriglyceridemia (fasting, ≥265 mg/dL) or hypofibrinogenemia (≤1.5g/L)
5) Hemophagocytosis in bone marrow, spleen, or lymph nodes
6) Low or absent natural killer cell activity
7) Serum ferritin > 500μg/L
8) Elevated CD25 (soluble IL-2 receptor) levels (>2400 U/mL)

Pathogenesis and Molecular Features

The precise mechanism on how T or NK cells lacking CD21, the primary receptor for EBV, are infected by EBV in EBV-associated HLH is still unknown. A previous report showed that CD21 is synaptically transferred to NK cells through conjugation to CD21+, EBV-infected B cells, thereby allowing EBV binding to NK cells (16, 17). T-cell receptor (TCR) gene rearrangement can be detected in about half of cases with EBV-associated HLH using conventional method (18). Furthermore, with the introduction of Biomed-2 multiplex PCR, the detection rate of T-cell clonality is notably increasing in EBV-associated HLH. It has been suggested that changes in T cell clonality pattern (monoclonal to polyclonal) could be helpful to predict the therapeutic response of patients (18).

Many predisposing genetic conditions of HLH are characterized by impaired cytotoxicity of cytotoxic T or NK cells. Familial HLH 2, 3, 4, and 5 are caused by mutations in *PRF1, UNC13D, STX11,* and *STXBP2,* respectively (19–22). In patients with these mutations, cytotoxic cells demonstrate functional impairment in the degranulation process including cytotoxic granule docking, priming, and fusion with the plasma membrane, when encountering susceptible target cells infected with virus (11, 23). As a result, target cells are not eliminated by cytotoxic lymphocytes, and persistently activate immune cells, ultimately leading to a hyperinflammatory HLH (11). Among these mutations, *PRF1* mutation induces total deficiency of functional perforin, which results in defective cytotoxicity of cytotoxic T or NK cells (24). The pathogenetic mechanism of XLP-associated HLH is more complicated. Patients with XLP type 1 harbor mutations in *SH2D1A* (Xq25) encoding signaling lymphocyte activation molecule-associated protein (SAP). Defective SAP induces serious immunological complications including impaired 2B4-mediated cytotoxicity of T or NK cells against EBV-infected cells, vigorous expansion of CD8+ T cells by a failure of T cell reactivation-induced cell death, and defects in the development of NKT cells (25, 26). XLP type 2-induced HLH is pathogenetically different from other genetic HLH, because cytotoxic lymphocyte-mediated cytotoxicity is apparently normal in patients with XLP type 2, which is caused by mutations of *XIAP/BIRC4* (27, 28). Instead, defective expression of XIAP

increases a susceptibility of lymphocytes to apoptosis in response to CD95 and tumor necrosis factor receptor–related apoptosis-inducing ligand receptor stimulation, and induces defective NOD2 signaling with dysregulation of inflammasome function (27, 29, 30). Due to normal cytotoxicity, the development of HLH in these patients seems to have a less strong association with EBV, compared to patients with XLP type 1.

CHRONIC ACTIVE EBV INFECTION OF T- AND NK- CELL TYPE, SYSTEMIC FORM

CAEBV of systemic form is characterized by persistent clinical symptoms and signs including fever, hepatosplenomegaly, hepatitis, and lymphadenopathy after infectious mononucleosis (IM). Originally, when first described by Straus et al., the required duration of IM-like symptoms was more than 6 months to fulfill the criteria for CAEBV; however, the revised criteria require now only 3 months (3, 31, 32). The current diagnostic criteria are as follows: (1) IM-like symptoms persisting more than 3 months; (2) increased EBV DNA ($>10^{2.5}$ copies/mg) in PB, (3) histological evidence of organ disease; and (4) demonstration of EBV RNA or viral protein in affected tissues (3). In addition, CAEBV should be diagnosed in patients without known immunodeficiency, malignancy or autoimmune disorders. Most cases have been reported in East Asia including Japan, South Korea, China, and Taiwan (33–36). Few reports come from Latin America. It appears to occur rarely in Western and African populations (37). CAEBV arises predominantly in pediatric and adolescent patients. If it develops in adults, it shows a more aggressive clinical course (38). No sex predilection is present.

Clinical Features

The typical IM-like manifestations including persistent fever, hepatosplenomegaly and lymphadenopathy are present in about half of the patients. Some patients with CAEBV may have variable and non-specific symptoms according to the organs affected by EBV-induced inflammation, which often causes the diagnosis to be delayed or misdiagnosed. Other relatively common symptoms and signs are severe mosquito bite allergy (33%), skin rash (26%), HV-like eruptions (10%), diarrhea (6%), and uveitis (5%) (39). Pancytopenia and liver dysfunction are common. High titers of anti-VCA IgG and anti-early antigen IgG are found in almost all patients, and IgA antibodies against VCA and early antigen are frequently revealed. Increased copies of EBV DNA are also identified in all patients with CAEBV (39, 40).

The clinical course is variable. Some patients show an indolent clinical course and remain stable over a long period, while other patients progressively deteriorate with serious complications including hemophagocytic syndrome (24%), disseminated intravascular coagulation (16%), hepatic failure (15%), digestive tract ulcer/perforation (11%), coronary artery aneurysm (9%), CNS involvement (9%), interstitial pneumonia (5%), and myocarditis (7%) (4, 33, 39). The variability of clinical behavior generally depends on the viral load of EBV DNA and the host immunity. The prognosis is associated with the predominant cell type infected with EBV. Patients with CAEBV of T-cell type shows significantly poorer survival rate than those with CAEBV of NK-cell type (probability of 5-year survival, 0.59 in CAEBV of T-cell type vs. 0.87 in CAEBV of NK-cell type) (39). Furthermore, both groups tend to have different clinical presentations. Patients with T-cell infection are more likely to show severe systemic symptoms, high titers of EBV specific antibodies and aggressive clinical behavior, whereas those with NK-cell infection commonly have mild systemic symptoms, high concentrations of IgE, relatively lower levels of EBV-specific antibody and skin lesions including rash and hypersensitivity to mosquito bites, probably reflecting the more favorable clinical course of this type (4, 39, 41). However, CAEBV of NK-type (23.1%) might eventually evolve into aggressive NK-cell leukemia or extranodal NK/T cell lymphoma, nasal type (39).

Morphology and Immunophenotypical Findings

The biopsy of affected tissues in CAEBV is characterized by reactive inflammatory changes with no histological evidence of a malignant lymphoproliferation. Because microscopic findings are similar to nonspecific inflammation, pathological diagnosis can be very difficult and overlooked without careful attention to the clinical history. The detection of EBV infection in the infiltrated lymphoid cells using EBER ISH is necessary for tissue confirmation, and very useful especially in patients with no clinical suspicion of having CAEBV, due to nonspecific and unusual presentations. Histological findings of LN include follicular hyperplasia, paracortical hyperplasia, focal necrosis, and small epithelioid granulomas (3). A polymorphic infiltrate can be noted occasionally in interfollicular areas. Atrophy of the white pulp and congestion of the red pulp are seen in the spleen. In liver biopsies, infiltration of small lymphocytes is shown in portal areas or sinusoids like in viral hepatitis. The BM appears to be microscopically normal, except cases with accompanying HLH. According to the affected organs, CAEBV can mimic interstitial pneumonia in lung biopsies, myocarditis in heart biopsies and dermatitis in skin biopsies. EBV-infected cells show either a T-cell immunophenotype (59%) or a NK-cell immunophetype (41%), and rarely EBV-infection in both T and NK cells (3%). T-cells are mostly CD4+ (21%), with a minority being either CD8+ (8%), or γδ T-cell type (5%). Ill-defined T-cell phenotypes occur in 25% of the cases. CAEBV of B-cell type occurs rarely (2–3%) and mainly in Western population (42).

Pathogenesis and Molecular Features

The pathogenesis remains unknown. Although CAEBV develops in immunocompetent hosts by definition, some patients have impaired activity in EBV-specific cytotoxic T cells (43, 44). Strong racial differences in susceptibility to CAEBV suggest that its occurrence is influenced by genetic polymorphisms in host immune response-related genes (34, 45). The viral latency pattern of CAEBV indicates latency type 2, because EBV-infected T or NK cells express only a few EBV-related genes including EBNA1, LMP1 and LMP2A (41, 44). EBV is mononclonal in 84%, oligoclonal in 11%, and polyclonal in 5% of the reported cases (4). The rearrangement pattern of the TCR genes seems to be monoclonal in about 50 % of the cases (4).

Therefore, monoclonality of EBV-infected cells does not warrant a diagnosis of malignant lymphoma in EBV-associated T and NK-cell LPDs. Chromosomal abnormalities have been noted in a minority of cases (4). A clinicopathological classification of EBV-associated T and NK-cell LPDs has been proposed according to morphological evaluation and clonality results, which consists of category A1 (polymorphic, polyclonal LPD), category A2 (polymorphic, monoclonal LPD), category A3 (monomorphic, monoclonal LPD), and category B (monomorphic, monoclonal LPD with fulminant course) (46). Categories A1–A3 represents a continuous spectrum of CAEBV and its evolution to overt lymphoma. Category B corresponds to systemic EBV-positive T-cell lymphoma of childhood in the 2016 revised WHO classification (3).

HYDROA VACCINIFORME-LIKE LYMPHOPROLIFERATIVE DISEASE

HV-like LPD is one of the cutaneous forms of CAEBV. HV-like LPD is considered as a chronic EBV+ LPD that affects mainly children, but it carries a risk of progression to clinically overt malignant lymphoma. HV was initially described in Western populations as a benign photodermatosis that presents with recurrent self-limited vesiculopapular eruption, which progresses to crusts with rupture and heals with vacciniform scarring (47). Skin lesions mimicking classic HV were identified with some different features including no association with photosensitivity in children and young adolescents from Asia and Latin America (48–51). These lesions were shown to be associated with EBV infection and frequently contained monoclonal TCR gene rearrangements in subsequent studies (49, 50, 52). Reflecting these results, HV-like lymphoma was included in the 2008 WHO classification as a new type of EBV-positive cutaneous T-cell lymphoma of childhood. However, because this term does not represent the diverse clinical spectrum from classic self-limited HV to HV-like lymphoma, the term HV-like lymphoma was changed to HV-like LPD in the 2016 revised WHO classification (3).

Clinical Features

HV-like LPD shows variable clinical presentations and behaviors (4, 53, 54). HV-like LPD was previously classified into the classic type and severe type, representing the two extremes of the clinical presentation. Patients with the classic HV have light-induced vesiculopapules in sun-exposed skin area including the face and arm without systemic symptoms, whereas the severe form is characterized by the complicated vesicles that progress to large skin ulcers and occasionally leave severe scarring with disfigurement in sun-exposed and unexposed areas, with frequent systemic symptoms such as fever, lymphadenopathy, and hepatosplenomegaly (55). Some mild cases are cured after photoprotection, but the majority of cases usually shows a very indolent clinical course and has multiple recurrences and remissions that may finally develop into a more severe disease including EBV-positive T or NK-cell lymphoma of skin. A seasonal variation is present with increased occurrences of episodes in spring and summer.

Morphology and Immunophenotypical Findings

Intraepidermal spongiotic vesicles are characteristically formed by epidermal reticular degeneration. However, in some case only a very subtle lymphoid infiltration might be observed (**Figures 1A–F**). These lymphoid infiltrates are predominantly present in the dermis with the frequent extension to the subcutaneous tissue, and often exhibit an angiocentric and periadnexal involvement with the pattern of septal or lobular panniculitis. The infiltrating cells are usually bland-looking small lymphocytes with no or mild atypia, and highlighted by EBV positivity (**Figures 1A,B,E**). These cells are CD8+ cytotoxic T cells in the majority of cases (**Figures 1C,D,F**), followed by CD56+ NK cells (about 30%) (4, 56, 57). γδ T cells are found in a few cases in immunophenotyping of skin-infiltrating T cells, whereas flow cytometry of PB shows clonal expansion of γδ T cells in most cases (56, 58, 59). CD30 is frequently positive in the lymphoid infiltrates, and LMP1 is mostly negative (56).

Pathogenesis and Molecular Features

The pathogenesis is unknown. The geographic and racial distribution pattern suggests that genetic predisposition related to a defective immune response to EBV may contribute to the susceptibility to HV-like LPD, like in other EBV-associated T, and NK-cell LPDs. Monoclonal rearrangement of the TCR genes is found in almost all cases with T-cell immunophenotype (4, 56). EBV-infected cells can be detected by EBER ISH. The number of EBV-positive cells is variable among cases, and there are only a small number of EBV-positive cells in some cases. Pathological parameters including T cell clonality or the quantity of EBV-positive cells are generally not associated with the clinical behavior or related to the risk to develop a systemic lymphoma (3). In contrast to LMP1 negativity shown by immunohistochemistry in tissues, LMP1 expression is mostly identified by PCR in PB, indicating an EBV latency type 2 (60).

SEVERE MOSQUITO BITE ALLERGY

Severe mosquito bite allergy is another cutaneous form of CAEBV. Severe mosquito bite allergy is an EBV+ NK-cell LPD involving skin and characterized by an exaggerated allergic reaction to mosquito bites. Patients with severe mosquito bite allergy may be complicated by HLH, HV-like LPD, or systemic CAEBV in the prolonged clinical course. Furthermore, they have a higher risk of developing overt NK/T-cell lymphoma or aggressive NK-cell leukemia.

Severe mosquito bite allergy is a rare disease, of which most have been described in Japan with a few cases from other East Asia (4, 61–65). It mainly affects children and young adolescents aged 0–18 years (mean onset age, 6.7 years) (66). No sex predilection is reported.

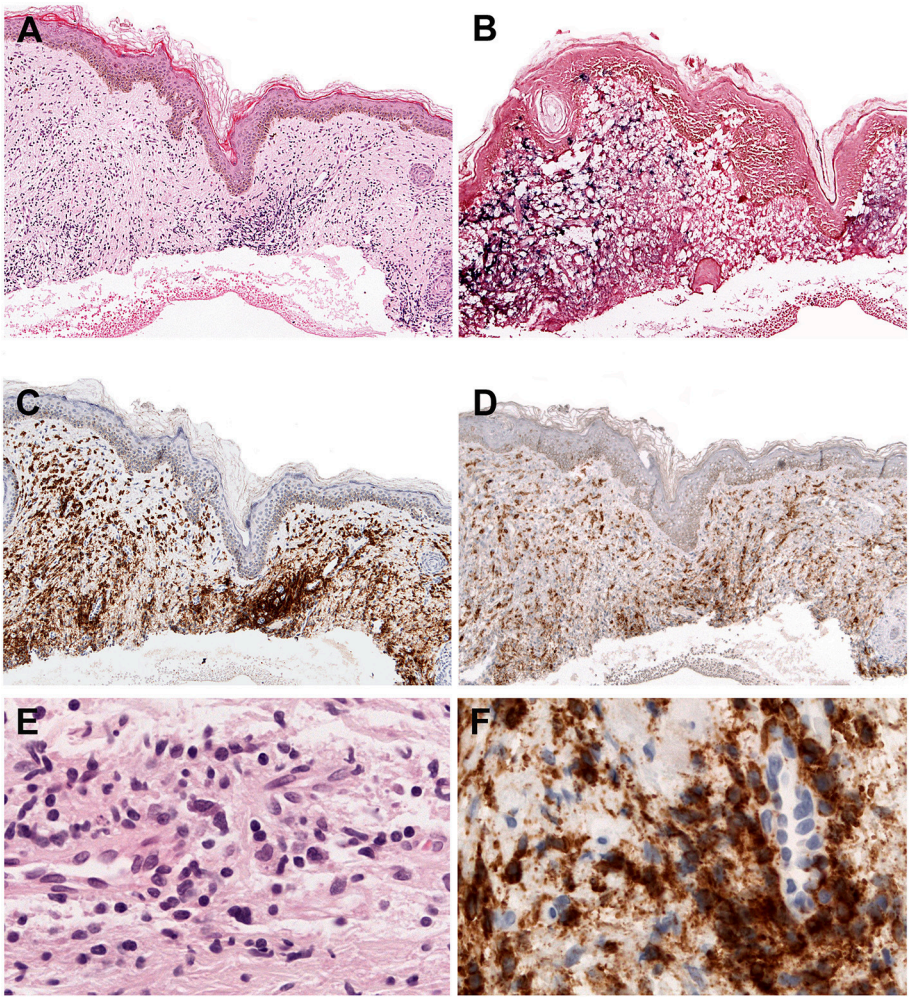

FIGURE 1 | Hydroa vacciniforme-like lymphoproliferative disorder. **(A)** A skin biopsy with a subtle dermal infiltrate surrounding adnexae and blood vessels (H&E, 25x); **(B)** The lymphoid cells are EBV positive, as demonstrated by *in situ* hybridization for EBV-encoded small RNA (EBER) (*in situ* hybridization, 25x); **(C)** CD8 is positive in the majority of the infiltrating cells (immunohistochemistry, 25x); **(D)** The infiltrating cells are negative for CD4. CD4 highlights the abundant histiocytes (immunohistochemistry, 25x); **(E)** The infiltrating cells are predominantly small, without atypia (H&E, 400x); **(F)** The infiltrating cells are surrounding a blood vessel highlighted by CD8 stain (immunohistochemistry, 400x).

Clinical Features

Hypersensitivity reactions after mosquito bites include localized cutaneous manifestations such as erythema, bullae, ulcers, and scar formation, and systemic findings including high fever, lymphadenopathy, liver abnormalities, and hepatosplenomegaly. Some patients experience similar hypersensitivity reactions at the injection site after vaccination (66). Most patients exhibit a high serum IgE level, high EBV load, and NK-cell lymphocytosis in the PB. After the hypersensitivity reaction resolves, the patients remain asymptomatic until the next mosquito bites.

Morphology and Immunophenotypical Findings

Epidermal necrosis, ulceration and bullae formation are present in the mosquito bite lesion. Skin biopsies from these lesions show a dense infiltrate of lymphoid cells that may extend into the subcutaneous tissue. The lymphoid infiltrate has a polymorphous composition consisting of small lymphocytes, large atypical cells, and other reactive inflammatory cells including histiocytes and eosinophils. The overall appearances are similar to HV-like LPD.

The infiltrating lymphoid cells are immunophenotypically CD3ε+, CD56+ NK cells with the expression of cytotoxic molecules TIA1 and granzyme B. Reactive CD4+ or CD8+ T cells are also present in the infiltrates. EBV-positive cells are often positive for CD30, and rarely positive for LMP1.

Pathogenesis and Molecular Features

The etiology remains unknown. Genetic predispositions and environmental factors may have an influence on the pathogenesis. Mosquito bites can induce the expression of LMP1 in NK cells through the proliferation of mosquito antigen-specific, CD4+ T cells, which are involved in the reactivation of

latent EBV in NK cells (67–69). EBV positivity is found only in a percentage of the infiltrating NK cells. EBV is monoclonal in almost all cases by EBV terminal repeat analysis (4). Like HV-like LPD, LMP1 expression is seen in PB by PCR, thereby, indicating type 2 EBV latency.

SYSTEMIC EBV-POSITIVE T-CELL LYMPHOMA OF CHILDHOOD

Systemic EBV-positive T-cell lymphoma of childhood is a rapidly progressive, fatal disease of children and young adults that is characterized by monoclonal expansion of EBV-positive T cells with an activated cytotoxic phenotype in tissues or PB. Historically, several different terms have been used to describe this disease, including fulminant EBV+ T-cell LPD of childhood, sporadic fatal IM, fulminant hemophagocytic syndrome in children, fatal EBV-associated hemophagocytic syndrome, and severe CAEBV. Systemic EBV-positive T-cell lymphoma is almost always accompanied by HLH, and shows a fulminant clinical course, rapidly progressing to multiple organ failure, sepsis, and finally death, within days to weeks. This disease was first incorporated as a LPD in the 2008 WHO classification; however, in the current 2016 revised WHO classification, it has been renamed as systemic EBV-positive T-cell lymphoma of childhood, reflecting its clinical severity. Systemic EBV-positive T-cell lymphoma of childhood occurs mainly in East Asia including Japan, Taiwan and China (33, 70–72). It also occurs in Latin America but it is rare in Western populations (73).

Clinical Features

Systemic EBV-positive T-cell lymphoma of childhood usually occurs shortly after acute primary EBV infection in previously healthy children or adolescents. Patients present with severe systemic findings such as fever, hepatosplenomegaly, pancytopenia, coagulopathy, and abnormal liver function, within days to weeks after IM symptoms due to primary EBV infection. Lymphadenopathy is occasionally seen. The patients are usually complicated by HLH, sepsis, and multiorgan dysfunction, ultimately leading to death within days to weeks.

In serological tests for EBV, anti-VCA IgM is often absent or barely detectable in the majority of patients, whereas IgG antibodies against VCA are positive. These abnormal results may be misleading and contribute to the delay in diagnosis, considering that they do not indicate acute or active EBV infection (74, 75).

Morphology and Immunophenotypical Findings

Systemic EBV-positive T-cell lymphoma of childhood is histologically characterized by increased infiltration of small lymphoid cells with histiocytic hyperplasia and striking hemophagocytosis in the BM, spleen, and liver. Tumor cells have no or minimal cytological atypia in most cases and may not be distinguishable from normal lymphocytes. However, some cases show atypical lymphoid infiltrates composed of pleomorphic medium to large-sized cells with frequent mitosis (72) (**Figure 2**). The liver reveals mild to marked infiltration of small lymphocytes in portal and sinusoidal area with cholestasis, steatosis, and focal necrosis (73). The spleen shows the depletion of white pulp with prominent sinusoidal and nodular lymphoid infiltrates. The LNs are usually unremarkable with preserved architecture. Early histological findings of lymph nodes include the depletion of B-cell areas and the expansion of paracortical/interfollicular areas that is infiltrated by a polymorphous population of lymphoid cells consisting of small to medium-sized lymphocytes and large atypical lymphocytes with irregular nuclei (**Figures 2A–F**). The LNs appear to be more depleted along the disease progression. The infiltrating lymphoid cells are mostly EBV+, CD8+ cytotoxic T cells that express CD2, CD3, TIA-1, and granzyme B, and lack CD56 (71, 73). In contrast, cases associated with CAEBV show CD4+ immunophenotype (73). Rare cases exhibit EBV-positive tumor cells with co- expression of CD4 and CD8 (73). LMP1 is usually negative by immunohistochemistry. EBNA2 is always negative. The confirmation of EBV infection by EBV ISH is very useful for diagnosis.

Pathogenesis and Molecular Features

The etiology of systemic EBV-positive T-cell lymphoma of childhood is unknown. However, its association with primary EBV infection and strong racial predisposition suggest a genetic defect in the host immune response to EBV. The infiltrating T cells show monoclonal rearrangements of the TCR genes. EBV is present in a clonal episomal form in all cases (33, 70, 72, 73, 76). Because systemic T-cell lymphoma of childhood have some clinicopathological characteristics overlapping with EBV-associated HLH, the distinction of both diseases is sometimes difficult. In a literature review of systemic T-cell lymphoma and EBV-associated HLH, the patients with chromosomal aberrations are 100% fatal, whereas cases with evidence for T-cell clonality are fatal in 62% (77). Therefore, karyotypic abnormalities can be more helpful to distinguish systemic T-cell lymphoma of childhood from EBV-associated HLH in ambiguous cases, compared to T cell clonality, which is demonstrated in about half of EBV-associated HLH cases.

AGGRESSIVE NK-CELL LEUKEMIA

Aggressive NK-cell leukemia is a very rare, fatal disease characterized by systemic neoplastic proliferations of NK cells in PB and BM and a strong association with EBV. However, leukemic NK cells are variably present, and sometimes can be sparse in the PB and BM. It was originally designated as aggressive NK-cell leukemia/lymphoma to emphasize the variable clinical manifestations, because there are some cases without leukemic phase, presenting with hepatosplenomegaly and peripheral lymphadenopathy (78, 79). To permit a clear distinction, and avoid confusion between this disease and extranodal NK/T-cell lymphoma, nasal type, the term aggressive NK-cell leukemia has been chosen in the WHO classification. Extranodal NK/T-cell lymphoma with systemic involvement at multiple sites has some clinicopathological features similar to those of aggressive NK-cell leukemia, and the distinction between the two diseases may be difficult. Aggressive NK-cell leukemia has been reported mainly in Asians (80). It occurs mostly in young to middle-aged adults with no definite sex predilection.

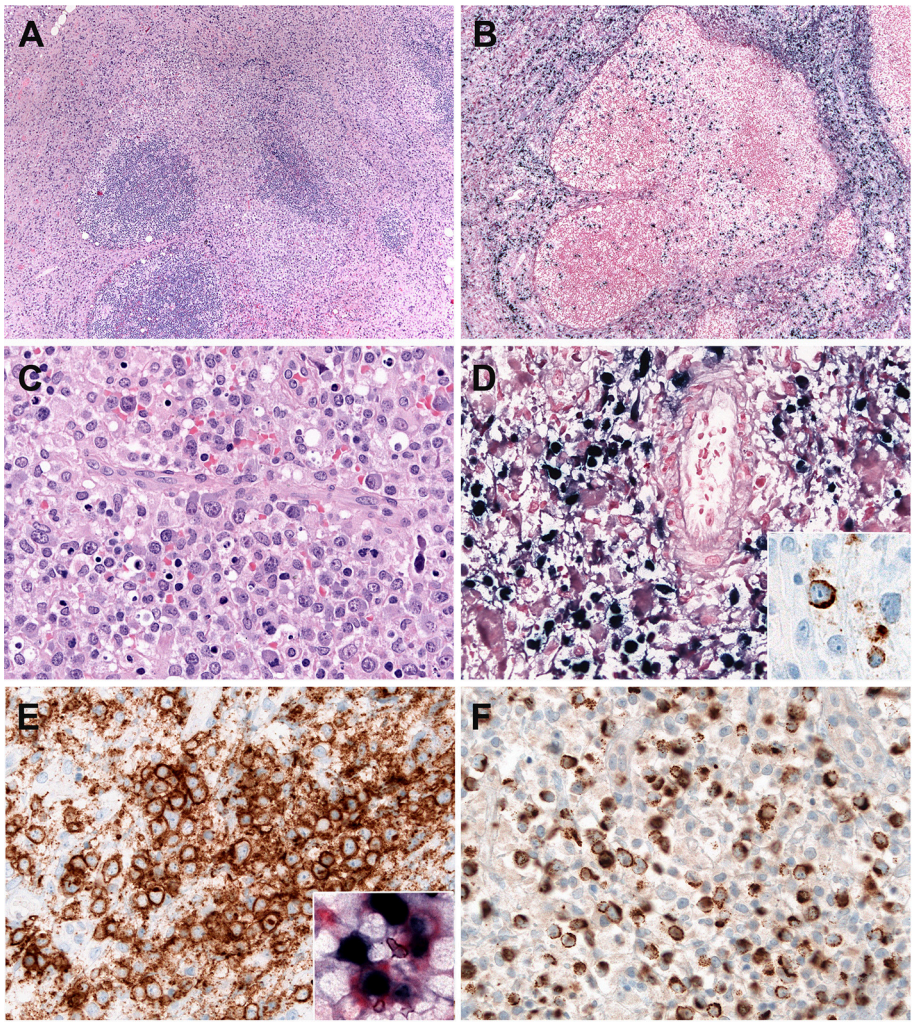

FIGURE 2 | Systemic Epstein-Bar virus (EBV)-positive T-cell lymphoma of childhood. **(A)** Lymph node with partial preservation of the architecture with depleted germinal centers and expansion of the interfollicular area (H&E, 50×); **(B)** Many of the lymphoid cells in the interfollicular area are EBV-positive, as demonstrated by *in situ* hybridization for EBV-encoded small RNA (EBER) (*in-situ* hybridization 100×); **(C)** The neoplastic cells are mostly medium to large-sized cells with irregular nuclei. Note the presence of apoptosis (H&E, 400×); **(D)** Many cells are EBER positive (*in-situ* hybridization, 400×). LMP1 is positive indicating an EBV latency type 2 (immunohistochemistry, insert, 400x); **(E)** The neoplastic cells are CD8 positive (immunohistochemistry, 400x). Double stainings show that the CD8-positive cells (red) are EBER-positive (Black) (Immunohistochemistry and in situ hybridization, insert, 400x) **(F)** TIA1 is positive in the infiltrating cells (immunostaining, 400×).

Clinical Features

Clinical presentations include high fever, general malaise, hepatosplenomegaly, hepatic failure, and pancytopenia. Variable numbers of leukemic NK cells are present in the PB, ranging from <5% to >80% of all leukocytes (75). Laboratory tests show high levels of serum lactate dehydrogenase (LDH) levels and circulating FAS ligand (FASL) (81). Lymphadenopathy is occasionally seen, and skin lesions are uncommon. This disease is frequently complicated by HLH and coagulopathy, and shows a fulminant clinical course with multiple organ failure (75). The overall prognosis is very poor with a median survival <2 months (82). Some cases develop in the setting of CAEBV infection of NK cell type, or evolve from extranodal NK/T-cell lymphoma or chronic LPD of NK cells (4, 33, 83–86). Aggressive NK-cell leukemia shares some clinicopathological features with systemic EBV-positive T-cell lymphoma of childhood, but the immunophenotype of the neoplastic cells is basically different between these two disease entities (CD56+ NK cells vs. CD56- T cells).

Morphology and Immunophenotypical Findings

Leukemic NK cells have a broad cytological spectrum ranging from normal large granular lymphocytes to atypical lymphocytes showing nuclear enlargement, irregular nuclear contours, open chromatin, or conspicuous nucleoli. The cytoplasm is pale or lightly basophilic, and relatively abundant with fine or coarse azurophilic granules. In the BM biopsy, the extent of neoplastic NK cells varies from extensive to focal, subtle infiltration. Some cases show minimal involvement of the

BM, indistinguishable from normal BM in conventional H&E stain. Increased infiltration of histiocytes is observed with hemophagocytosis in the BM. The liver, spleen, and LNs show varying degrees of tumor cell infiltration, displaying massive, patchy, or subtle involvement, like the BM (**Figures 3A–E**). The immunophenotype of the tumor cells is that of a mature CD56+ NK-cell with positivity for CD2, CD3ε, and cytotoxic granules TIA1 and granzyme B, and negative for surface CD3 and CD5. Aggressive NK-cell leukemia frequently expresses CD16 (in 75%), which is different from CD16-negative extranodal NK/T-cell lymphoma (82). The neoplastic cells express FASL, but usually lack CD57. The early diagnosis of aggressive NK-cell leukemia can be difficult due to unusual pathological findings including lack of lymphocytosis in PB, interstitial infiltration pattern in BM, rarely EBER negativity and aberrant immunophenotype such as CD3 negativity by immunohistochemistry (87).

Pathogenesis and Molecular Features

Although the etiology remains unknown, the strong association with EBV has been suggested to be central in pathogenesis. EBV infection has been reported in 85–100% of cases (88–90). EBV exists in clonal episomal form. However, some cases of EBV-negative aggressive NK-cell leukemia have been described, and show the clinicopathological features similar to those of EBV-positive cases, except for the fact that EBV-negative cases tend to occur in older patients with no obvious racial predilection (87, 91). Some of the EBV-negative cases may evolve from chronic LPD of NK cells (82, 91, 92). The genetic comparison of aggressive NK-cell leukemia and extranodal NK/T-cell lymphoma show some significant differences. Aggressive NK-cell leukemia shows gains of 1q23.1–q24.2 and 1q31.3–q44 and losses of 7p15.1–p22.3 and 17p13.1 more frequently than extranodal NK/T-cell lymphoma (93). A previous study reported that EBV-negative aggressive NK-cell leukemia was negative for JAK-STAT pathway-associated gene mutations, known as recurrently mutated in extranodal NK/T-cell lymphoma, suggesting different molecular pathogenesis between these two diseases (87). However, because the mutations of the JAK-STAT pathway-associated genes have been also reported in EBV-positive aggressive NK-cell leukemia (94), further investigations are warranted to reveal the mutational landscape of the NK-cell malignancies.

EXTRANODAL NK/T-CELL LYMPHOMA, NASAL TYPE

Extranodal NK/T-cell lymphoma, nasal type, is an EBV-positive aggressive lymphoma characterized by prominent necrosis,

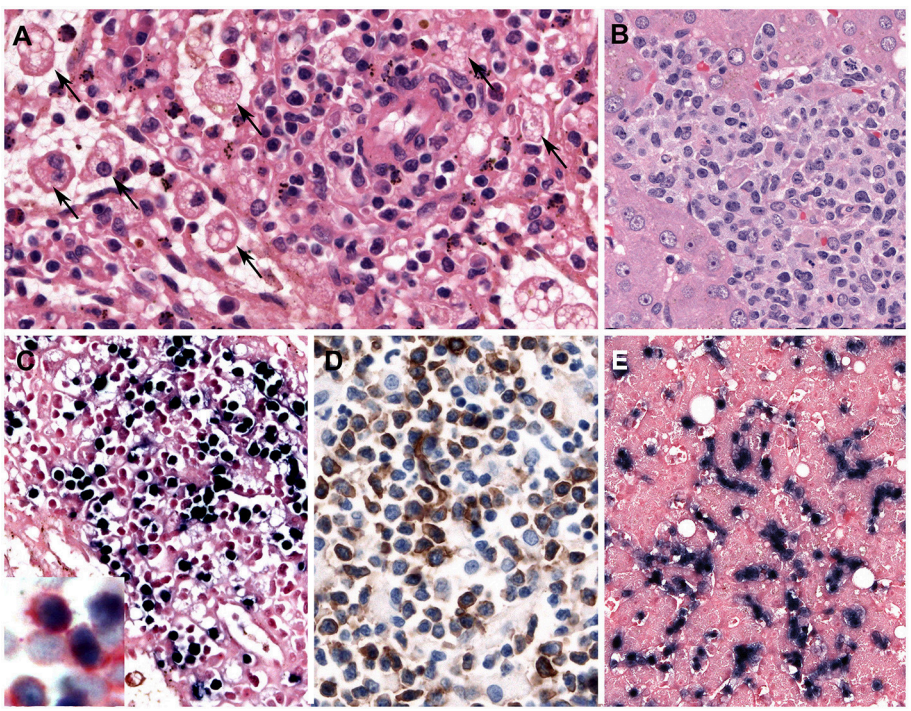

FIGURE 3 | Aggressive NK-cell leukemia. **(A)** The spleen shows a scant atypical lymphoid infiltrate of small cells with bland cytology surrounding blood vessels. Note the striking erythrophagocytosis (arrows) (H&E, 400×); **(B)** The liver shows an atypical infiltrate in the sinusoids composed of medium-sized cells with irregular nuclei and pale cytoplasm; **(C)** Neoplastic cells in the spleen are stained positively with EBER (*in-situ* hybridization 400×). Insert shows double staining of CD56 (red) and EBER (black) demonstrating that the NK cells are infected by EBV (Immunohistochemistry and *in situ* hybridization 400x); and **(D)** The infiltrating cells are CD56 positive (immunohistochemistry 400×); **(E)** The neoplastic cells in the liver are EBER positive. Note the intrasinusoidal infiltration characteristic of the disease (*in-situ* hybridization, 400×).

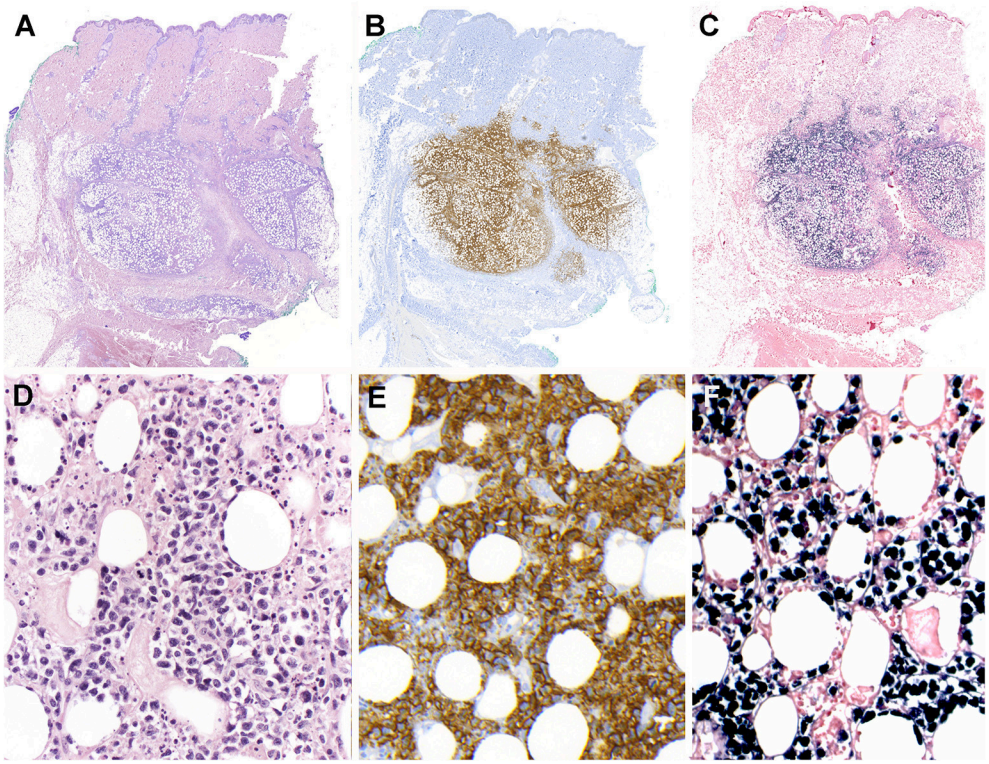

FIGURE 4 | Extranodal, NK/T-cell lymphoma, nasal type in the skin. **(A)** Panoramic view of a skin biopsy shows a partially circumscribed nodule located in the subcutaneous tissue (H&E, scanned slide); **(B)** The tumor cells are CD56 positive (immunohistochemistry, scanned slide). **(C)** The lymphoid cells are positive for EBV-encoded small RNA *in situ* hybridization (EBER) (*in situ* hybridization) **(D)** The infiltrate is composed of large atypical cells with irregular nuclei. The tumor cells surround the adipocytes revealing a "lace-like pattern" mimicking panniculitis-like T-cell lymphoma. Numerous apoptotic bodies are observed (H&E, 400×); **(E,F)** Higher magnification demonstrates that the neoplastic cells are positive for CD56 and EBER, (immunohistochemistry and *in situ* hybridization 400×).

angioinvasion, and cytotoxic phenotype. It is derived from NK cells and uncommonly cytotoxic T cells. Extranodal NK/T-cell lymphoma was called "lethal midline granuloma" in the past, because of the destruction of midline facial structures due to vascular damage and subsequent ischemic necrosis. Extranodal NK/T-cell lymphoma, nasal type, occurs commonly in East Asians and the Native Americans in Central and South America, but rarely in Western populations (95). It accounts for ∼6–8% of all lymphomas in East Asia and some Latin American countries, but <1% in Western populations (96–100). It affects males more commonly than females. It occurs frequently in middle-aged adults.

Clinical Features

Extranodal NK/T-cell lymphoma arises from extranodal sites in almost all cases, most commonly involving upper aerodigestive tract (UAT) including nasal cavity, paranasal sinuses, nasopharynx, oropharynx, oral cavity, and palates. It also occurs in non-UAT sites including skin, soft tissue, gastrointestinal tract, testis, lung, and CNS. Nasal NK/T-cell lymphoma is defined as a primary tumor involving the nasal and nasopharyngeal region, regardless of dissemination to other sites. Patients with nasal NK/T-cell lymphoma present initially with nonspecific localized symptoms including nasal obstruction, purulent nasal discharge, and epistaxis. In later stages, nasal NK/T-cell lymphoma can extend to adjacent UAT tissues, or cause extensive necrotic lesions in the midline facial area. The BM is infrequently involved. Some patients may be complicated by HLH and extranasal dissemination to various non-UAT sites. The prognosis of nasal NK/T-cell lymphoma has been reported to be poor with the overall survival rate of 30–40%, but has recently improved with the introduction of new chemotherapy regimens such as L-asparaginase-based chemotherapy (95, 101). Extranasal NK/T-cell lymphoma indicates a primary tumor in a non-UAT site at first clinical presentation, and occurs in ∼20–30% of cases (**Figure 4**) (100, 102). These lymphomas usually present with advanced stage at diagnosis, multiple sites of involvement, elevated LDH levels, and poor performance status. They are often refractory to treatment and show an inferior prognosis compared to nasal NK/T-cell lymphoma (101). Skin lesions commonly present as nodular ulcerative lesions. Gastrointestinal involvement frequently results in ulcer, bleeding, or perforation. Lymph nodes can be secondarily involved by dissemination. As some extranasal cases may harbor occult nasal lymphoma, it is important to inspect the UAT regions carefully.

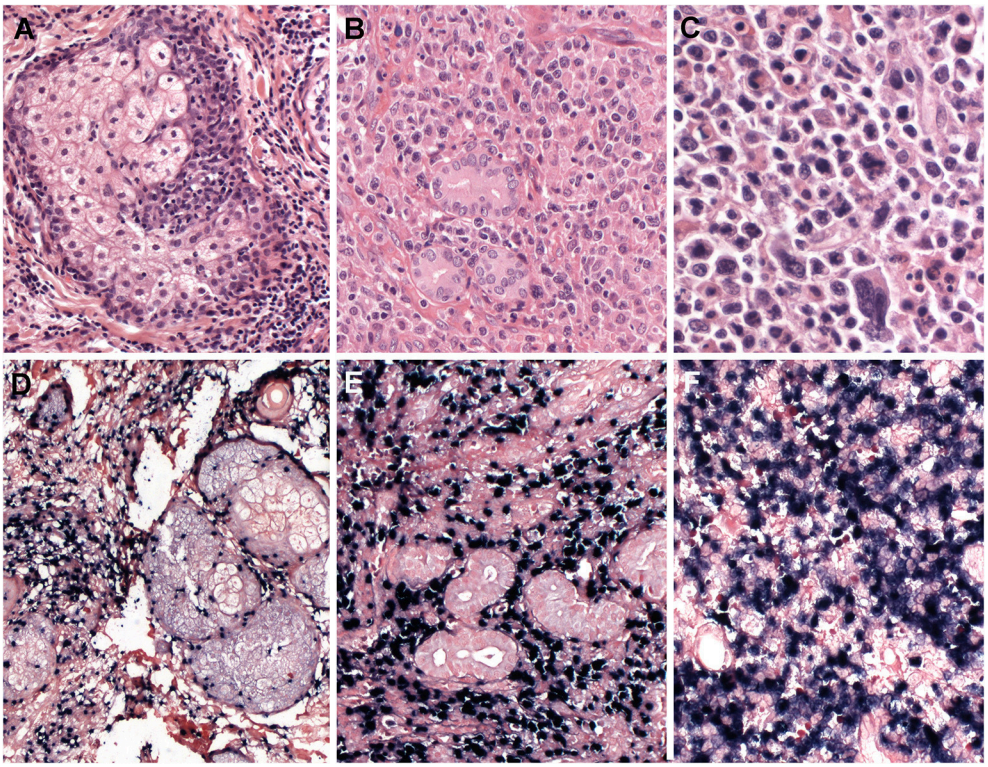

FIGURE 5 | Extranodal, NK/T-cell lymphoma, nasal type. **(A–C)** Nasal biopsies displaying the morphological spectrum of ENKTCL. **(A)** Infiltrate of small lymphoid cells with bland cytology surrounding the sebaceous gland, mimicking a reactive lesion (H&E, 400×);**(B)** Dense lymphoid infiltrate of intermediate-sized cells, showing nuclear irregularity and pale to clear cytoplasm (H&E, 400×); **(C)** Atypical cell infiltrate, of pleomorphic large cells admixed with intermediate-sized cells (H&E, 400×); **(D–F)** Lymphoma cells are positive for EBV-encoded small RNA *in situ* hybridization (EBER, 400×).

Morphology and Immunophenotypical Findings

Involvement of mucosal sites frequently presents with extensive ulceration. Histological findings show diffuse infiltration of atypical lymphoid cells with angiocentricity and angiodestruction, leading to vascular obstruction and the consequent ischemic, coagulative necrosis. Involvement of non-mucosal sites also shows similar morphological changes. Tumor cells have a broad cytological spectrum, ranging from bland-looking small lymphocytes to large pleomorphic cells (**Figures 4A–F**). Most cases show a relatively monotonous population of medium-sized cells or a polymorphous pattern composed of small and large cells. Variable amounts of reactive inflammatory cells are admixed with tumor cells, mimicking an inflammatory lesion. Tumor cells have often irregularly folded nuclei with indistinct nucleoli and moderate amount of cytoplasm. Mitotic figures are easily identified. The most common immunophenotype is CD3ε+, CD56+, CD2+ and cytotoxic molecules (granzyme B, perforin and TIA1), but lacks surface CD3, CD4 and CD5 (**Figures 4**, **5**). Tumor cells often express CD25, FAS, FASL, and HLA-DR. CD30 expression is identified in about 30–40% of cases (103–106). The minority of cases shows a CD3+, CD56- cytotoxic T-cell phenotype, expressing CD8, CD5,TCR (γδ or αβ type), and cytotoxic molecules. These cases account for 15–20% of all cases and represent a real T-cell phenotype of the tumor cells (104, 107). There are no significant differences in the clinicopathological features between CD56+ and CD56- cases (88). EBV infection should be confirmed in virtually all cases to render a diagnosis of extranodal NK/T-cell lymphoma. Therefore, when CD3ε+, CD56+ nasal lymphomas do not show EBV positivity, other types of T-cell lymphoma should be considered in the diagnosis. Because of a strong association with EBV, the immune microenvironment has some prognostic implication. High quantity of tumor-infiltrating FOXP3+ regulatory T cells or PD-L1 expression on tumor cells independently predict better prognosis, suggesting that inhibitory immunomodulation might suppress lymphoma cells, as well as immune cells related with antitumor immune response (108, 109).

Pathogenesis and Molecular Features

EBV has a pathogenetic role in the development of extranodal NK/T-cell lymphoma, nasal type. EBV exists in a clonal episomal form with EBV type 2 latency. Most patients are infected with EBV subtype A with some geographic variations (110–112). EBV has frequently a 30-base pair deletion in LMP1 gene, which may contribute to lymphomagenesis through

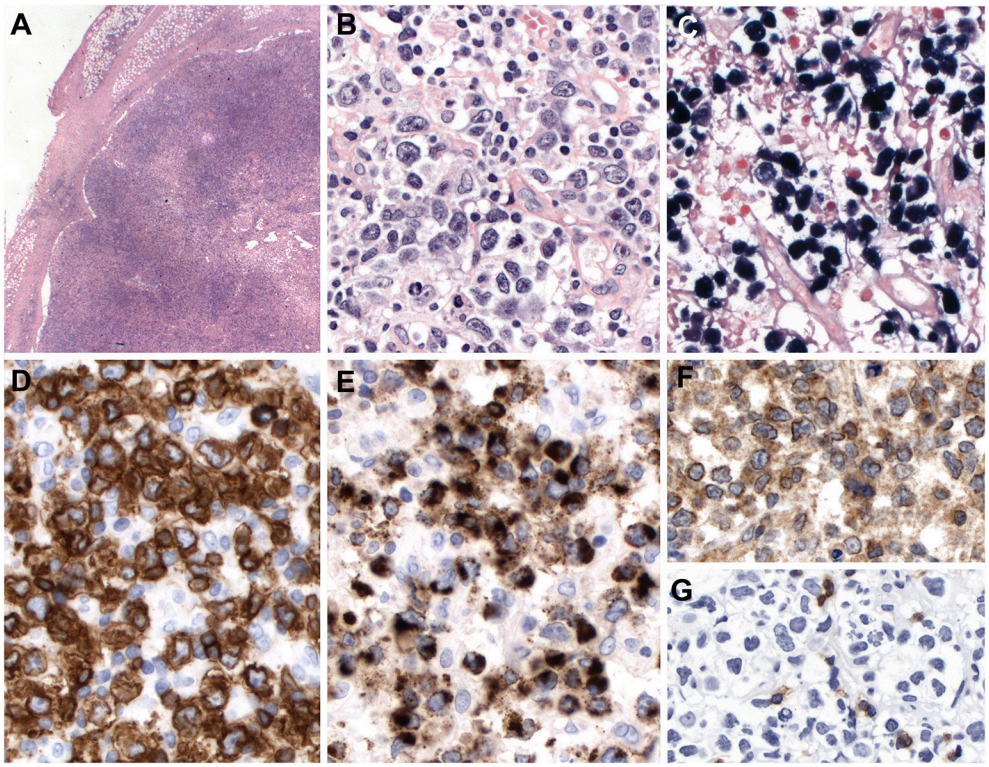

FIGURE 6 | Primary EBV-positive nodal T and NK-cell lymphoma. **(A)** Lymph node with complete effacement of the architecture by a diffuse infiltrate that extends beyond the capsule and infiltrate the perinodal fat (H&E, 12,5×); **(B)** Neoplastic cells are large, pleomorphic with irregular nuclei and clear or pale cytoplasm (HE,400×); **(C–E)** The neoplastic cells EBER, CD56 and TIA-1 positive (*in-situ* hybridization and immunohistochemistry, 400×); **(F)** TCR-gamma immunostain demonstrates the gamma-delta derivation of the tumor cells (immunohistochemistry, 400×); **(G)** TCR alpha-beta (BetaF1) is negative in the tumor cells but positive in the reactive T cells (immunohistochemistry, 400×).

decrease in immune recognition (113–115). The quantity of circulating EBV DNA reflects the tumor load and activity, because EBV DNA is released into the blood from apoptotic tumor cells. Elevated EBV DNA copies are correlated to adverse clinical parameters, poor response to treatment and inferior clinical outcomes (116, 117). Most cases show a germline configuration of TCR genes. Monoclonal rearrangements of the TCR genes are found in 10–40% of cases, which may be derived from cytotoxic T cells (100, 107, 118). In the gene expression profiling, extranodal NK/T-cell lymphomas cluster together, regardless of NK cell or γδ T-cell phenotype, and show patterns similar to non-hepatosplenic γδ T-cell lymphomas (119). The most frequent chromosomal aberrations in extranodal NK/T-cell lymphoma are deletion of chromosome 6q at q21–23 region, which contains some tumor suppressor gene such as *HACE1*, *PRMD1*, *FOXO3*, and *PTPRK* (120–122). This deletion is also commonly found in aggressive NK-cell leukemia, suggesting a genetic link between these two diseases. However, it is unknown whether this genetic alteration develops as primary pathogenetic event or secondary progression-related event (123, 124). Mutation analyses have shown that activating mutations of *JAK3* (5–35%), *STAT3* (6–27%) and *STAT5B* (2–6%) are commonly found, suggesting that the *JAK-STAT* pathway can be a therapeutic target (125–127). Other mutations include the RNA helicase *DDX3X*, the tumor suppressor gene *TP53*, the transcription corepressor BCOR, and genes involved in epigenetic pathways (*MLL2*, *ASXL3*, *ARID1A*, and *EP300*) (127, 128).

PRIMARY EBV-POSITIVE NODAL T AND NK-CELL LYMPHOMA

Primary EBV-positive nodal T and NK-cell lymphoma is a rare type of peripheral T-cell lymphoma that primarily involves the LNs without nasal or other extranodal site involvement. It has been included in the current 2016 WHO classification as a new provisional group within peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS). It affects mainly elderly patients with a median age of 61 years, showing an older age distribution than extranodal NK/T-cell lymphoma (129). There is a male predilection (129, 130).

Clinical Features

Most patients present with generalized lymphadenopathy. Extranodal involvement may be present in a limited number of sites, but the nasal cavity and adjacent structures should not be involved by definition. This disease shows an aggressive clinical course with adverse clinical features including advanced stage,

systemic symptoms, and high International Prognostic Index scores. The prognosis is very poor with a median survival <4 months (129, 130).

Morphology and Immunophenotypical Findings

Most cases show relatively monomorphic proliferation of large atypical cells with centroblastic features or diffuse proliferation of pleomorphic cells composed of small, medium, to large atypical cells mimicking Hodgkin cells and Reed-Sternberg cells (**Figure 6**). Necrosis is occasionally seen with granulomatous or epithelioid histiocytic reactions. Angiodestructive pattern is rare. The immunophenotype of the tumor cells is mostly that of a cytotoxic T cell with expression of CD3, CD8, and cytotoxic molecules. Expression of CD56 (7.5–15%) or CD4 (15–20%) is rarely observed (129, 130). The majority of cases are of αβ T cell phenotype (46–64%), followed by TCR-silent T cells (negative for both TCRβF1 and TCRγ; 21–26%) and other T cells (130). Cases with NK-cell phenotype are found in 6.6–15%. CD30 expression is frequently expressed in TCR-silent cases, which raises the differential diagnosis with anaplastic large cell lymphoma (ALCL).

Pathogenesis and Molecular Features

Monoclonal rearrangements of the TCR genes are found in most cases. EBV infection is diffusely detected with high density by EBER ISH, and LMP1 expression indicates EBV latency type 2. In a recent gene expression profiling and cytogenetic analysis, primary EBV-positive nodal T/NK-cell lymphoma showed a distinct molecular signature characterized by upregulation of PD-L1 and T-cell-related genes, including CD2 and CD8, and downregulation of CD56, compared to extranodal NK/T-cell lymphoma (131). A cytogenetic deletion frequently found is loss of chromosome 14q11.2 indicating loss of TCR loci as evidence for the T-cell origin of this lymphoma (131).

CONCLUSION

EBV-associated T and NK-cell LPDs are a group of diseases including reactive LPDs, as well as overt hematolymphoid malignancies. Some categories have an indolent clinical course with pathological features mimicking a reactive disorder delaying the appropriate diagnosis and treatment. It is crucial to have good clinical information to render the correct diagnosis since some of these disorders have overlapping morphological features. It is recommended to perform EBER ISH in biopsies from extranodal sites with "atypical" infiltrations of T and NK cells regardless of the severity of the infiltration to confirm the presence of EBV infection. EBV+ T and NK cell LPDs frequently express CD30, which might be misleading raising the diagnosis of ALK- ALCL. New genetic studies suggest that the new provisional group of primary nodal T and NK cell lymphomas might be a distinct entity among the EBV+ LPDs.

AUTHOR CONTRIBUTIONS

WYK performed the literature review and wrote the manuscript. IM-M prepared all photographs and images. FF helped writing the manuscript. LQ-M supervised the work and helped writing the manuscript.

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REVIEW
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Advances in the Study of Chronic Active Epstein-Barr Virus Infection: Clinical Features Under the 2016 WHO Classification and Mechanisms of Development

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Chronic active Epstein-Barr virus infection (CAEBV) is one of the Epstein-Barr virus (EBV)-positive T- or NK-lymphoproliferative diseases. It is considered rare and geographically limited to Japan and East Asia. However, CAEBV is drawing international attention, and the number of case reported worldwide is increasing, after its classification in the EBV-positive T- or NK-cell neoplasms, in the 2016 WHO classification. In this article, I review current advances in the study of CAEBV under the new definition and show future directions. In CAEBV, EBV-infected T or NK cells clonally proliferate and infiltrate multiple organs, leading to their failure. These characteristics define CAEBV as a lymphoid neoplasm. However, the main symptom of CAEBV is inflammation. Recently, the mechanisms underlying the development of CAEBV have gradually become clearer. EBV infection of T or NK cells can occur during the acute phase of primary infection with a high EBV load in the peripheral blood. In addition, it was reported that cytotoxic T cells decreased in numbers or showed dysfunction in CAEBV. These findings suggest that undetermined immunosuppressive disorders may underlie persistent infection of T or NK cells. Furthermore, EBV itself contributes to the survival of host cells. *In vitro* EBV infection of T cells induced intercellular survival-promoting pathways. Constitutive activation of NF-kB and STAT3 was observed in EBV-positive T or NK cells in CAEBV, promoting not only cell survival but also CAEBV development. During the disease course, CAEBV can lead to two lethal conditions: hemophagocytic lymphohistiocytosis and chemotherapy-resistant lymphoma. It is necessary to start treatment before these conditions develop. At present, the only effective treatment strategy for eradicating EBV-infected T or NK cells is allogeneic stem cell transplantation (allo-HSCT). However, patients with an active disease, in which the condition is accompanied by fever, liver dysfunction, progressive skin lesions, vasculitis, or uveitis, had worse outcomes after allo-HSCT, than patients with an inactive disease had. Unfortunately, current chemotherapies are insufficient to improve the activity of CAEBV. Based on the molecular mechanisms for the development of the disease, the NF-kB, or JAK/STAT mediating pathways are attractive candidate targets for new treatments.

Keywords: epstein-barr virus, chronic active EBV infection, NF-κB, STAT3, T-or NK-lymphoproliferative disease, inflammation, hematopoietic stem cell transplantation, lymphoma

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INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous double-stranded DNA virus, categorized under the human herpes virus family. The virus was discovered in 1964 in cells affected by endemic Burkitt lymphoma (1). Once EBV infects human beings, it cannot be eradicated and latently infects B cells throughout the lifespan. EBV immortalizes infected B cells and can be a cause of B-cell neoplasms under immunocompromised conditions, which can promote the proliferation of EBV-infected B cells. The vast majority of immunodeficiency-associated lymphoproliferative disorders are classified into these categories.

B cells are not the only targets of EBV. Epithelial cells may also test positive for the EBV genome, leading to the development of nasopharyngeal cell carcinoma. Furthermore, the EBV genome is also positive in T- or NK-lymphoid neoplasms. Chronic active EBV infection is one of the EBV-positive T- or NK-lymphoproliferative diseases (EBV-T/NK-LPDs). It was originally reported in Western countries but has been primarily reported and studied in Japan and neighboring countries. In 2016, CAEBV was classified under EBV-positive T- or NK-cell neoplasms in the revised WHO classification of tumors of hematopoietic and lymphoid tissues (2). Since then, CAEBV has been drawing international attention, and the number of case reports on the topic is increasing worldwide. Although CAEBV can be lethal, some patients have recently achieved long-term survival by allogeneic hematopoietic stem cell transplantation (allo-HSCT). Furthermore, the mechanisms by which EBV infects T or NK cells in the small number of patients who develop CAEBV are currently being clarified. In this review, I describe the current status of CAEBV based on its new definition, focusing on the mechanisms underlying its development, the diagnostic and therapeutic procedures for the disease and future directions.

HISTORY OF CAEBV AND ITS RELATED DISORDERS

To the best of my knowledge, the first report of suspected CAEBV was in the U.S. in 1948 (3), documenting 53 cases with fever and splenomegaly "from 3 months to longer than 4 years after the initial attack" as chronic infectious mononucleosis (IM). Three of the patients developed "lymphoblastoma." Subsequently, other researchers reported similar cases (4). They considered the disease to be sustained IM and named it CAEBV. However, this disease was not equivalent to sustained IM. In 1988, Jones and colleagues reported that EBV-infected and clonally proliferating T cells were detected in CAEBV (5). Similar reports followed, mainly from Japan and East Asia. These reports indicated that EBV-positive NK cells were also detected in CAEBV. After the 1980s, it was confirmed that CAEBV was a progressive disease and that EBV-infected cells infiltrate multiple organs, leading to their dysfunction. Furthermore, conditions with characteristic skin lesions, such as severe mosquito bite allergy (sMBA) or hydroa vacciniforme (HV), have EBV-infected T or NK cells and show disease courses similar to that of CAEBV. In 2005, Okano et.al suggested the first diagnostic guidelines for CAEBV: persistent and recurrent IM-like symptoms; an unusual pattern of anti-EBV antibodies, with raised levels of anti-VCA and anti-EA; detection of increased EBV genomes in affected tissues, including the peripheral blood (PB); and chronic illness that cannot be explained by other known disease processes at diagnosis (6). It also mentioned that hemophagocytic lymphohistiocytosis (HLH) or LPD/lymphoma originated from the T- or NK-cell lineage, often developed during the disease course. It should be noted that the guidelines clearly defined CAEBV as a disease distinct from known immunosuppressive conditions. Three years later, Ohshima and colleagues reported that CAEBV patients showed clonal evolution of the infected cells and eventually developed T- or NK-cell lymphoma or leukemia from the viewpoint of pathologist (7). They suggested multistage lymphomagenesis of EBV-T/NK-LPDs. Subsequently, the WHO classification of tumors of hematopoietic and lymphoid tissues, which was revised in 2008, first described CAEBV as a systemic EBV-T-LPD of childhood (8). This was a major event that identified CAEBV as a neoplastic disorder. However, the classification had some issues. First, CAEBV with an EBV-infected NK-cell type was not described. Second, the term childhood reminds us that the disease is a pediatric disorder. Kimura and colleagues performed a prospective assay of 108 patients with EBV-T/NK-LPDs, which was accompanied by sustained inflammation with the EBV infection of T or NK cells (9). They categorized these disorders into 4 subtypes: CAEBV, sMBA, HV-like lymphoproliferative disorder (HV-LPD), and EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH). In the report, CAEBV was described as a disease harboring systemic inflammation, and sMBA and HV-LPD were defined as diseases with lesions limited to the skin. According to these studies, the new WHO classification revised in 2016 defined CAEBV as an EBV-T/NK-LPD (2). In Japan, the research group, Measures against Intractable Diseases by the Ministry of Health, Labor and Welfare of Japan, suggested that the diagnostic criteria for CAEBV with EBV infection of T- or NK cells be added to Okano's guidelines (**Table 1**). The criteria matched the new WHO classification.

Cohen and colleagues, an American research group, reported 19 patients with CAEBV in the U.S (10). The types and frequencies of EBV-infected cells were as follows: T or NK cells, 4, B cells 11, T cells 3, and NK cells 1. Thus, the majority had B-cell type. Interestingly, most patients with B-cell type also had hypogammaglobulinemia prior to B-cell suppressing therapy, such as rituximab or cytotoxic reagents, whereas T- or NK-cell type patients did not. Thus, B-cell type CAEBV may be a different disorder from T- or NK-cell type CAEBV.

EPIDEMIOLOGY OF CAEBV

The rate of the onset of CAEBV in Japan was 23.8/year, according to the annual report of the research group of Measures against Intractable Diseases by the Ministry of Health, Labor and Welfare of Japan. CAEBV had been considered a childhood disease, as mentioned previously. However, as shown in **Figure 1**, the Japanese nationwide CAEBV survey showed that more than

TABLE 1 | Diagnostic criteria of Chronic active Epstein-Barr virus infection.

(1) Sustained or recurrent IM-like symptoms persist for more than 3 months
(2) Elevated EBV genome load in the peripheral blood (PB) or the tissue lesion
(3) EBV infection of T or NK cells in the affected tissues or the PB
(4) Exclusion of other possible diagnoses: primary infection of EBV (infectious mononucleosis), autoimmune diseases, congenital immunodeficiencies, HIV, and other immunodeficiencies requiring immunosuppressive therapies or underlying diseases with potential immunosuppression
Patients who fulfilled criteria (1–4) were diagnosed with CAEBV.

(1) IM-like symptoms generally include fever, swelling of lymph nodes, and hepatosplenomegaly; additional complications include hematological, gastroenterological , neurological, pulmonary, ocular, dermal, and/or cardiovascular disorders (including aneurysm and valvular disease), which have mostly been reported in patients with IM. EBV-HLH accompanied by primary infection of EBV and HV, whose symptoms are limited to those in the skin, should be excluded. Even if EBV-HLH or EBV-positive T- or NK-cell lymphoma/leukemia develops during the disease course, the original diagnosis of CAEBV does not change.
(2) A standard for elevated EBV DNA load by quantitative PCR in the PB is more than $10^{2.5}$ copies/μg DNA.
(3) For detection of EBV-infected cells, it is recommended to perform a combination analysis of detecting the phenotypes of the infected cells (immune fluorescent staining, immune histological staining, magnetic bead sorting) and detecting EBV (EBNA staining, EBV-encoded small RNA in situ hybridization, PCR for EBV DNA).
(4) Patients who were diagnosed with congenital immune deficiencies, autoimmune diseases, collagen diseases; patients who were pathologically diagnosed with malignant lymphomas [Hodgkin lymphoma; extranodal NK/T-cell lymphoma, nasal type (ENKL); angioimmunoblastic T-cell lymphoma; peripheral T-cell lymphoma (PTCL); aggressive NK-cell leukemia (ANKL)]; and patients who were diagnosed with an iatrogenic immunosuppressive condition, either concurrently or prior to CAEBV diagnosis, were also excluded from CAEBV.

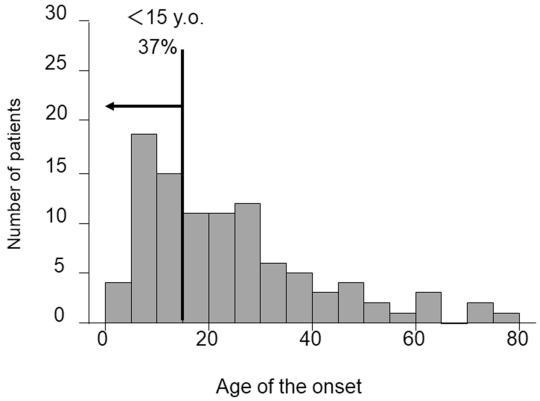

FIGURE 1 | Distribution of age at diagnosis of CAEBV patients. Patient data were collected by means of a nationwide survey of the Japanese study group of the Japan Agency for Medical Research and Development, AMED. Patients had been newly diagnosed with CAEBV between January 2003 and March 2016.

half of the patients were adults. Several reports indicated that the prognosis of adult-onset patients was poorer than that of childhood-onset patients, suggesting that adults and children might have different disorders (9, 11, 12). In addition, most cases to date have been reported in Japan and East Asia. Some investigators have suggested that there are genetic issues in the development of CAEBV (13). However, there has been little evidence to support this hypothesis. Further studies with a large number of patients are necessary to determine the detailed epidemiology of CAEBV.

CLINICAL FEATURES OF CAEBV

CAEBV has two characteristics: systemic inflammation and neoplastic disease. In CAEBV, EBV-infected T or NK cells clonally proliferate and infiltrate systemic organs, leading to their failure. These characteristics define CAEBV as a lymphoid neoplasm. However, CAEBV rarely has solid tumors. In fact, the main clinical finding of CAEBV is inflammation. According to Kimura's report, major clinical findings of CAEBV were fever, liver dysfunction, thrombocytopenia, anemia, and their incidences were 91, 77, 59, 44, and 43%, respectively (9). Furthermore, CAEBV causes vasculitis due to the direct invasion of the infected cells, as well as an immune reaction caused by activation of the cells. Every organ can be a target. Vasculitis can lead to the development of vascular aneurysms, ischemic organ damage and uveitis. As a result, patients with CAEBV can visit any hospital department. Every clinician should be aware of CAEBV and consider it a differential diagnosis in cases of sustained inflammation of unknown origin.

Two skin conditions are well-known CAEBV-related disorders. sMBA is characterized by local skin inflammation followed by high fever, lymphadenopathy and liver dysfunction following the bites of *Aedes (Stegomyia) albopictus*—also known as the Asian tiger mosquito. The puncture sites ulcerate, and although they can be cured within a month, they often leave scars. sMBA occurs due to the hyperreactive response of the patients' lymphocytes to the mosquito's saliva (14). In 1997, Ishihara et al. detected a monoclonal proliferation of EBV-positive T and NK cells in the PB of sMBA patients (15). The following reports indicated that sMBA could lead to the development of fatal disorders such as T- or NK-cell lymphoma or HLH. Therefore, sMBA is now classified as one of the EBV-T/NK-LPDs (2). HV, which is characterized by light-induced vesicles, can be accompanied by systemic inflammation with detection of EBV-infected clonally proliferating T or NK cells (16), and was defined as an HV-LPD in WHO 2016 (2). The diagnoses of sMBA and HV-LPD are made for conditions in which the lesions are limited to the skin. Some CAEBV patients have hyper sensitivity for mosquito bites or HV-like eruptions. The difference of pathogenesis between these skin-limited diseases and CAEBV has not been clarified due to the rarity of these diseases. The analysis of a large number of patients under the unified diagnostic criteria is critical to better understand these diseases.

CAEBV is a progressive disease with two characteristics, specifically, systemic inflammation and the development of neoplasms during the disease course, leading to two lethal conditions, namely, HLH and chemotherapy-resistant lymphoma, respectively. The duration from disease onset to the development of these conditions ranges from several months

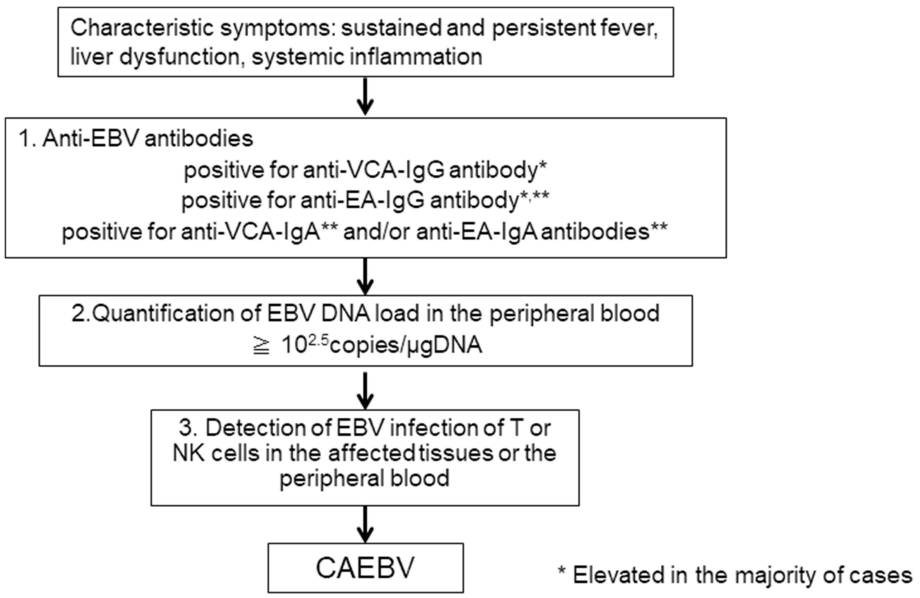

FIGURE 2 | A flowchart of the diagnosis of chronic active EBV infection.

to several decades. Establishing how to predict and prevent the development of these conditions is an urgent issue.

DIAGNOSTIC PROCEDURES FOR CAEBV

When clinical doctors see patients suffering from sustained inflammation, CAEBV as a differential diagnosis is rarely considered, because they are either unaware of the disease or because of the rarity of the disease. According to our report, the mean length of time from onset to diagnosis in CAEBV patients was 20 months (11). The first step to correctly diagnosing CAEBV is to suspect the disease. CAEBV should be frequently considered in cases of sustained inflammation of unknown origin. **Table 1** presents the diagnostic criteria of CAEBV suggested by the Japanese study group in 2015, based on previous reports. **Figure 2** shows a flow chart of the diagnosis of CAEBV. It is not difficult to make a diagnosis of CAEBV under the criteria and the procedure. However, some issues need to be resolved.

Anti-EBV Antibodies

If CAEBV is suspected, anti-EBV antibodies should first be examined. An anti-VCA-IgG antibody is necessary to confirm EBV infection. Many CAEBV cases show high anti-VCA-IgG antibody levels of more than × 640 (6). According to the previous guidelines for the diagnosis, the majority of cases show high levels of anti-EA-IgG antibody and positive for anti-VCA-IgA and anti-EA-IgA antibodies that are originally positive in the acute phase of primary EBV infection (6). Therefore, it is important to rule out primary infection of EBV, IM. The clinical and laboratory findings of IM and CAEBV are quite similar and sometimes difficult to distinguish. Importantly, most IM cases resolve spontaneously, whereas CAEBV needs intensive treatment, including allo-HSCT, to be cured. An anti-EBNA antibody is not useful because some cases of CAEBV are negative for anti-EBNA antibody. An anti-VCA IgM antibody is more useful to exclude primary infection of EBV, but it is not infallible. It is important to check a patient's accurate clinical history to rule out primary EBV infection.

EBV-DNA Load in the Peripheral Blood

If a patient with sustained inflammation of an unknown cause shows elevated levels of anti-VCA-IgG, anti-EA-IgG antibodies, and is positive for anti-VCA-IgA or anti-EA-IgA antibodies, and if IM can be ruled out, the next step to making a diagnosis of CAEBV is the quantification of the EBV DNA load in the PB by PCR. In CAEBV, EBV-infected T or NK cells can be detected in the PB and are characteristic of the disease. Therefore, EBV DNA is detected in the fraction containing mononuclear cells in the blood (17). A cut-off value for the EBV DNA load in the PB for CAEBV is $10^{2.5}$ copies/μgDNA (18), and the EBV DNA load can become undetectable after successful allo-HSCT (19). On the other hand, EBV DNA is usually detected in the serum of EBV-positive lymphomas, including the following: extranodal NK/T-cell lymphoma, nasal type (ENKL), EBV-positive Hodgkin lymphoma; and nasopharyngeal cell carcinoma (20–22). In 2018, the quantification of the EBV DNA load in the PB by RT-PCR was approved as an examination of CAEBV and is covered by health insurance in Japan.

Detection of EBV-Infection of T or NK Cells

If EBV DNA is detected in PB-containing mononuclear cells, the next step is to detect EBV infection of T or NK cells in the affected tissues or the PB. If pathological specimens of EBV-infected

cell infiltrating organs are available, histological examination by immune staining and *in situ* hybridization of Epstein-Barr virus-encoded mRNA (EBER) is performed to detect the phenotypes. However, CAEBV rarely develops solid tumors. As mentioned above, EBV-infected cells can be detected in the PB of CAEBV. Therefore, the phenotypes of EBV-infected cells were determined using unfixed PB in most patients (9, 11, 23). This procedure is costly and requires skilled examiners. In addition, institutes that are capable of performing the examination are limited. This issue is serious and makes the diagnosis of CAEBV difficult. It is indispensable to establish more convenient procedures to determine phenotypes of EBV-infected cells.

THE SUGGESTED MECHANISMS OF THE DEVELOPMENT OF CAEBV

EBV is a common virus; almost all adults have been infected with the virus worldwide. Why does EBV infect T or NK cells, which leads to the development of CAEBV in specific patients? Recently, the mechanisms have gradually become clearer.

There has been a geographical concentration of the reports of CAEBV in Japan and East Asia, indicating that CAEBV is an Asian endemic disorder and that patients may have a common genetic background. However, this hypothesis is controversial. In Western countries, CAEBV patients certainly exist, even in the Caucasian population. Currently, members of the Japanese study group are investigating genetic factors contributing to the development of CAEBV using next-generation sequencing.

How does EBV infect T or NK cells? EBV infects its target B cells by associating with CD21 on the cell surface as a receptor. It has been reported that weak expression of CD21 can be detected on T cells (24). In addition, an *in vitro* examination reported that activated NK cells that were conjugated to CD21-positive EBV-infected B cells transiently acquired weak CD21 expression by the synaptic transfer of a few receptor molecules onto their surface (25). A similar mechanism also exists in T cells (26). Furthermore, another *in vitro* infection assay using a high EBV load showed that EBV infection of T or NK cells could be established (27, 28). Additionally, *in vivo* EBV infection of T or NK cells can be detected in the rapid phase of IM patients. These findings indicated that under a high viral load, EBV can infect T or NK cells (29). Although it is unknown whether the infection is transient or the appearance of the infected cells is transient, EBV-positive T cells disappeared 1 year after onset in IM (30). Why can EBV infection of T or NK cells be sustained in CAEBV? Two mechanisms can be suggested: suppressed immune reaction to the infected cells or characteristics of the virus. It was reported that cytotoxic T cells (CTL) decreased in numbers or showed dysfunction in CAEBV (31, 32). In addition, some congenital immunosuppressive disorders, such as the case of autoimmune lymphoproliferative disorder (ALPS) with FAS gene mutation (33) or the case of perforin mutation (34), can be complicated by CAEBV-like conditions. CAEBV is not accompanied by known primary immunodeficiency disorders (35), however undetermined immunosuppressive disorders may co-occur. Virus-related factors may also play a role. Although characteristic viral strains of CAEBV have not yet been determined, Japanese groups are currently working to clarify these issues through genome-wide analyses. The results are highly anticipated.

EBV infects B cells and immortalizes them. The next question is how EBV-infected T or NK cells become neoplastic cells. Several studies have reported that survival-promoting molecules or pathways are activated by EBV infection. Imadome et al. found that EBV-infected T or NK cells obtained from CAEBV patients expressed CD40 (36). They performed *in vitro* EBV infection of T cells and observed inducible CD40 expression on the surface (27). CD40 was originally expressed on activated B cells. Because the ligand of CD40, CD40L, is originally expressed on the surface of activated T cells, it was hypothesized that inducible CD40 expression was associated with CD40L on the T-cell surface and activated intracellular signaling molecules such as NF-kB. They also confirmed that CD40 on EBV-infected T cells activated CD40L-mediating signaling in an autocrine or paracrine manner and suppressing their apoptosis (27). Another costimulatory molecule, CD137, may contribute to promoting the survival of EBV-infected T cells. Yoshimori et al. reported that CD137 was also expressed in EBV-infected T or NK cells in CAEBV, and its expression could be induced by *in vitro* EBV infection on T cells (23). Stimulation of CD137 by CD137 ligand suppressed etoposide-induced cell apoptosis. Furthermore, Takada et al. found that NF-κB, a transcription factor mediating cell survival signals, was constitutively activated in EBV-infected T or NK cells in CAEBV (37). They also reported that *in vitro* EBV infection of T cells induced constitutive activation of NF-κB and suppressed serum depletion and etoposide-induced apoptosis of the infected cells. NF-κB exists downstream of CD40 and CD137. These findings suggest that EBV infection directly induces cell survival of T or NK cells via survival-promoting pathways such as NF-κB.

EBV may contribute not only to promoting cell survival but also to inducing gene mutations in EBV-infected cells. Nakamura et al. observed activation-induced cytidine deaminase (AID) in the peripheral blood mononuclear cells of EBV-T/NK-LPD patients (38). AID is essential for the somatic hypermutation and class switch recombination of immunoglobulin genes (39). Deregulated AID expression acts as a genomic mutator, leading to the development of B-cell lymphoma (40). In addition, EBV infection induces AID expression in B cells (41). These findings suggest that AID plays a role in EBV-induced lymphomagenesis in B cells. Further studies are expected to determine whether AID has the same roles in CAEBV development.

Recently, interesting findings have been reported by some investigators using next generation sequencing. Okuno et al. performed whole-exome sequencing (WES) on T-, B-, and NK-cell subsets from CAEBV patients. They reported that the most frequently mutated gene was *DDX3X*, an RNA helicase gene detected in 16% (14/83) (42). They also reported that patients carrying *DDX3X* mutation at diagnosis demonstrated significantly shorter overall survival (OS) in comparison with patients without the mutation. Interestingly, Jiang et al. preformed WES for tumor cells from ENKL and reported that *DDX3X* was frequently mutated in them (20%, 21/105) (43). They also determined that the mutant exhibited growth promoting

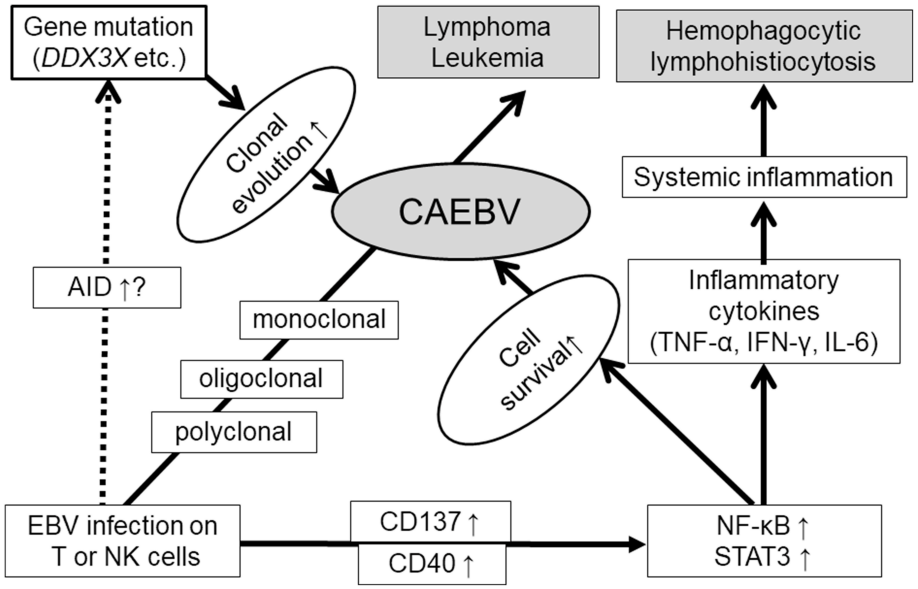

FIGURE 3 | Suggested mechanisms of CAEBV development.

effects on NK cells in comparison with the wild-type protein. *DDX3X* mutation was also detected in Burkitt lymphoma (44). Many reports have focused on *DDX3X* and their association with cancers (45), and the mutation has a possibility of a common driver mutation of EBV-positive neoplasms. Other than *DDX3X*, however, various mutations were detected in CAEBV by Okuno et.al: *KMT2D* (4.8%), *BCOR*/*BCORL1* (3.6%), *KDM6A* (3.6%), and *TET2* (2.4%) (42). Furthermore, they reported the detection rate of at least one somatic mutation in CAEBV by WES was 52% as a whole. These findings indicate a diverse background of CAEBV.

CAEBV has common characteristics of inflammatory disorders. In patients with CAEBV, the serum levels of inflammatory cytokines, namely, IFN-γ, TNF-α, and IL-6, are higher than those in healthy people (46, 47). Their elevated serum levels are associated with the status of the disease. In addition, Onozawa et al. reported that the mRNA of these cytokines was increased in EBV-infected T or NK cells obtained from CAEBV patients (46). The expression of these inflammatory cytokines can be induced by NF-κB (48). Constitutive activation of NF-κB in CAEBV may contribute to the production of cytokines.

STAT3 is a transactivation factor that mediates proliferation and anti-apoptotic signaling. It is activated in various cancer cells and contributes to their transformation (49). STAT3 also mediates intracellular signaling downstream of cytokines and regulates inflammation (50). We observed that STAT3 was constitutively activated in EBV-positive T or NK cells, not only in EBV-positive T or NK cell lines established from EBV-T/NK-lymphoid neoplasms, but also in neoplastic EBV-positive T or NK cells obtained from CAEBV patients (51). Since there was no mutation in the SH2 domain of the STAT3 gene, an essential site for activation of the molecules serving as hot spots of activating mutations in other T or NK cell tumors, upstream molecules might contribute to the activation. Several studies have suggested that STAT3 is activated downstream of LMP1 through the activation of NF-κB (52, 53). Interestingly, Onozawa et al. found that inhibition of a tyrosine kinase, JAK1/2, which phosphorylates STAT3 by its inhibitor ruxolitinib, inhibited STAT3 activation in EBV-positive T or NK cell lines. Furthermore, ruxolitinib suppressed proliferation and induced apoptosis of these cells (51). It was also determined that ruxolitinib suppressed the mRNA expression of IFN-γ, TNF-α, and IL-6 in EBV-T/NK cells. These results indicated that the JAK1/2 STAT3 pathway contributed to the development of both the inflammatory and neoplastic aspects of CAEBV.

In summary, EBV infection of T or NK cells can occur during the acute phase of primary infection with a high EBV load. Furthermore, EBV itself contributes to the survival of host cells by inducing CD40 and CD137 expression and constitutive activation of NF-κB in infected cells. The upregulated AID expression, and accumulation of gene mutations of the infected cells during the course were reported. Gain of function mutation of *DDX3X* may be a responsible driver mutation. Once driver mutations occur, high malignant disorders, such as lymphoma or leukemia, can develop. Furthermore, constitutive activation of STAT3 promotes not only cell survival but also the production of inflammatory cytokines. These suggested mechanisms of CAEBV development are summarized in **Figure 3**.

CURRENT TREATMENT STRATEGY FOR CAEBV

The purpose of the treatment for CAEBV is to control two faces of the disease: a neoplasm and an inflammatory disease. Once HLH or lymphoma develops, CAEBV can be fatal. Therefore,

it is recommended that treatment starts before these diseases develop. According to Kimura's report, the survival of EBV-T/NK-LPDs from onset was 44%, the median follow-up period was 46 months (9). Unfortunately, chemotherapy that can eradicate EBV-infected T or NK cells for CAEBV has not yet been established. The only effective treatment strategy for a cure currently is allo-HSCT. Fifteen-year OS from onset among the patients treated with allo-HSCT was 60.6%, whereas that of those without allo-HSCT was 25.7% (9). The OS with allo-HSCT was significantly longer than those without allo-HSCT. Furthermore, Kawa et al. retrospectively analyzed the prognosis of patients treated with allo-HSCT in their hospital and reported that the 3-year OS after allo-HSCT among patients receiving reduced intensity conditioning (RIC), was 85%, significantly higher than that of patients receiving myeloablative conditioning (54.5%) (54). These findings indicate that the effects of allo-HSCT were partially due to the replacement and reconstruction of the hematopoietic and immune system by allogeneic grafts, rather than the antitumor effects of chemo- and radiotherapies. In other words, immunological dysfunction plays a pivotal role in the development of CAEBV.

The inflammatory symptoms of CABEV are closely associated with the outcomes. Two reports indicated that patients with active CAEBV had poorer outcomes after allo-HSCT (9, 19). An active disease has been defined as a condition accompanied by any of the following: fever, liver dysfunction, progressive skin lesions, or vasculitis. Condition without any of these clinical findings has been defined as an inactive disease. From reports, it is critical to establish chemotherapy that effectively resolves disease activity. What, then, is the most effective chemotherapy to reduce the activity? Sawada and his colleagues from the Osaka Medical Center and Research Institute for Maternal and Child Health, suggested a sequential treatment strategy consisting of prednisolone, cyclosporine A, and etoposide, a so-called cooling therapy as the first step, followed by combination chemotherapies, CHOP, and ESCAP (55). The last step suggested was RIC followed by allo-HSCT. Unfortunately, the rates of resolution of CAEBV disease activity by the chemotherapies were very low, approximately 10% (our manuscript in preparation). To improve outcomes of CAEBV, it is indispensable to establish a more effective chemotherapy for CAEBV.

As mentioned before, CTL disturbance was indicated for CAEBV (31, 32). Based on these findings, Bollard and her colleagues generated EBV-specific CTLs and used them for the treatment for EBV-positive lymphoid tumors, including T-cell type CAEBV (56, 57). Although significant effects of direct reduction of tumor cells have yet to be achieved, induced CTL infusion has potential as an adjuvant therapy to restore EBV-specific T-cell immunity and prevent disease progression.

Some reagents are candidates for new treatments of CAEBV based on the molecular mechanisms of CAEBV development. As mentioned in the previous section, NF-κB is constitutively activated in EBV-positive T or NK cells and may contribute to suppressed apoptosis of the infected cells (37). A proteasome inhibitor, bortezomib, suppresses NF-κB activation in B-cell neoplasms and was approved as a medicine for multiple myeloma and mantle cell lymphoma. Iwata et al. reported that bortezomib suppressed proliferation and induced apoptosis in EBV-infected cell lines, including T or NK cells (58). The JAK/STAT pathway can also be a target. This pathway is commonly used by multiple cytokine receptors and functions to establish inflammation. As mentioned above, the JAK inhibitor ruxolitinib suppresses the proliferation and cytokine production of EBV-positive T or NK cells. JAK inhibitors are clinically used and effective for cytokine-associated inflammatory diseases, such as RA, UC, and GVHD (59–61). Since CAEBV shows sustained inflammation accompanied by hypercytokinemia, the effects of JAK inhibitors on inflammatory symptoms and disease activity are highly anticipated.

SUMMARY

CAEBV has been recognized as an endemic disease in East Asia. However, the number of reported cases is increasing worldwide, due to the 2016 WHO classification and the determination of diagnostic criteria. The prognosis of CAEBV is still insufficient. It is necessary to clarify the molecular mechanisms of the disease development to establish effective treatment strategies.

AUTHOR CONTRIBUTIONS

AA planned, wrote, and reviewed the manuscript.

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Epstein-Barr Virus-Positive T/NK-Cell Lymphoproliferative Diseases in Chinese Mainland

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Epstein-Barr virus-positive T/NK-cell lymphoproliferative disorders (EBV$^+$ T/NK LPD) encompass a heterogeneous group of disorders, including chronic active Epstein–Barr virus infection (CAEBV), Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH), systemic EBV$^+$ T-cell lymphoma of childhood and hydroa vacciniforme-like lymphoproliferative disorder (HVLPD) and so on, predominantly affecting children and young adults with high mortality. Patients with EBV$^+$ T/NK LPD have overlapping clinical symptoms as well as histologic and immunophenotypic features. In this review, we summarized the clinical features of EBV$^+$ T/NK LPD in Chinese patients from the published articles.

Keywords: Epstein-Barr virus, T/natural killer cell, lymphoproliferative disorder, clinical feature, China

INTRODUCTION

Epstein-Barr virus (EBV)-positive T/natural killer (NK)-cell lymphoproliferative disorder (EBV$^+$ T/NK LPD) encompasses a heterogeneous group of disorders, including chronic active Epstein–Barr virus infection (CAEBV), Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH), systemic EBV$^+$ T-cell lymphoma of childhood and hydroa vacciniforme-like lymphoproliferative disorder (HVLPD) and so on. EBV$^+$ T/NK LPD are rare, predominantly affect children and young adults, and associated with high mortality. To date, only hematopoietic stem cell transplantation (HSCT) has been shown to be promising for EBV$^+$ T/NK LPD patients, including those not yet having progressed to lymphoma (1). In this review, we summarized the clinical features of EBV$^+$ T/NK LPD in Chinese patients including CAEBV, EBV-HLH, systemic EBV$^+$ T-cell lymphoma of childhood and HVLPD.

CHRONIC ACTIVE EPSTEIN–BARR VIRUS INFECTION (CAEBV)

CAEBV has been defined as a systemic EBV-positive lymphoproliferative disease (EBV$^+$ LPD) characterized by fever, lymphadenopathy, and splenomegaly developing after EBV infection in patients without known immunodeficiency. CAEBV is more common in children than in adults.

In China, there were only two retrospective studies on the clinical features of CAEBV systematically, one is about pediatric patients and the other is about adult cases (2, 3). In total, eighty one CAEBV patients were reported, including 53 children with a mean age of 6.3 years (ranging from 6 months to 15 years) and 28 adults with a median age of 45 years (ranging from 20 to 81 years). The male to female ratios were 2.12 and 0.75 in children and adults, respectively.

The clinical features and complications of CAEBV in Chinese patients are summarized in **Table S1**. The most frequent signs and symptoms of CAEBV were fever, splenomegaly, hepatomegaly and lymphadenopathy. Life-threatening complications mainly included hemophagocytic syndrome, hepatic failure, and interstitial pneumonia. The peripheral blood count depletions are common in CAEBV patients. In China, one study about pediatric CAEBV patients showed that there was an imbalance in lymphocyte subsets and disturbance in cellular immunity. The number of lymphocytes, NK cell, B cell, total T cell, $CD4^+$ T cell, and $CD8^+$ T cell in CAEBV were lower than that in acute EBV infection (4). In adult onset CAEBV patients, the B cell, NK cell, $CD4^+$ T cell and $CD8^+$ T cell counts were also decreased (3).

The clinical characteristics of pediatric CAEBV cases were different from that of adult patients in China. The prevalence of hemophagocytic syndrome was lower in pediatric patients than in adult patients. Unlike pediatric cases reported, the manifestations of cardiovascular diseases in adult patients included pulmonary arterial hypertension, decreased cardiac function and aorta vasculitis. A comparison with Japanese CAEBV (5) was also made in **Table S1**. The incidences of lymphadenopathy and interstitial pneumonitis were comparatively higher and the prevalence of hypersensitivity to mosquito bites was comparatively lower in Chinese patients than in Japanese patients.

CAEBV can be classified into the T-cell, NK-cell and B-cell types, depending on which lymphocyte subset is mainly infected with EBV. However, in Chinese CAEBV patients, EBV infected cell types were analyzed in only small fraction of patients. In a study, in seven out of 10 CAEBV patients the virus infected cell type was detected, with six cases in T cells, and one in NK cells (6).

Although CAEBV occurred in individuals without apparent immunodeficiency, primary HLH associated immune gene mutations were detected in some CAEBV patients in China, such as heterozygous mutations in *PRF1*, *UNC13D*, and *STXBP2* (7). Furthermore, a pediatric patient with atypical primary immunodeficiency (PID) was reported with CAEBV as the initial symptom (8). Thus, genetic background may play a role in this disease.

There were few studies on the treatment of CAEBV in China. Retrospective studies showed that the prognosis of CAEBV is poor. Without HSCT, only 12.0% patients (5/42) experienced remission for 1 to 3 years after the onset of the disease and 26.2% (11/42) patients died 7 months to 3 years after onset because of the life-threatening complications, such as hemophagocytic syndrome, malignant lymphoma and hepatic failure and so on (2).

EPSTEIN-BARR VIRUS-ASSOCIATED HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS (EBV-HLH)

HLH is an immune disorder characterized by uncontrolled T lymphocyte and macrophage activation and an excessive production of inflammatory cytokines. EBV-HLH is the most frequent subtype of secondary HLH triggered by infections. Eligibility criteria for EBV-HLH were as follows: (1) meeting HLH-2004 diagnostic criteria (9), (2) high level of EBV viral load in the peripheral blood or tissues or number of cells containing EBV-encoded small RNA (EBER) in the peripheral blood or tissues.

In China, most of the studies on EBV-HLH were retrospective (10–12). There was no exact number of EBV-HLH cases in China because the overlap among different studies conducted in the same hospital. EBV-HLH was more common in pediatric patients than in adults, with the age of onset from 2 months to 78 years (12, 13). Over all, male was more likely to develop EBV-HLH than female.

Active EBV-HLH develops rapidly with a high mortality rate if reasonable and effective interventions are not undertaken. The initial therapies of EBV-HLH used in China included antiviral therapy, glucocorticosteroid, symptomatic therapy, HLH-94, and HLH-04 regimen. Antiviral therapy was also used in some EBV-HLH patients in China (10, 12, 13), but the exact benefit of antiviral therapy was not shown in these studies. The treatments of EBV-HLH in China are shown in **Table 1**. The response rate showed that HLH-94 and HLH-04 regimens were more effective. Without chemotherapy, the prognosis of EBV-HLH was very poor.

In refractory EBV-HLH after the therapy of HLH-94, a salvage therapy DEP regimen (including liposomal doxorubicin, etoposide, and high-dose methylprednisolone) was used and achieved better efficacy with overall response rates (complete and partial response) of 72.7% (14). However, the duration of response after DEP regimen is relatively short and there is a significant risk of gastrointestinal bleeding. A modified PEG-aspargase and DEP regimen combination therapy (L-DEP) was used in refractory EBV-HLH as the salvage therapy (15). The overall response rate of L-DEP regimen was 85.7%. It seems that L-DEP is a safe and effective salvage therapy prior to allo-HSCT (allogeneic hematopoietic stem-cell transplantation) for refractory EBV-HLH and increases the possibility of such patients receiving allo-HSCT. A prospective multicenter large-scale clinical trial that aims to validate the L-DEP regimen for refractory EBV-HLH is currently underway (ClinicalTrails.gov Identifier: NCT02631109) (15).

For refractory EBV-HLH, allo-HSCT should be used as early as possible. In China, the survival rates of allo-HSCT were 64.3 and 76.9% after the HLH-94 and L-DEP regimen, respectively (15, 16). Haploidentical HSCT was also used in Chinese adult EBV-HLH patients. The 3-year overall survival rate of haploidentical HSCT was 63.3% (17).

Abbreviations: DEP, doxorubicin-etoposide-methylprednisolone, L-DEP, DEP regimen in combination with PEG-aspargase.

TABLE 1 | Treatment of EBV-HLH in China.

Treatments	Number of cases (*n*)	Gender (male/female)	Median age (range), y	Previous treatment of HLH	Disease status before treatment	Prognosis	Response/survival rate (%)	References
Glucocorticoid	21	/	/	No	Initial diagnosed	3 years OS: (36.2 ± 14.7)%	36.2±14.7	(11)
Symptomatic treatment	18	/	/	No	Initial diagnosed	3 years OS: 0	0	(11)
HLH-94	33	/	/	No	Initial diagnosed	5 CR, 12 PR, 16 NR	51.5	(12)
HLH-04	16	/	/	No	Initial diagnosed	1 CR, 6 PR, 9 NR	43.8	(11)
	44	/	/	No	Initial diagnosed	3 years OS: (55.8±7.9)%	55.8±7.9	(12)
DEP	22	16/6	30.5 (18–57)	HLH-94± rituximab	refractory	5 CR, 11 PR , 6 NR,	72.7	(14)
L-DEP	28	22/6	24 (7–50)	HLH-94± rituximab	refractory	9 CR, 15 PR, 4 NR and dead	85.7	(15)
Allo-HSCT	14	9/5	19 (14–55)	HLH-94	10 remission,	9 alive, 5 dead	64.3	(16)
	13	/	/	L-DEP	4 unremission 9 CR,4 PR	10 alive, 3 dead	76.9	(15)
Haploid HSCT	30	20/10	32 (18–55)	HLH-94, salvage therapies	10 CR, 10 PR, 10 NR	19 survival, 11 dead	63.3	(17)

/, not reported; OS, overall survival; CR, complete response; PR, partial response; NR, no response; allo-HSCT, allogeneic hematopoietic stem-cell transplantation; salvage therapies: DEP, pegaspargase-DEP, or CHOP; Response rate: CR+PR or survival.

SYSTEMIC EBV$^+$ T-CELL LYMPHOMA OF CHILDHOOD

Systemic EBV$^+$ T-cell lymphoma of childhood is a life-threatening illness in children and young adults, and is characterized by the clonal proliferation of EBV infected T cells with an activated cytotoxic phenotype. Its name used to be systemic EBV$^+$ T-cell LPD of childhood in the 2008 World Health Organization (WHO) classification of lymphomas (18) and changed to systemic EBV$^+$ T-cell lymphoma of childhood in 2016 WHO Classification of lymphoid neoplasms (19).

In China, 3 pediatric cases with systemic EBV$^+$ T-cell lymphoma were reported (20, 21) (**Table S2**). The common clinical features of this disease were fever and hepatosplenomegaly. A special patient manifested as gastrointestinal disorders and skin lesion progressed from CAEBV (T-cell type) to systemic EBV$^+$ T-cell lymphoma of childhood was reported (21).

The prognosis of systemic EBV$^+$ T-cell lymphoma is poor. Among the 3 cases reported in China, 2 of them died. One patient experienced rapid progression and died within 5 months of onset (20). The other one died of intestinal hemorrhea (21).

HYDROA VACCINIFORME-LIKE LYMPHOPROLIFERATIVE DISORDER (HVLPD)

HVLPD is a rare type of EBV$^+$ lymphoproliferative disorder of cytotoxic T-cell or NK-cell origin that mainly affect children, characterized by a vesicopapular skin eruption that clinically resemble hydroa vacciniforme (HV). The disease is reported to more frequently affect Asians and Latin Americans. Its name used to be hydroa vacciniforme-like lymphoma in 2008 World Health Organization (WHO) classification of lymphomas (18) and changed to HVLPD in 2016 WHO classification of lymphoid neoplasms (19).

In China, 31 patients with HVLPD were reported and their clinical features were summarized in **Table 2** (22–31). Among them, 20 patients were children with the age ranging from 3 to 15 years old, and 11 patients were adult with the age ranging from 18 to 74 years old.

HVLPD patients often had a long history of recurrent skin lesions before systemic manifestations. In Chinese HVLPD patients, the history of recurrent skin lesions ranged from 2 months to 13 years (22). Many therapies have been applied for the treatment of HVLPD, including interferon (IFN), traditional Chinese medicine, acitretin, acyclovir, prednisone, prednisolone, and chemotherapy. IFN and glucocorticoid were used more commonly and always made an improvement of the disease (22–24, 26). Chemotherapy is uncommonly used because the temporary improvement of disease and the worsened condition of patients after the use of it (22, 23, 25). The prognosis of HVLPD was not well. In all patients reported in China, 20 cases (64.5%) had condition improved after therapy, 6 cases (19.4%) died, 3 cases (9.7%) got worse after therapy, 1 case (3.2%) had no change after therapy and 1 case (3.2%) lost follow up.

In conclusion, EBV-positive T/NK-cell lymphoproliferative disorders encompass a heterogeneous group of disorders which have a common feature with excessive lymphoid proliferation of mainly T cells and/or NK cells. They often have overlapping

TABLE 2 | Clinical features of hydroa vacciniforme-like lymphoproliferative disorder (HVLPD) reported in China.

Number of cases	Age (year)	Gender	History of recurrent skin lesions (year)	Treatment/prognosis	References
7	Ranging from 6 to 14	5M/2F	Ranging from 0.17 to 13	Patient 1: acitretin, prednisone and traditional Chinese medicine/alive with occasional skin eruptions; patient 2: prednisone and traditional Chinese medicine/alive with occasional skin eruptions; patient 3: prednisone and chemotherapy/dead of disease; patient 4: traditional Chinese medicine/alive with occasional skin eruptions; patient 5: IFN/alive with occasional skin eruption; patient 6: prednisone/alive with disease with no changes; patient 7: prednisone and IFN/alive with disease with condition improved	(22)
7	Mean 7.43 (ranging from 3 to 12)	2 M/5F	Mean 2.79 (ranging from 0.5 to 6)	Four cases were treated with IFN-α with skin eruptions improved; and 2 patients were treated with chemotherapy with condition got worse; one case lost follow up	(23)
2	8, 11	M, F	2, 3	One was treated with IFN-α with remarkable clinical improvement at the 6-month follow up; and another was treated with Tibetan medicine with 6-month follow-up, part of her skin eruptions had become smaller and regressed	(24)
1	6	F	3	Treated with cyclosporine and CHOP with condition stable for 6 months	(25)
1	14	F	6	Treated with acyclovir and IFN-α with marked improvement	(26)
2	15, 14	M, M	3, 6	One received glucocorticoid treatment with an improvement of the edema and the cutaneous lesion, and another received glucocorticoid treatment with no sign of recurrence or extracutaneous involvement during 36 months follow up	(27)
6	Ranging from 20 to 58	4M/2F	Ranging from 0.25 to 7	Patient 1: lost to follow up/dead of disease; patient 2: prednisone, IFN, rapamycin, and etoposide/dead of disease; patient 3: prednisone and IFN/dead of disease; patient 4: prednisone/alive with partial remission; patient 5: chemotherapy, IFN, cyclophosphamide/dead of disease; patient 6: IFN/alive with occasional skin eruptions	(22)
2	19, 18	F,F	6, 10	One was treated with chemotherapy and died of disease; Another was treated with chemotherapy and alive with disease	(28)
1	74	M	Over a year	Treated with topical corticosteroids and with good improvement	(29)
1	19	M	2	Treated with Chinese homeopathic medicine, ketotifen, IFN-α, Levofloxacin, sirolimus, and prednisone, died 3 month later	(30)
1	48	M	0.58	Treated with hydroxychloroquine, IFN-α, and prednisone with symptoms began to regress gradually	(31)

M, male; F, female; IFN, interferon; IFN-α, interferon-α.

clinical symptoms as well as histologic and immunophenotypic features because both T and NK lymphoid cell types derive from a common precursor.

LIMITATION OF THE MINI REVIEW

There were some limitations in this mini review. First, in China EBV infected lymphocyte lineages were only characterized in small part of CAEBV patients, not characterized in EBV-HLH and HVLPD patients. It has been shown that clinical feature of CAEBV are different between T-cell type and NK-cell type. So it is difficult to compare the clinical features of Chinese CAEBV with Japanese CAEBV without considering EBV infected cell types. Second, the treatment and outcome of some CAEBV cases were not fully described in references.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2018.00289/full#supplementary-material

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MINI REVIEW
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Aggressive NK-Cell Leukemia

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Aggressive NK cell leukemia (ANKL) is a rare malignant lymphoproliferative disorder of mature NK cells closely associated with Epstein-Barr virus (EBV) and more common in East Asia than in other areas. Significant variations exist in the morphology of ANKL tumor cells, from typical large granular lymphocyte morphology to highly atypical features with basophilic cytoplasm containing azurophilc granules. The main involved sites are hepatosplenic lesions, bone marrow and peripheral blood, and nasal or skin lesions are infrequent. A fever and liver dysfunction with an often rapidly progressive course are the main clinical symptoms, including hemophagocytic syndrome and disseminated intravascular coagulation. Although the outcome had been dismal for decades, with a median survival of less than three months, the introduction of combined chemotherapy including L-asparaginase and allogeneic hematopoietic cell transplantation has helped achieve a complete response and potential cure for some patients. With the advent of next-generation sequencing technologies, molecular alterations of ANKL have been elucidated, and dysfunctions in several signaling pathways, including the JAK/STAT pathway, have been identified. Novel target approaches to managing these abnormalities might help improve the prognosis of patients with ANKL.

Keywords: NK cell, L-asparaginase, JAK/STAT, allogeneic hematopoietic cell transplantation, Epstein-Barr virus, LGL

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INTRODUCTION

Aggressive NK cell leukemia (ANKL) is a rare malignant lymphoproliferative disease of mature NK cell type (1–3). ANKL is prevalent among Eastern Asian populations compared with Western countries and develops mainly in the relatively young (4–6). ANKL is closely associated with Epstein-Barr virus (EBV) (7, 8), with only 10% of ANKL cases negative for EBV (9). With the advent of next-generation sequencing technologies, the molecular basis of ANKL has been considerably elucidated. However, the prognosis of ANKL is still quite poor, with a median survival duration shorter than one year (6, 10). I will discuss several molecular and clinical issues associated with ANKL in this review.

TERMINOLOGY

ANKL has been categorized as a distinct entity since the third WHO classification and has been also classified as a large granular lymphocyte (LGL) leukemia based on its morphological features (3, 11). ANKL is further mentioned as a leukemic type of mature NK cell lymphoproliferative disorder, another type of which was designated chronic lymphoproliferative disorders of NK cell (CLPD-NK) in WHO 2017 classification, provisionally. ANKL is also discussed in relation to NK cell neoplasms, especially extranodal NK/T cell lymphoma, nasal type (ENKL), based on its immunophenotype.

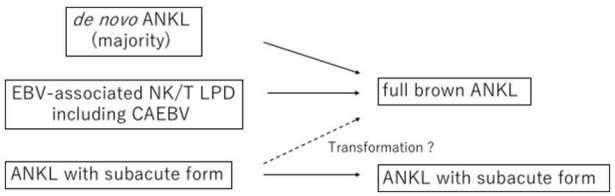

FIGURE 1 | Development of aggressive NK-cell leukemia (ANKL). Most of aggressive NK-cell leukemia (ANKL) develops in *de novo* form and in some younger patients, ANKL evolves from EB virus-associated NK/T cell lymphoproliferative disorders including chronic active EB virus infection (CAEBV) disease. Subacute form of ANKL has been also reported, although it is uncertain whether subacute ANKL transforms into full brown ANKL.

It is thus appropriate that ANKL be classified as an EBV-associated mature malignant NK cell neoplasm and discussed in special relation to ENKL and/or EBV-positive T cell and NK-cell lymphoproliferative disease of childhood, rather than indolent LGL leukemia, such as T cell LGL leukemia or CLPD-NK, based on the current molecular and clinical recognition of ANKL.

EPIDEMIOLOGY

ANKL develops mainly in patients between 20 and 50 years of age but has also been reported in teenagers and patients in their 70s (5, 6, 12). Transformation into ANKL from EBV-associated NK/T lymphoproliferative disorders, such as chronic active EBV infection, is particularly prone to occur in patients of younger age (13, 14) (**Figure 1**). Therefore, ANKL should be recognized as a malignancy among adolescent and young adult (AYA) populations. Approximately 400 cases have been reported, with an increasing number of patients being reported from Caucasian populations and Latin American areas (4–6, 12, 15–20). Whether or not clinical characteristics differ geographically or with ethnic backgrounds is unclear at present.

MOLECULAR PATHOGENESIS

Genetic alterations of ANKL have been largely unclear, in contrast to the wealth of information available for ENKL, a closely related disease of ANKL. With target sequencing of a limited number of ANKL patients, including EBV-negative cases, mutations in *STAT3* or *STAT5B*, molecules of the JAK/STAT signaling system, have been identified, but not in *JAK3*, the molecule in which recurrent mutations were first identified in ENKL (18, 21, 22).

Two groups recently performed a comprehensive genetic analysis of ANKL with next-generation sequencing technology (**Supplementary Table**). Dufva et al. analyzed 14 patients of ANKL with whole-exome sequencing (23). The median number of non-synonymous somatic mutations was 105. Frequent genetic mutations were recognized in the signal transduction system, including the JAK/STAT and RAS-MAPK systems. In 21% of cases, *STAT3* was mutated. In approximately half of the cases, mutations in epigenetic regulatory molecules and or histone modification molecules were also detected, including four cases with *DDX3X*, an RNA helicase. Huang et al. analyzed 8 patients with ANKL by whole-genome sequencing and 29 patients by target sequencing (24). The mean number of non-synonymous mutations was 40. In 48% of cases, mutations in molecules of the JAK-STAT system were detected almost mutually exclusively, and *STAT3* mutations were the most frequent at 17%. In epigenetic modification-related genes, *TET2* (28%), *CREBBP* (21%) and *MLL2* (21%) mutations were found. *TP53* mutations were also recognized in 34% of cases. In contrast, *DDX3X* and *BOCR* were less frequently mutated. Because of the high frequency of mutations in the JAK-STAT system, the authors examined the levels of inflammatory cytokines and found that the plasma IL-10 levels were significantly elevated in ANKL patients and that activation of the JAK/STAT system in ANKL led to an increased expression of MYC, implying the importance of IL-10-STAT3-MYC transcription regulation in ANKL.

Certain chemokine receptors expressed on ANKL cells, especially CXCR1 and CCR5, might be associated with organ damage, including liver damage, in ANKL along with corresponding chemokines (25). Indeed, the serum levels of IL-8, MIP-1α, and MIP1β were significantly elevated in ANKL (26). Interferon γ is also an important regulator in ANKL (27, 28).

IS ANKL THE SAME AS ADVANCED-STAGE ENKL?

There has been debate regarding the relationship between ANKL and advanced-stage ENKL—namely, whether or not ANKL is a leukemic form of ENKL—since several common features exist between these two diseases, such as their cellular phenotypes and close association with EBV. Furthermore, almost the same therapeutic strategies are currently applied for these diseases.

Genetic Abnormalities

An earlier study reported on the genetic differences between ANKL and ENKL. An array comparative genomic hybridization analysis showed that gain of 1q23.1-q23.2 and 1q31.3-q44 and loss of 7p15.1-q22.3 and 17p13.1 were more frequently recognized in ANKL than in ENKL (29). Furthermore, the loss of 6q16.1-q27, which was reported as a common region in NK-cell malignancies in a previous study (30), was frequently recognized in ENKL but less so in ANKL (29). On next-generation sequencing, ANKL showed a different mutation signature from ENKL, with fewer TP53 mutations and more mutations in RAS-MAPK signaling pathway genes, although several common mutations with ENKL were identified, and the frequencies of *TP53* mutations differed (23).

Biological Aspects

An immunophenotypic analysis showed that the CD16 expression on tumor cells was positive in 75% of ANKL cases, but only in 22% of ENKL cases (31). Commonly involved sites were the nose and skin in ENKL and the liver, spleen, peripheral blood and bone marrow in ANKL (31), in which the disease definitions including lesions of these two diseases might reflect.

Granulysin, a cytotoxic molecule in the cytotoxic granules of NK cells, can be detected in the sera. The granulysin levels were significantly elevated in ANKL compared to stage IV ENKL and normal subjects (32).

EBV-NEGATIVE ANKL

ENKL is essentially positive for the EBV genome in all cases. In contrast, a certain proportion of ANKL patients (∼10%) are negative for EBV. Initial reports have suggested a better prognosis of EBV-negative ANKL than EBV-positive ANKL (9), but findings are not consistent (6, 18). No morphologic or immunophenotypic features reliably discriminate between EBV-positive and EBV-negative ANKL (9, 18).

THE DIAGNOSIS OF ANKL

The diagnosis of ANKL should be made based on three factors: the cellular characteristics, involved sites, and clinical features (5, 6). The tumor cells morphologically resemble large granular lymphocytes and are sometimes pleomorphic and large and immunophenotypically CD2+ surface CD3-, CD3epsilon+, CD16+, and CD56+, with a lack of myeloid and B-cell markers. The T-cell receptor genes are in a germline configuration, and the EBV genome is usually positive. The main involved sites are the bone marrow, peripheral blood, liver, and spleen. The clinical features consist of a fever, hemophagocytosis, liver dysfunction, disseminated intravascular coagulation and a progressive course of weeks or sometimes months. In some patients, the diagnosis of ANKL has been delayed and challenging because of the presence of non-specific clinical symptoms and fewer tumor cells in the peripheral blood and/or bone marrow on presentation. Appropriate diagnostic methods for ANKL must be established.

DO SOME ANKL PATIENTS SHOW A DIFFERENT CLINICAL TIME FRAME FROM OTHERS?

Some patients with ANKL are known to present with indolent characteristics and later develop aggressive disease or have a slowly progressive course (33). Tang et al. defined the subacute form of ANKL as ≥90 days of infectious mononucleosis-like symptoms, and 16% of their patients corresponded to this form (17). They compared the gene mutation profiles between the subacute forms and the other patients and found that the patients with the subacute form possessed fewer TP53 mutations and had a better prognosis than those with the typical form, which further implies the heterogeneity of ANKL.

CHEMOTHERAPY FOR ANKL

The optimum chemotherapy regimen as an initial treatment for ANKL has not been established. There have been no prospective clinical trials conducted solely for ANKL. With anthracycline-containing chemotherapy, some patients have achieved a complete response (5). *In vitro*, ANKL cell lines and tumor cells from these patients were shown to be sensitive to L-asparaginase, leading to apoptosis (34). Furthermore, an improved outcome for ANKL with L-asparaginase-containing chemotherapy has been shown (6). Subsequently, the significant efficacy of L-asparaginase against ANKL has been recognized (10, 21). Treatments with L-asparaginase have contributed to an improved survival. Various L-asparaginase-containing regimens, such as SMILE (dexamethasone, methotrexate, ifosfamide, etoposide, and L-asparaginase), AspaMetDex (L-asparaginase, methotrexate, and dexamethasone) or VIDL (etoposide, ifosfamide, dexamethasone, and L-asparaginase) in addition to L-asparaginase monotherapy have been utilized, although no study has compared these regimens for ANKL (10, 35, 36). The formulation of L-asparaginase—as native *E. coli* asparaginase, pegylated-asparaginase or *Erwinase* asparaginase—has also varied. A report implied the effectiveness of gemcitabine, cisplatin, and dexamethasone (GDP) in selected patients (37). A complete response, including negativity for EBV DNA in the blood after treatments, was associated with a better outcome, including the overall survival (10). However, even patients who achieved a CR after chemotherapy including L-asparaginase rarely survived for more than one year without further treatments, especially allogeneic hematopoietic cell transplantation (HCT) (6, 10, 38).

Several prognostic factors for ANKL, such as the patient age, serum lactose dehydrogenase levels and serum total bilirubin level (17), have been proposed, but none have been validated.

ROLE OF HEMATOPOIETIC CELL TRANSPLANTATION FOR ANKL

Although patients have responded to chemotherapy including L-asparaginase, almost all patients eventually died of their disease. Therefore, in order to improve the outcome, hematopoietic cell transplantation has been applied in select patients with ANKL (35, 39, 40). Among the eight patients with a non-CR condition before HCT who received allogeneic HCT, four reached CR, and two of them survived for several years (6). Subsequent studies have also shown the significant efficacy of allogeneic HCT for ANKL (10, 17). A total of 21 ANKL patients registered between 2000 and 2014 in the International Bone Marrow Transplantation Registry (IBMTR) database underwent allogeneic HCT, with most receiving L-asparaginase-containing chemotherapy before proceeding to HCT (38). Patients with a CR prior to HCT showed a significantly better survival after two years than those without a CR (38 vs. 0%). However, 76% of all patients died in the long run, mostly due to ANKL.

Allogeneic HCT for ANKL might help extend the survival, including achieving a cure, in some patients, but the success is limited. Myeloablative conditioning regimens were used in most cases, and the use of non-myeloablative regimens has been increasing. The ideal donor source has not been defined, but performing HCT as early as possible has been suggested to lead to a better outcome in some studies (6, 38), which might be worth

considering. The role of autologous HCT in ANKL is uncertain. Given the retrospective nature of studies on HCT for ANKL, selection bias might also affect these results.

DEVELOPMENTS OF NEW THERAPIES FOR ANKL

Immune Checkpoint Inhibitors

Immune checkpoint inhibitors, which have been widely applied in various malignancies and shown to be highly efficacious, were also demonstrated to be effective in patients with ENKL (41, 42). These patients were resistant to multiple chemotherapies, including L-asparaginase, and had very limited therapeutic options with a short life expectancy. Among the 10 patients with refractory or relapsed ENKL who were resistant to L-asparaginase-containing therapy, a 50–100% response to pembrolizumab or nivolumab was observed, including a CR. No unexpected adverse effects were recognized. While these were retrospective and small-sized studies, it would be of interest to examine the efficacy of immune checkpoint inhibitors in ANKL, a disease very closely related to ENKL.

Other Candidate Agents

Several novel agents have shown significantly efficacy against ANKL cell lines *in vitro*. Decitabine, a hypomethylating agent, and vorinostat, an HDAC inhibitor, showed significant suppressive effects on select cell lines (43). A panel of drug sensitivity and resistance testing showed the JAK inhibitor ruxolitinib and the BCL2 inhibitor navitoclax to be highly effective against malignant NK cell lines. In addition, synergic relationships were observed between ruxolitinib and the BCL2 inhibitor venotoclax or the aurora kinase inhibitor alisertib, which further supports the notion that JAK-STAT alteration is a potential therapeutic approach to ANKL (23). Most of these drugs have already been approved for the treatment of other hematological malignancies, and their efficacy against ANKL must be proven in future studies before they can be applied in clinical practice.

CONCLUSIONS

Significant progress has been made in understanding the molecular pathogenesis and clinical characteristics of ANKL; however, the outcomes of ANKL patients remain poor. Novel approaches, including targeted therapy, such as that for JAK-STAT signaling systems, and immune therapy, such as immune checkpoint inhibitors, HCT and cellular immune therapy with CAR-T cells, or combinations of these approaches may help improve the prognosis of this devastating disease.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2018.00292/full#supplementary-material

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REVIEW
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Extranodal Natural Killer/T-Cell Lymphoma, Nasal Type: Basic Science and Clinical Progress

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Extranodal natural killer (NK)/T-cell lymphoma, nasal type (NNKTL) has very unique epidemiological, etiologic, histologic, and clinical characteristics. It is commonly observed in Eastern Asia, but quite rare in the United States and Europe. The progressive necrotic lesions mainly in the nasal cavity, poor prognosis caused by rapid local progression with distant metastases, and angiocentric and polymorphous lymphoreticular infiltrates are the main clinical and histologic features. Phenotypic and genotypic studies revealed that the lymphoma is originated from either NK- or γδ T-cell, both of which express CD56. In 1990, the authors first reported the presence of Epstein-Barr virus (EBV)-DNA and EBV-oncogenic proteins, and EBV has now been recognized to play an etiological role in NNKTL. *in vitro* studies revealed that a wide variety of cytokines, chemokines, and micro RNAs, which may be produced by EBV-oncogenic proteins in the lymphoma cells, play important roles for tumor progression in NNKTL, and could be therapeutic targets. In addition, it was revealed that the interaction between NNKTL cells and immune cells such as monocytes and macrophages in NNKTL tissues contribute to lymphoma progression. For diagnosis, monitoring the clinical course and predicting prognosis, the measurements of EBV-DNAs and EBV-micro RNAs in sera are very useful. For treatment with early stage, novel concomitant chemoradiotherapy such as DeVIC regimen with local radiotherapy and MPVIC-P regimen using intra-arterial infusion developed with concomitant radiotherapy and the prognosis became noticeably better. However, the prognosis of patients with advanced stage was still poor. Establishment of novel treatments such as the usage of immune checkpoint inhibitor or peptide vaccine with molecular targeting therapy will be necessary. This review addresses recent advances in the molecular understanding of NNKTL to establish novel treatments, in addition to the epidemiologic, clinical, pathological, and EBV features.

Keywords: nasal natural killer /T-cell lymphoma, cytokine, chemokine, Epstein-Barr virus, ICAM-1, CCR4, PD-L1, MPVIC-P

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INTRODUCTION

Extranodal natural killer (NK)/T-cell lymphoma, nasal type (NNKTL) has very unique epidemiological, etiologic, histologic, and clinical characteristics. The lymphoma is commonly observed in Eastern Asia (1–4) and Latin America (4, 5) but quite rare in United States and Europe (6–8). The progressive necrotic lesion mainly in the nasal cavity is one of the main

clinical features of this disease, which is often characterized by a poor prognosis because of rapid local progression and distant metastases (2, 9). The histological feature shows angiocentric and polymorphous lymphoreticular infiltrate, and the disease has been previously called polymorphic reticulosis (10, 11). Phenotypic and genotypic studies revealed that the lymphoma is originated from either NK- or γδ T-cell, both of which express CD56 (2, 8, 12–16). In the late 20th century, the authors first demonstrated the presence of Epstein-Barr virus (EBV) DNA, EBV oncogenic proteins, and the clonotypic EBV genome in NNKTL (1, 2, 16, 17). Based on these findings, EBV has been recognized to play an etiological role in NNKTL (16), and EBV DNA has been applied as a clinical progression/recurrence marker (18). Although NNKTL generally occurs in adult patients, a few cases of pediatric NNKTL are reported (19). In this article, the authors summarize the current understandings of clinical, pathological, biological, and virological characteristics of this lymphoma.

HISTORICAL BACKGROUNDS

McBride (20) first reported, in 1897, the rapid destruction of the nose and face (midline) with progressing necrotic granuloma. The clinical course was generally aggressive and lethal, this disease was initially termed as "rhinitis gangrenosa progressiva " (21) in Europe or "lethal midline granuloma" (22, 23) in the United States. The histologic features show angiocentric and polymorphous lymphoreticular infiltrates with necrotic granuloma. Therefore, the disease had been called many histopathologic terms such as "reticulum cell sarcoma," "midline malignant reticulosis", "polymorphic reticulosis" (11), and "malignant histiocytosis" (24). Since the late twentieth century, this disease had been coined as nasal T-cell lymphoma based on the finding that these tumor cells had a T-cell phenotype (9, 25). Subsequently, the expression of NK-cell marker CD56 was also reported. Accordingly, the term of this lymphoma has been determined as nasal NK/T-cell lymphoma (NNKTL) (26).

Etiologically, Harabuchi et al. (1) first found the presence of EBV-DNA and EBV-determined nuclear antigen (EBNA1) in the lymphoma cells from 5 Japanese patients. These EBV-findings were also verified in Western countries (8, 27–29). Accordingly, NNKTL is now classified as one of the EBV-associated malignancies (16).

EPIDEMIOLOGY

There is a clear geographic deviation in NNKTL prevalence. In Asia and South America, NNKTL consists of 3-10% of non-Hodgkin lymphoma, whereas less than 1% in Western countries (30–33). In Peru, the percentage of NNKTL in non-Hodgkin lymphoma was 8%, respectively (34). Aozasa et al. (35) estimated that the incidence rate of NNKTL is higher in Asia by 10-fold compared to Europe. Because the common race in Asia and South America is mongoloid, the genetic background may play a role in the onset of NNKTL. Although the specific genetic feature of NNKTL patients remains to be elucidated, there is a possibility that a specific type of HLA has the disadvantage to present EBV-associated epitope to T cells. Indeed, HLA-B46 is a risk factor in nasopharyngeal cancer, an EBV-associated malignancy (36).

Another explanation of the skewed distribution of NNKTL could be EBV strain and/or environmental factors. Because the subtype of EBV in NNKTL (Type A/F/C) is similar to healthy donors (37), the high-risk EBV subtype has not been identified in this lymphoma. Nagamine et al. (38) previously demonstrated that the EBV strain in NNKTL has an amino acid mutation in the CD8 T-cell epitope. Furthermore, Nagamine et al. (39) investigated the full length of the LMP1 sequence in NNKTL and found the amino acid changes at codon 126 and 129 in an HLA-A2 restricted CD8 T cell epitope LMP1 125–133 from all patients. Our group (40) previously showed that the frequency of HLA-A*0201 was significantly lower in NNKTL than in the healthy population, suggesting that the HLA-A*0201-restricted CTL responses may inhibit the development of the lymphoma. It is possible that the mutated EBV allows infected cells to escape from cytolysis by immune cells. The precise characteristics of EBV in NNKTL are described below.

The environmental factors also influence the pathogenesis of NNKTL. Our group (41) previously demonstrated that the exposure to pesticides and chemical solvents could be a significant risk factor in NNKTL by a case-control study. Every type of pesticide, herbicide, insecticide, and fungicide was related to the NNKTL incidence. Moreover, the use of gloves or mask to circumvent the pesticide pollution was effective to reduce the incidence of NNKTL. Kojya et al. (42) also reported the case of familial development of NNKTL who are exposed to the pesticide. Taken together, the genetic background, EBV strain, and environmental factors concordantly affect the geographic distribution of NNKTL.

CLINICAL FEATURES

The lymphoma is initially found as progressive ulceration and necrotic granuloma in the nasal cavity, palate, and nasopharynx (1, 2) (**Figure 1**). The tumor frequently invades around tissues such as facial skin, paranasal sinus, and orbits, and then develops extensive destruction of midline lesions (1, 20, 23). The most common symptoms at the time of diagnosis are nasal obstruction and bloody rhinorrhea (2, 43). The swelling of cheek or orbit, sore throat, and hoarseness are also major symptoms of NNKTL (2, 43). In addition, systemic symptoms such as prolonged fever and weight loss are commonly seen (2, 43, 44).

The clinical characteristics of NNKTL from different countries are summarized in **Table 1** (3, 43–47). The disease developed around 40 to 50 years of age, and there is no significant difference between the sexes. The patients over 60 years old were not common (18–35%). Most patients (69–100%) were diagnosed in early stages, Stage I or II. As mentioned above, the most common symptoms were nasal obstruction (70–80%), bloody rhinorrhea (44–47%) and B symptoms (31–53%). The tumor directly invaded several tissues including nasal cavity, hard plate, facial skin, and pharynx. Lymph nodes and distant tissues such as liver, lung, digestive tracts, and bone marrow were also

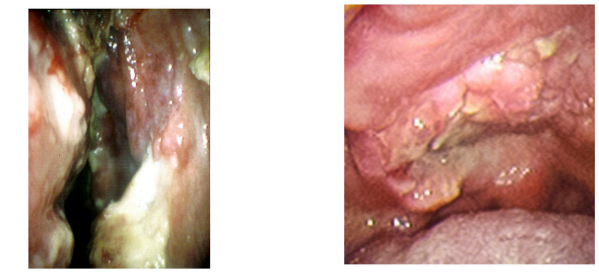

FIGURE 1 | The representative local findings of NNKTL. **(A)** Necrotic granulation in nasal cavity. **(B)** Necrotic ulceration in hard palate.

involved. Twenty to forty percentages of patients had high lactate dehydrogenase (LDH), whereas 38% of patients had high soluble IL-2 receptor in serum. More than 90% of tumors expressed T cell markers such as CD3 or NK marker CD56 on the surface. EBV LMP1 was found in 47% of patients. T-cell receptor (TCR) gene rearrangement was observed in 10–35% of patients. The precise role of EBV, surface markers, or TCR gene rearrangement is described later in this review.

PATHOLOGY

Pathologic characteristics of NNKTL show diffused infiltrates of lymphoma cells, which have a diverse size, pleomorphic large or small cells with mitosis, together with various inflammatory cells such as granulocytes, macrophages and plasma cells, in the necrotic background. The lymphoma had been called as polymorphic reticulosis or angiocentric lymphoma because angiocentric and angio-invasive infiltrates are commonly found (48). The lymphoma cells express T-cell markers such as CD2, cytoplasmic CD3 (CD3ε), and CD45 as well as NK-cell marker CD56. Perforin, Fas ligand, and intercellular adhesion molecule-1 (ICAM-1) are also shown in the NNKTL cells (49).

The lymphoma cell was initially thought to be originated from NK-cells alone by reason that the gene rearrangement of T-cell receptors (TCR) was not found out (50). However, a number of cases with TCR rearrangement reported by Harabuchi et al. (2) and others (51, 52) indicated that some NNKTL are derived from T-cell lineage. This is evidenced by Nagata et al. (13), who succeeded in establishing two NNKTL cell lines from patients, NK-cell lineage without TCR rearrangement and γδ T-cell lineage with γδ TCR rearrangement. Therefore, the current concept of the origin of NNKTL is NK- or γδ T-cells lineage (14, 15) as first proposed by Harabuchi et al. (2).

EBV CHARACTERISTICS

We first reported the close association of EBV with NKTCL in 1990 (1), because EBV genomic DNA and EBNA1 were identified in the nuclei of the lymphoma cells. Subsequently, we demonstrated clonotypic EBV genome (2), suggesting that EBV plays a role in the lymphomagenesis. The other studies also confirmed the etiological role of EBV for NNKTL (1, 8, 28, 29). The lymphoma cells express EBNA1 but not the other EBNAs (2, 53). We detected the mRNA of LMP 1 in all patients, but found the protein of LMP 1 in only half of the patients, because of the methylation of LMP coding sequences (2, 17, 44). Therefore, NNKTL is categorized to the type II latency infection of EBV. Moreover, we performed a southern blot analysis of terminal repeats of the EBV genome and found a single fused terminal fragment, indicating that EBV infection may occur at the early stage of lymphomageneses, and EBV infection in these cells is not from contamination (2, 17, 53).

To discover the oncogenic strains of EBV, several efforts have been made. In the sequence analysis of LMP1, the 30-bp deletion in the codon 343–352 of the B95-8 strain was found in the vast majority of the patients (39, 54). Moreover, we found that several amino acid changes in the LMP1 and LMP2 sequence coding major HLA-A2 restricted CTL epitopes (38, 39). These data suggest that the mutation in EBV endows EBV-infected cells with the ability to escape from immune surveillance by CTLs, which may play an important role in lymphomagenesis.

GENE MUTATIONS

The genetic abnormalities, which may have pathogenic importance, have been reported in NNKTL. The deletion in the chromosome 6q21–25 was frequently found (55–58). The aberrant activation of the JAK/STAT3 pathway, which supports the growth of tumors, has been reported (59). The gene mutations in the cell surface receptor Fas (Apo-1/CD95), which transmits an apoptosis signal, were detected in more than half of the patients (60, 61). Our group found a different frequency of p53, K-ras, and c-kit mutations between NNKTL in Korea and Japan (62). In NNKTL, the p53 mutations were detected in 20–50% of patients (44, 62–65). However, our group showed that mutations of the Ras, c-kit, and β-catenin were not frequent (44, 62, 64). Regarding relation to prognosis, Takahara et al. (44) showed that the p53 missense mutation had a prognostic value predicting poor survival.

PROLIFERATION AND INVASION FACTORS OF NNKTL CELLS

Based on the success of establishing two EBV-positive NNKTL cell lines from NK- and γδ T-cell lineage origins (13), the gene or protein expressions of these cell lines have been discovered. Nagato et al. (66) investigated gene expression patterns of these NNKTL cell lines using cDNA arrays, and (66) found that both the IL-9 mRNA and protein were specifically expressed in NNKTL cell lines (66). NNKTL cell lines also expressed IL-9 receptor. Anti-IL-9 neutralizing antibody decreased proliferation of the cells and recombinant IL-9 increased, suggesting that NNKTL cells use IL-9 as a proliferation factor in an autocrine manner. IL-9 was present in clinical specimens and NNKTL patient sera. EBER induces IL-9 expression (67), suggesting that EBV may play a role for IL-9 expression in the lymphoma.

TABLE 1 | Clinical characteristics of NNKTL from different countries.

Reported country		Japan	Japan	China	Korea	Korea	Brazil
Reported year		2016	2010	2008	2006	2005	2011
Authors		Our institution	Suzuki et al (45)	Wu et al. (43)	Lee et al. (3)	Kim et al. (46)	Gualco et al. (47)
Case number		62	123	115	262	114	122
Age	Range (mean)	20–85(53)	14–89(52)			(47)	9–89(45)
	>60	22(35%)		20(18%)	55(21%)	20(18%)	
Sex	Male/Female	43/19	81/42	78/29	170/92	72/42	85/37
CLINICAL STAGE							
	I/II/III/IV	44/13/1/4	55/29/8/31	61/26/8/12		83/31/0/0	23/2/2/4
	I+II(%)	57(92%)	84(68%)	87(76%)	200(76%)	114(100%)	25(81%)
Symptom	Nasal obstruction	49(70%)		84(73%)			97(80%)
	Bloody rhinorrhea	29(47%)		50(44%)			
	B symptom	32(52%)	56(46%)	57(53%)	92(35%)	35(31%)	
Invaded tissues	Nasal cavity	60(97%)	111(90%)	115(100%)		73(64%)	97(80%)
	Hard plate	11(18%)		8(7%)		15(13%)	
	Facial skin	13(21%)	19(15%)				
	Pharynx	13(21%)	28(23%)	27(23%)		21(18%)	
	Lymph nodes	10(16%)	31(25%)	21(18%)			
	Skin	9(15%)					
	Liver	9(15%)	10(8%)		4(2%)		
	Lung	10(16%)	10(8%)		4(2%)		
	Digestive tracts	5(8%)			10(4%)		
	Bone marrow	3(5%)	9(7%)	3(3%)	16(6%)		
	VAHS	3(5%)					
SEROLOGIC FINDINGS (HIGH CASES/TOTAL CASES)							
	High LDH	22/61(36%)	52(43%)	28(26%)	96(33%)	34(31%)	
	High sIL-2R	9/24(38%)					
PATHOLOGIC FINDINGS (POSITIVE CASES/TOTAL CASES)							
CD3		25/47(53%)	68/86(79%)	105/108(97%)		104(98%)	116/122(95%)
CD43		31/35(89%)	15/17(88%)				
CD45RO		25/35(71%)	44/49(90%)	103/110(94%)		61/62(98%)	
CD20		0/59(0%)	1/14(7%)	0/115(0%)		0/106(0%)	
CD56		61/62(98%)	115/120(96%)	95/105(91%)	262(100%)	94/106(89%)	103/122(84%)
CD16		5/11(45%)	9/40(23%)				
EBER		59/62(95%)	93/94(99%)	106/110(96%)	262(100%)	46/61(75%)	74/74(100%)
LMP1		25/53(47%)					10/122(8%)
TCR rearrangement		12/34(35%)					7/74(10%)

In addition to IL-9, Takahara et al. (68) found that IL-10 was also secreted by NNKTL cells. Exogenous IL-10 increased CD25 (IL-2 receptor) and LMP1 expressions, and then enhanced cell growth of NNKTL. IL-10 treated cells required lower amounts of IL-2 for proliferation. This effect was seen only with the EBV-positive NK-cell lines, in which CD25 and LMP1 were overexpressed, suggesting that IL-10 induces IL-2 receptor expression via enhancement of LMP1 expression, resulting in the proliferation of NNKTL cells.

Chemokines play a huge role in proliferating/recruiting tumor cells and immune cells. Moriai et al. (69) analyzed the expression of chemokines in these NNKTL cell lines using a protein array analysis. We found that the interferon-gamma-inducible protein-10 (IP-10), i.e., CXCL10 was produced in NNKTL cell lines. The amount of IP-10 was significantly larger in NNKTL cell lines than in EBV-negative NK-cell lines. IP-10 was also determined in the biopsy samples and sera from NNKTL patients. The receptor of IP-10, CXCR3, was also expressed in NNKTL cells. *In vitro* studies showed that exogenous IP-10 enhanced invasion of the NNKTL cells, on the other hand, the neutralizing antibodies to IP-10 and CXCR3 inhibited, suggesting that NNKTL cells use IP-10/CXCR3 to invade in an autocrine manner.

Subsequently, Kumai et al. (70) found that NNKTL cells produced chemokine (C-C motif) ligand (CCL) 17 and CCL22. CCL17 and CCL22 were also observed in the NNKTL patients' sera. Moreover, CCR4, which is the receptor for CCL17 and

CCL22, was expressed on the NNKTL cell lines and tissues. Anti-CCR4 antibody efficiently induced antibody-dependent cellular cytotoxicity mediated by NK-cells against NNKTL cell lines. Because anti-CCR4 antibody mogamulizumab has shown clinical efficiency in cutaneous T-cell lymphoma (71), this antibody could also be a useful option in NNKTL treatment.

Metalloelastase is a family of extracellular matrix-degrading enzymes. Metalloelastase degrades several substrates such as elastin, laminin, collagen, fibronectin, and casein. Because MMP-9 was expressed in NNKTL samples (16, 72), NNKTL cells might use this enzyme to invade into surrounding tissues.

CD70, a ligand of CD27, is expressed on activated T-cells, B-cells, and lymphoma. Because lymphoma expressed a higher level of CD70 than lymphocytes, anti-CD70 antibodies might be a possible treatment for CD70 positive lymphomas (73). Yoshino et al. (74) found that NNKTL cell lines specifically expressed CD70, but not EBV-positive NK-cell lines without LMP1 did not. Exogenous soluble CD27, which is the ligand for CD70, enhanced cell proliferation of NNKTL cells in a dose-dependent fashion. In the clinical samples, CD70 was expressed on the NNKTL tissues, and soluble CD27 was detected in patients' sera at higher levels. These results suggest that soluble CD27/CD70 signaling, possibly up-regulated by LMP-1 (75), supports lymphoma progression, and anti-CD70 antibody may be a candidate for the NNKTL treatment.

Intercellular adhesion molecule (ICAM)-1, a ligand for LFA-1, attracts macrophage and create precancerous environment (76). Harabuchi et al. (49) have previously shown that ICAM-1 and soluble ICAM-1 (sICAM-1) was expressed in NNKTL cells and in NNKTL patient sera, respectively. To elucidate the functional role of ICAM-1 in NNKTL, Takahara et al. (77) examined the NNKTL proliferation with sICAM-1. As a result, exogenous sICAM-1 enhanced the proliferation of NNKTL cells, whereas LFA-1/ICAM-1 blockade by anti-ICAM-1 antibody, anti-LFA-1 antibody, or LFA-1 inhibitor simvastatin reduced the number of viable NNKTL cells. In the NNKTL tissues, we confirmed that NNKTL cells also expressed LFA-1. Accordingly, the blockade of LFA-1/ICAM-1 by simvastatin may be a potential agent for NNKTL.

Micro RNAs (miR) play an important role in the carcinogenesis of several malignancies by regulating gene expression. Komabayashi et al. (78) performed MiR array and quantitative RT-PCR analyses and then found that miR-15a was downregulated, while the expression of MYB and cyclin D1 was elevated in NNKTL cells. On the other hand, transfected NNKTL cells with miR-15a precursor downregulated MYB and cyclin D1 levels, resulting in blocking G1/S cell cycle transition and cell proliferation. In NNKTL tissues, we found that the reduced miR-15a expression, which correlated with MYB and cyclin D1 expression, was associated with poor prognosis of NNKTL patients. Knockdown of LMP1 significantly increased miR-15a expression in NNKTL cells, suggesting that LMP1 downregulate miR-15a and then induce cell proliferation via MYB and cyclin D1. Therefore, miR-15a may be useful as a target for anti-tumor therapy as well as a prognostic factor for NNKTL patients.

Together, these results suggest that cytokines, chemokines, and miR, which may be produced by EBV-oncogenic proteins in the lymphoma cells, play important roles for tumor progression in NNKTL (**Figure 2**), and could be therapeutic targets in NNKTL patients.

INTERACTION BETWEEN NNKTL CELLS AND IMMUNE CELLS

Histological features of NNKTL are characterized by diffused infiltrates of lymphoma cells, together with various inflammatory cells such as granulocytes, monocytes, macrophages, and lymphocytes. Accordingly, it is rational to consider that there is an interaction between NNKTL cells and surrounding immune cells. Ishii et al. (79) co-cultured the NNKTL cells with monocytes or granulocytes to examine whether proliferation, survival and LMP1 expression of NNKTL cells are affected by immune cells. Although granulocytes had no effect on proliferation, survival, or LMP1 expression, co-cultured monocytes enhanced proliferation, LMP1 expression and IP-10 production of NNKTL cells. Being not observed when monocytes were placed in a separate chamber, this interaction was mediated in a contact-dependent manner. Because the monocyte-induced proliferation and LMP1 expression of NNKTL cells were inhibited by anti-IL15 antibody, monocytes might support NNKTL cells via membrane-bound IL-15/IL-15 receptor α complex. Because NNKTL cells secrete IP-10 (69), CCL2 and CCL22 (70) that are monocyte-attractant chemokines, a positive feedback loop by the interaction between NNKTL cells and monocytes may contribute to lymphoma progression *in vivo* (79).

Recently, the detrimental effects of negative immune checkpoints have been considered as a druggable target (80). Nagato et al. (81) detected the PD-L1 expression on NNKTL cells and PD-1 expression on the macrophages infiltrated the NNKTL tissues. Soluble PD-L1 was also detected in sera of NNKTL patients at higher levels. Patients with higher soluble PD-L1 in sera showed worse prognosis. Furthermore, Nagato et al. (81) elucidated that NNKTL cell lines expressed and secreted PD-L1 *in vitro*. Because IL-10 converts macrophage to tumor-associated macrophages (82), it is possible that NNKTL cells educate macrophage to be tumor-supportive. The clinical significance of PD-1/PD-L1 blockade is mentioned below.

DIAGNOSIS

Early diagnosis of NNKTL is essential to treat patients promptly (2, 16). Although pathological examination (detection of tumor cells with CD56 and EBER1) is indispensable, the surrounding necrotic tissue may lead to the difficulty of NNKTL diagnosis.

Circulating cell free EBV DNA levels measured by RT-PCR is previously reported to be useful as a tumor marker of EBV-associated malignancies (83). Nagato et al. (18) measured both Bam HI W DNA and LMP1 DNA levels in sera and showed that measurement of both DNAs is more useful as a predictor for prognosis and as a monitoring marker for the clinical course

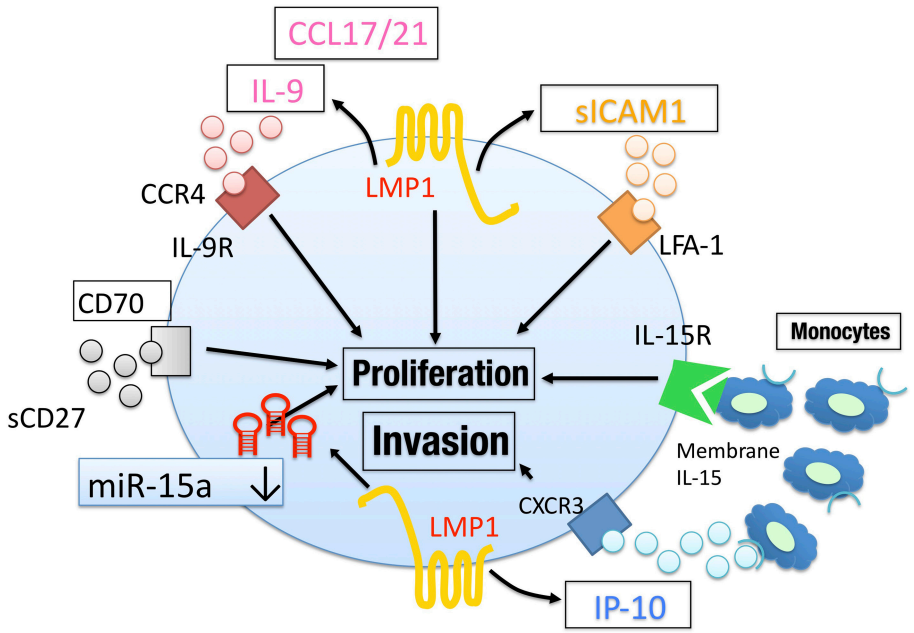

FIGURE 2 | The tumor microenvironment in NNKTL. NNKTL utilize CCL17/21, IL-9, IP-10, and soluble ICAM1 to proliferate/invade in an autocrine manner. These factors may be regulated by EBV LMP1. CD70 activation via soluble CD27 also mediate tumor proliferation. The downregulation of miR-15a mediates tumor progression by regulating surviving. Surrounding immune cells such as monocytes support NNKTL through IL-15 signaling.

of NNKTL than measurement of Bam HI W DNA alone. These DNAs were decreased with the treatment and increased at relapse in NNKTL patients. Patients with high pre-treatment EBV DNAs showed an aggressive clinical course. Multivariate analysis revealed that high pre-treatment level of both EBV DNAs has the most value as an independent prognostic factor. Because the detection of serum EBV DNA reflects the residual lymphoma cells, further treatment should be considered to achieve a complete remission in NNKTL patients with detectable serum EBV DNAs even after the initial therapy (18). This is supported by prospective measurement of serum EBV-DNA in NNKTL patients (84).

Epstein-Barr virus encodes viral miRNAs (miRs). Komabayashi et al. (85) investigated whether the circulating EBV-miRs level was useful as biomarkers for NNKTL. As a result, the serum levels of miR-BART2-5p, miR-BART7-3p, miR-BART13-3p, and miR-BART1-5p could distinguish NNKTL patients from normal donors. *In vitro* studies confirmed that these EBV-miRs were secreted from NNKTL cells. In NNKTL patients, these levels significantly decreased after treatment. Moreover, a high circulating miR-BART2-5p level correlated with poor prognosis. Thus, circulating EBV-miRs, particularly miR-BART2-5p, are useful as diagnostic and prognostic biomarkers in NNKTL patients.

As described above, Nagato et al. (81) clearly showed that the level of serum sPD-L1 was elevated and consistent with disease prognosis in NNKTL patients. Collectively, serum EBV DNA, miRs, and sPD-L1 must be useful biomarkers in NNKTL treatment.

NNKTL TREATMENT

Because a high recurrence rate was reported in the radiation therapy alone (86), chemoradiotherapy is the main strategy to treat NNKTL, but even in early clinical stages, five-year survival rates had been around 50% (44, 87). To improve the treatment outcome, the phase I/II trial (JCOG0211), which consists of three course of DeVIC chemotherapy (dexamethasone, etoposide, ifosfamide, and carboplatin) concomitant with local radiotherapy (50 Gy) for localized NNKTL, conducted in Japan and then showed a good clinical outcome for NNKTL (88). Ifosfamide and carboplatin were chosen because they are not affected by multidrug resistance genes 1, which is frequently expressed in the NNKTL cells (89). Etoposide was used to prevent virus-associated hemophagocytic syndrome (VAHS) (90). Toxicities of the therapy were comparable to those in a previous trial. With a median follow-up of 32 months, 2-year overall survival was 78% (91).

Other regimens such as SMILE (steroid, methotrexate, ifosfamide, L-asparaginase, and etoposide) showed a promising clinical outcome even in a late stage of NNKTL patients (92). Due to the high rate of progression, asparaginase needs to be combined in the regimen with ifosfamide, methotrexate, etoposide, and prednisolone (93). The similar regimen without ifosfamide but sandwiched with radiotherapy also displayed a favorable result (94). The necessity of methotrexate has been examined in the ongoing clinical trial (NCT00283985). The same regimen without radiotherapy has shown satisfactory results (95). Another regimen of the sandwich protocol was reported,

Jiang et al. presented the protocol using L-asparaginase, cisplatin, dexamethasone and etoposide sandwiched with radiotherapy (96). The overall response rate in a phase 2 study of sequential radiation therapy followed by gemcitabine, dexamethasone, and cisplatin was 97.5% in early stage NNKTL (97). Another chemotherapy combining gemcitabine (DDPG: cisplatin, dexamethasone, gemcitabine and pegaspargase) without radiotherapy has also shown promising results (98). Li et al. demonstrated that DDPG chemotherapy showed an improved response rate without severe toxicity like the SMILE regimen (99). GELAD (gemcitabine, etoposide, pegaspargase, and dexamethasone, NCT02733458), GDP (gemcitabine, cisplatin, dexamethasone) with radiotherapy (NCT02276248), P-Gemox (gemcitabine, oxaliplatin, and pegaspargase) have been tested in the clinical trials.

Bone marrow transplant is another approach to treat NNKTL. Despite the expectation, the outcome of autologous or allogenic bone marrow transplant is controversial (100–102). Because improved treatment approaches were needed for localized NNKTL exhibiting elevated pretreatment soluble interleukin-2 receptor (103), it is mandatory to develop novel treatment approaches in NNKTL.

Recently, Takahara et al. (104) have developed a novel arterial infusion chemotherapy from a superficial temporal artery in combination with radiotherapy. The regimen for the arterial infusion consists of methotrexate, peplomycin, etoposide, ifosfamide, carboplatin, and prednisolone (MPVIC-P), which is not influenced by multidrug resistance genes 1 (except for etoposide) as well as a DeVIC regimen. Chemotherapy and concomitant radiotherapy were performed for 3 cycles and over 54Gy, respectively. We administered 12 Japanese patients with stage I-II. During the observation period from 39 to 111 months after the treatment (median: 81 months), all 12 patients achieved complete remission and have remained tumor-free. Common adverse effects were mucositis (83%) and myelosuppression (33%), both of which were manageable. Thus, this MPVIC-P regimen using intra-arterial infusion with concomitant radiotherapy is an effective treatment for early stage NNKTL with adaptable toxicity.

ESTABLISHMENT OF NOVEL TREATMENTS FOR NNKTL

Immune Checkpoint Inhibitor

One of the negative immune checkpoints, the PD-1/PD-L1 pathway, plays an important role in immune evasion of tumor cells through T-cell exhaustion. Because of the expression of PD-L1 on tumor cells (81), PD-1 inhibitor is a promising therapeutic armamentarium in NNKTL. Although the general clinical outcome of NNKTL patients failing chemotherapy is fatal, PD-1 inhibitor has shown remission in these patients (105, 106). The adverse events were tolerable. Several clinical trials with PD-1/PD-L1 blockade are ongoing (NCT03595657, 03107962,

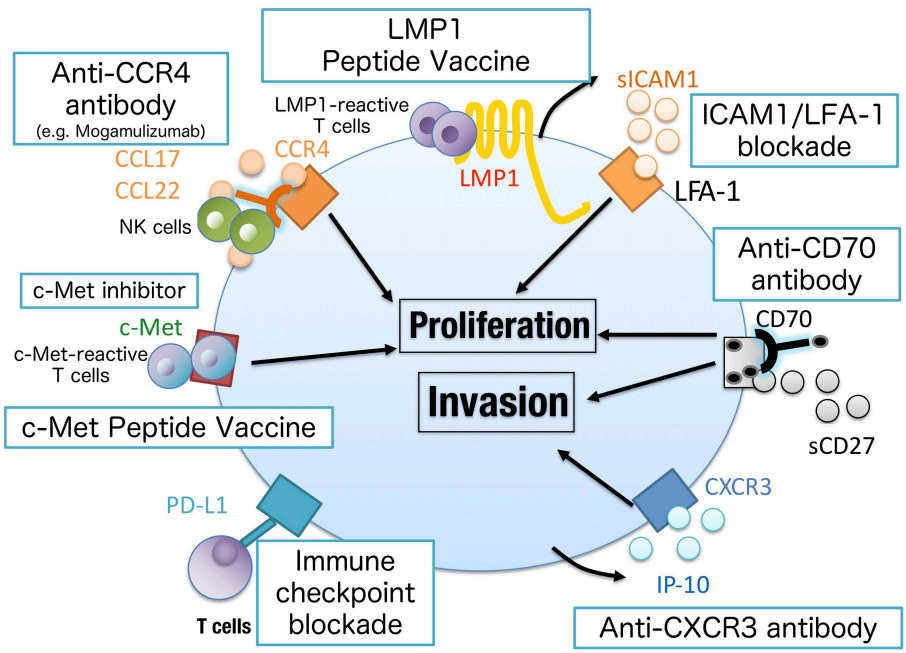

FIGURE 3 | Novel approaches to treat NNKTL. Chemokine/cytokine blockade may inhibit the growth of NNKTL cells (IL-9, IL-10, CXCR3, or LFA-1 blockade) as well as c-Met inhibitor. The antibody against surface markers on NNKTL can directly lyse tumor cells by antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. CCR4 or CD70 could be a promising target in this approach. Mogamulizumab, an anti-CCR4 antibody, has been clinically approved to treat cutaneous T cell lymphoma. LMP1 or c-Met peptide vaccine is useful to elicit tumor-specific T cell responses. Because NNKTL cells express PD-L1 to attenuate antitumor T cell responses, immune checkpoint blockades have shown clinical activity in NNKTL patients.

03439501). Thus, PD-1/PD-L1 blockade could be a favorable treatment in chemotherapy-resistant NNKTL patients.

Peptide Vaccine With Molecular Targeting Therapy

Among immunotherapy, peptide vaccine is a promising treatment to target virus-associated malignancy because these types of tumor express non-self viral antigens that are not ignored by immune cells (107). EBV-related proteins are ideal antigens for the peptide vaccine in NNKTL treatment. Kobayashi et al. (108) previously found an epitope peptide, which could bind to promiscuous MHC Class II (HLA-DR9, HLA-DR53, or HLA-DR15), by computer-based peptide algorithm from EBV LMP1. This peptide was naturally processed and expressed on NNKTL cells and could elicit peptide-specific helper T cells, which displayed Th1 phenotype and cytotoxic activity against NNKTL cell. Because this LMP1 epitope peptide overlaps with an HLA-A2–restricted CD8 T cell epitope, this peptide might have the ability to simultaneously induce antitumor CD4 and CD8 T cells against NNKTL cells.

HGF and its receptor c-Met play an essential role in cell proliferation and are involved in various malignancies. Kumai et al. (109) found that both HGF and c-Met were expressed in NNKTL cells and NNKTL tissues, and this pathway activated the proliferation of NNKTL cells in an autocrine manner. c-Met was also responsible for TGF-b production, a negative regulator of immune cells, from NNKTL cells. Kumai et al. (109) further found that several c-Met-derived helper T cell epitope peptides, which are restricted by various HLA-DR molecules. These peptides could elicit c-Met-reactive CD4 T cells that have a cytolytic ability to NNKTL and several solid tumor cells.

Taken together, LMP-1 and c-Met are promising antigens for a peptide vaccine to treat NNKTK patients, and c-Met blockade may augment the antitumor function of peptide-reactive T cells by suppressing TGF-b production from NNKTL cells.

Future Prospective

The pathogenesis and molecular biology of NNKTL have been gradually revealed as mentioned above. Some of these findings have been considered as direct evidence to establish current promising treatment in NNKTL (81, 105, 106). Although prospective clinical trials are required, novel chemotherapeutic approaches such as MPVIC-P and SMILE have shown favorable clinical outcomes (92, 104). Despite these successes, further basic and translational researches are required to improve the prognosis of NNKTL patients. The blockade of cytokine or chemokine (IL-9, IL-10, CCL17, or CCL21) to inhibit NNKTL proliferation can be an attractive method to treat NNKTL. Recently, several antibodies against cytokine have been approved in the clinic. Mogamulizumab, a clinical-grade anti-CCR4 antibody is a promising candidate to treat NNKTL as shown by Kumai et al. (70). It is mandatory to test the safety of these novel agents in an *in vivo* model. The development of immune deficient mice, in which we succeeded to engraft NNKTL cells, enable us to investigate the effect and safety of these agents (81, 110).

The ligands to pattern-recognition receptors have been recognized as efficient adjuvants in peptide vaccines (111). EBV LMP-1- or c-Met-derived peptide can be a useful peptide vaccine to treat NNKTL patients when combined with adjuvants such as poly-IC or gardiquimod (109, 111). The combination of c-Met or checkpoint blockade with peptide vaccine would be an attracting treatment option in NNKTL. The potential of immunotherapy against NNKTL has been summarized in a review article (112). Because NNKTL is an EBV-related disease, gene therapy to knockout EBV-related proteins or miR would be a fundamental solution to remove NNKTL cells. Since EBV is a widely disseminated virus, the prophylactic vaccine is difficult to establish. However, there is a possibility that high risk EBV subtypes with gene mutation cause EBV-associated malignancies including NNKTL (55–58). Thus, the vaccine that targets high risk EBV can be a preventive vaccine for NNKTL as well as other EBV-associated malignancies.

In conclusion, we demonstrated that recent advances in the molecular understanding of NNKTL have led us to establish novel approaches to treat NNKTL patients (**Figure 3**). We believe that further investigation will make NNKTL a curable 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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Transcriptomic Abnormalities in Epstein Barr Virus Associated T/NK Lymphoproliferative Disorders

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Epstein Barr virus positive T/NK lymphoproliferative disorders (EBV-TNKLPD) comprise a spectrum of neoplasms ranging from cutaneous lymphoid proliferations to aggressive lymphomas. The spectrum includes extranodal NK/T-cell lymphoma (ENKTL), aggressive NK-cell leukemia, and a group of EBV-TNKLPDs affecting children which are poorly characterized in terms of their molecular biology. Gene and miRNA expression profiling has elucidated RNA abnormalities which impact on disease biology, classification, and treatment of EBV-TNKLPD. Pathways promoting proliferation, such as Janus associated kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) and nuclear factor kB, are upregulated in ENKTL while upregulation of survivin and deregulation of p53 inhibit apoptosis in both ENKTL and chronic active EBV infection (CAEBV). Importantly, immune evasion via the programmed cell death-1 and its ligand, PD-1/PD-L1 checkpoint pathway, has been demonstrated to play an important role in ENKTL. Other pathogenic mechanisms involve EBV genes, microRNA deregulation, and a variety of other oncogenic signaling pathways. The identification of EBV-positive Peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) as a tumor with a distinct molecular signature and clinical characteristics highlights the important contribution of the knowledge derived from gene and miRNA expression profiling in disease classification. Novel therapeutic targets identified through the study of RNA abnormalities provide hope for patients with EBV-TNKLPD, which often has a poor prognosis. Immune checkpoint inhibition and JAK inhibition in particular have shown promise and are being evaluated in clinical trials. In this review, we provide an overview of the key transcriptomic aberrancies in EBV-TNKLPD and discuss their translational potential.

Keywords: Epstein–Barr virus, extranodal NK/T cell lymphoma, chronic active EBV infection, T cell lymphoma, RNA

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus with B-cell tropism and the ability to transform infected B lymphocytes into continuously proliferating lymphoblastoid cells. Infrequently, EBV infects T cells and Natural Killer (NK) cells, which can result in a wide spectrum of EBV-positive cytotoxic T/NK cell lymphoproliferative diseases (EBV-TNKLPD). The current classification of EBV-TNKLPD includes (i) systemic chronic active EBV infection of T- and NK-cell type (CAEBV), (ii) cutaneous CAEBV, which includes hydroa vacciniforme-like lymphoproliferative disorder (HV-LPD) and severe mosquito bite allergy (MBA), (iii) systemic EBV-positive T-cell lymphoma of childhood (STCL), (iv) aggressive NK-cell leukemia (ANKL), (v) extranodal NK/T-cell lymphoma, nasal-type (ENKTL), and (vi) nodal peripheral T-cell lymphoma, EBV-positive (EBV-PTCL) (1–3). ENKTL and ANKL are the prototypic examples of EBV-TNKLPD and are well-recognized (4). On the other hand, the classification of EBV-TNKLPD occurring in childhood (EBV-TNKLPD-childhood) has evolved and was recently updated in the revised 4th edition of the WHO classification (1). It includes a spectrum of diseases with heterogeneous clinical manifestations, a broad range of morphology from polymorphic to monomorphic lymphoid proliferations, and indolent behavior to systemic and aggressive diseases (5). EBV-TNKLPD involving primarily lymph nodes is uncommon but shows characteristic clinical and molecular features distinct from ENKTL (6). This group of nodal EBV-TNKLPD is currently classified as an EBV-positive variant of PTCL, not otherwise specified (EBV-PTCL), as it is presently unclear whether they represent a distinct entity (2).

The spectrum of EBV-TNKLPD remains a challenging group of diseases to study and this is often attributed to the rarity of disease and limited tissue availability (7, 8). Due to significant overlap in morphology and phenotype, the precise distinction of each of the entities can be challenging (9). Furthermore, the nomenclature and classification of EBV-TNKLPD occurring in childhood has been confusing and has suffered from a lack of well-established diagnostic criteria until recently (3, 10).

Genome-wide gene expression profiling (GEP) has revolutionized and greatly improved our understanding of the molecular biology of lymphoma (11, 12). Similarly, GEP has identified robust molecular signatures for subtypes of PTCL and ENKTL and uncovered actionable therapeutic targets (13, 14). Novel insights gleaned from recent genome-wide high throughout techniques have also significantly advanced our understanding of ENKTL (8, 15–18). On the other hand, knowledge of the molecular biology and genomics of EBV-TNKLPD of childhood (EBV-TNKLPD-childhood), such as CAEBV, is only slowly unraveling and there remains a paucity of large-scale gene or miRNA profiling of the diseases in childhood. In this brief review, we will summarize current insights on the transcriptomics abnormalities in EBV-TNKLPD and focus on those with translational impact on (i) understanding molecular biology and disease pathogenesis, (ii) disease classification and refining diagnosis, (iii) disease prognosis, and (iv) therapy. An overview of these abnormalities and their role in the pathogenesis of EBV-TNKLPD is presented in **Figure 1**.

IMPACT OF TRANSCRIPTOMIC ABNORMALITIES ON UNDERSTANDING DISEASE PATHOGENESIS OR DEREGULATED PATHWAYS

Cellular Proliferation

Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling has recently been shown to play a prominent role in the pathogenesis of ENKTL and ANKL (19, 20). GEP studies have revealed that components of this pathway are differentially expressed in ENKTL compared to normal NK cells (15, 21). Transcriptomic sequencing and integrated genomic analysis of ANKL showed that JAK/STAT mutations resulted in overexpression of MYC and its interacting proteins (20). These data suggest that JAK/STAT signaling promotes proliferation of malignant NK cells not only through its known pro-proliferative function but also through interaction with oncogenes, such as MYC. MYC is upregulated in ENKTL, and this is associated with a corresponding overexpression of its transcriptional targets. (17). Among these MYC targets is RUNX3, which promotes proliferation and survival in ENTKL (22). Inhibition of MYC leads to downregulation of RUNX3 and apoptosis in ENTKL cells, which supports a potential therapeutic role of MYC in ENKTL (22).

Enhancer of Zeste Homolog 2 (EZH2), a component of the polycomb repressive complex 2 (PRC2), has also been shown to be upregulated in ENKTL (17, 23). This may be explained by Myc-induced downregulation of microRNAs, miR-26a, and miR-101, which negatively regulate EZH2 expression (23). In ENKTL, EZH2 does not function as an epigenetic regulator. Instead, it acts as a transcriptional co-activator via a non-canonical pathway (24), and this switch of function is mediated by JAK3 via phosphorylation of EZH2. Inhibition of JAK3 has been demonstrated to reduce the proliferation of ENKTL cells, indicating that targeting this pathway may be a potential therapeutic strategy (25). EZH2 has also been shown to be upregulated in EBV-TNKLPD-childhood and, similar to ENKTL, downregulation of EZH2 using a PRC2 inhibitor induces apoptosis in CAEBV cell lines (23, 26).

Nuclear factor (NF) kB is a transcription factor with pro-survival and anti-apoptotic functions known to be upregulated in lymphoid malignancies (27). NF-kB and its target genes were shown to be overexpressed in ENKTL by GEP in two studies (15, 17) and treatment of ENKTL cell lines with NF-kB inhibitors resulted in induction of apoptosis (28). However, these results were not replicated in another study comparing ENKTL to PTCL (16). Aurora kinase A (AURKA) is a mitotic kinase important for cell proliferation. AURKA is also overexpressed in ENKTL and targeted inhibition induced significant growth arrest in ENKTL cell lines (16, 17). Recent data suggest that AURKA interacts with MYC and WNT pathways to promote cell proliferation (29, 30). Further studies are required to better delineate the role of AURKA in ENKTL pathogenesis.

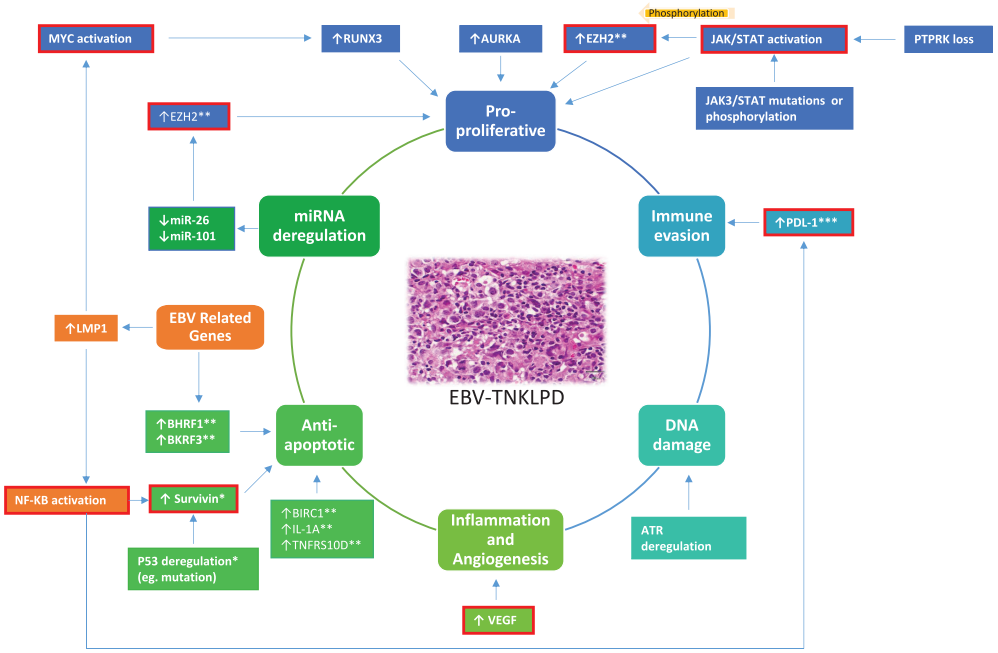

FIGURE 1 | A proposed model highlighting an overview of the main RNA abnormalities in EBV-TNKLPD. The key processes involved in the lymphomagenesis of EBV-TNKLPD are indicated in the central circle of the figure. The RNA abnormalities are grouped and color-coded according to the postulated processes and those which are likely to contribute to multiple processes are highlighted in orange. RNA abnormalities which have potential translational impact are outlined with red. All the above RNA abnormalities are present in ENKTL. *Refers to RNA abnormalities which are present in ENKTL, Systemic EBV+ T-Cell Lymphoma and CAEBV. **Refers to RNA abnormalities which are present in ENKTL and CAEBV. ***Refers to RNA abnormalities which are present in ENKTL and EBV+ PTCL NOS.

Murakami et al. performed GEP on CAEBV and ENKTL samples and identified upregulation of interleukin-2 (IL-2), IL-10, interferon gamma receptor 1 (IFNGR1), and Inhibin beta A (INHBA) (31). It has been proposed that EBV-infected T cells secrete IL-2 which functions via an autocrine loop to promote proliferation of T-cells in CAEBV. The binding of IFN-γ to IFNGR1 leads to activation of the JAK-STAT pathway, which is amenable to targeted therapy. (32, 33) The precise role of INHBA and IL-10 in EBV-TNKLPD remains unknown (31). Other genes that may confer a proliferative signature and are upregulated in ENKTL and CAEBV cell lines include cyclin dependent kinase 2 (CDK2), a regulator of cell cycle progression, and heat shock 90kDa protein 1-alpha (HSPCA), which is important for normal protein folding and survival of cancer cells (34).

In summary, there are multiple pathways which may provide mitogenic signals and allow the neoplastic cells to survive and proliferate in ENKTL and CAEBV. Among the pathways described above, JAK/STAT and NF-kB are the best studied in ENKTL. EZH2 and AURKA have promising translational impact and may serve as potential therapeutic targets in ENKTL. The evaluation of JAK3 inhibitors as modulators of non-canonical EZH2 activity in clinical trials is warranted.

Deregulation of Apoptosis and the DNA Damage Response

Resistance to apoptosis is a known hallmark of cancer and development of drug resistance (35). Several protein families that act as negative regulators of apoptosis by inhibiting cell death signaling pathways have been reported to be upregulated in CAEBV and ENKTL, including BIRC1, interleukin 1 alpha (IL1A), tumor necrosis factor receptor superfamily member 10d (TNFRS10D), survivin, p53, and NF-kB (15, 17, 26, 34, 36). Survivin is an anti-apoptotic protein that is overexpressed in the majority of ENKTL and EBV-TNKLPD-childhood (17, 26). Treatment of ENKTL cells with a survivin inhibitor, terameprocol, leads to increased apoptosis, suggesting this may be a therapeutic target. p53 is upregulated in ENKTL and EBV-TNKLPD-childhood and this is associated with deregulation of genes which are normally controlled by p53 (17, 26). p53 deregulation in ENKTL may be caused by mutations as reported by Quintanilla-Martinez et al. (36).

Appropriate response to DNA damage is essential for the maintenance of genome stability. Ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia-related (ATR) kinases are central regulators of the DNA damage response signaling pathway (37). Deregulation of ATR protein has been reported in ENKTL and CAEBV due to deletions resulting from aberrant splicing and leads to an abnormal response to DNA damage (38). ATR and related cell cycle checkpoint genes were also found to be overexpressed in ENKTL in another study by Ng et al. (17).

These data suggest that a defective DNA damage response along with inhibition of apoptosis may contribute to lymphomagenesis of EBV-TNKLPD. Survivin and p53 stand out as key players in the pathogenesis of EBV-TNKLPD. Survivin

in particular may have potential as a therapeutic target based on the pre-clinical data discussed. Non-apoptotic cell death pathways are also a potential research arena for drug discovery and targeted therapies and are especially important for the circumvention of drug resistance (35).

Immune Evasion

Programmed cell death-1 (PD-1) inhibition has changed the landscape of immunotherapy for lymphoid malignancies (39, 40). GEP studies have shown that PD-Ligand 1 (PD-L1, also known as CD274) mRNA is upregulated in ENTKL compared to control tissues (6, 8). Overexpression of PD-L1 in ENKTL has been proposed to be mediated by LMP1 via MAPK, NF-kB, and STAT3 signaling (41, 42). The glycoprotein, CD38, is expressed in the majority of ENKTL and its expression is associated with an inferior outcome (8, 43). We have demonstrated through GEP study that CD38 is upregulated in ENKTL compared to control tissues (GEO database GSE90597) (8). Recent *in vitro* studies revealed that daratumumab, a humanized monoclonal antibody approved for the treatment of relapsed multiple myeloma, has good efficacy against ENKTL (44). Our current understanding of the role of PD1 and CD38 in EBV-TNKLPD remains incomplete. Novel regulators of PD1 such as CMTM6 (45) warrant investigation in this context while the function of CD38 in lymphomagenesis requires further study.

Whole-transcriptome microarray studies have identified a unique set of 30 genes which are dysregulated in CAEBV (46). These include several phagocytosis-associated genes such as C1QC, FGL232, and PSTPIP233 as well as monocyte markers FCGR1A and FCGR1B (CD64A/B), suggesting a relatively hyperactive phagocytosis and monocyte-mediated antibody-dependent cellular cytotoxicity in CAEBV (46). The expression of many CAEBV-unique genes was highly correlated with the level of CD64, indicating an important role for monocytes in the cellular immune response to CAEBV (46). Understanding the immune microenvironment of EBV-TNKLPD will be helpful in the incorporation of immunotherapy in this group of diseases. The PD-1/PD-L1 pathway is the most important transcriptomic abnormality from a biological and translational point of view. The potential of this pathway as a therapeutic target is discussed below.

Tumor Promoting Inflammation and Angiogenesis

Chronic inflammation is a known driver of malignancy and angiogenesis is critical for tumor growth and metastasis (47). Vascular endothelial growth factor (VEGF) promotes tumor vascularization and growth in a variety of malignancies (48). VEGF is upregulated in ENKTL and has been proposed as a therapeutic target (7, 49). Guanylate-binding protein 1 (GBP1), a G protein involved in the chronic inflammatory response and strongly induced in endothelial cells and lymphocytes, was found to be overexpressed in CAEBV cells (50). It is postulated that the upregulation of IFNGR1 in CAEBV may result in the overexpression of GBP1, which in turn contributes to vascular dysfunction in chronic inflammation (31). Tumor necrosis factor alpha-induced protein 6 (TNFAIP6) is an adhesion molecule that plays multiple roles in chronic inflammation and tissue remodeling. TNFAIP6 is upregulated in CAEBV and postulated to play a similar role to GBP1 in this context (50). Activated T-cells in CAEBV express higher levels of interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and IFN-γ (51), with the expression of IL-10 and TGF-β being proportional to the EBV viral load in T cells (51). These data suggest that a complex deregulation of pro-inflammatory cytokines driven by EBV as well as a potent angiogenic drive play a crucial role in the pathogenesis of EBV-TNKLPD. VEGF appears to have the greatest translational potential among the deregulated angiogenic pathways discussed and requires further study.

EBV Related Genes

EBV mediated oncogenesis is thought to be driven by genes expressed during latency, such as LMP1 (52). The expression of EBV-related lytic genes, such as BHRF1 and BKRF3, was found to be increased in ENKTL cell lines and may have an anti-apoptotic role as BHRF1 has sequence homology with human BCL-2 (34). BZLF1, which encodes the immediate-early gene product Zta, was preferentially expressed in CAEBV compared to ENKTL cell lines (34). Given the critical role of EBV, further studies are required to fully understand the mechanistic underpinnings of the virus in the lymphomagenesis of this spectrum of disease with the aim of developing therapeutic targets.

Other Oncogenic Signaling Pathways

Other signaling pathways reported to play a pathogenic role in ENKTL include PDGFRα, AKT, and NOTCH-1 (7, 15). The availability of inhibitors to the NOTCH and AKT signaling pathways is currently under evaluation, and their clinical efficacy in ENKTL remains to be established (53, 54).

MicroRNA Deregulation

MicroRNAs (miRNA) have a critical role in the regulation of gene expression in cancer and have been proposed to play an important role in ENKTL and CAEBV (55, 56). miRNAs in ENKTL are predominantly downregulated compared to normal NK cells, specific examples include miR-150, miR-101, miR-26a, miR-26b, miR-28-5, miR-363, and miR-146 (57, 58). The targets of these miRNAs include genes in critical pathways such as p53, MAPK, and EZH2 (23, 57). Less commonly, miRNAs with pro-oncogenic functions, such as miR-21 and miR-155, are upregulated in ENKTL (59). miRNA profiles have also been suggested to have prognostic significance. For instance, downregulation of miR-146a and upregulation of miR221 are associated with poor prognosis (60, 61). The utility of miRNAs as a therapeutic target is, however, unknown and requires further studies.

Less is known about the miRNA profile of CAEBV than ENKTL. In a study of patients with CAEBV compared to those with infectious mononucleosis and healthy controls, miR-BART 1-5p, 2-5p, 5, and 22 were found to be upregulated in CAEBV patients (62). Interestingly, miR-BART 13, miR-BART 2-5p, and 15 levels were higher in patients with active compared to inactive disease, suggestive of a potential role in monitoring disease activity (56, 62).

TABLE 1 | Transcriptomic abnormalities in EBV associated NK and T lymphoproliferative disorders.

Transcriptomic abnormality	Role in lymphoma biology	Subtypes of EBV+TNKLPD	References	Clinical significance for therapeutics	References
JAK/STAT	Upregulated via mutation or phosphorylation. Transcriptomic sequencing and integrated genomic analysis of ANKL showed that JAK/STAT mutations resulted in overexpression of MYC and its interacting proteins.	ENKTL ANKL	(20); (8)	Anti-tumor activity of JAK-3 and STAT-3 inhibition in pre-clinical/*in vitro* models. Clinical trials evaluating JAK inhibitors in ENKTL ongoing.	(25); (72)
RUNX3	Upregulated and has oncogenic role promoting proliferation and survival in ENTKL.	ENKTL	(22)	MYC inhibition *in vitro* leads to down-regulation of RUNX3 and apoptosis, suggesting MYC as potential therapeutic target.	(22)
EZH2	Upregulated and functions as a transcriptional co-activator via a non-canonical pathway in ENKTL.	ENKTL and systemic EBV+T-cell lymphoma	(24); (26)	Targeting EZH2 using a PCR2 inhibitor induces apoptosis in ENKTL.	(23); (17)
NF-kB	Upregulated and promotes survival and proliferation.	ENKTL	(15); (17)	Bortezomib in ongoing early phase clinical trials for ENKTL.	(28); (74); (75)
AURKA	Upregulated, promotes cell proliferation.	ENKTL	(16); (17)	*in vitro* inhibition of AURKA induced apoptosis	(16)
IL-2	Upregulated. Promotes T-cell proliferation.	ENKTL and CAEBV	(31)	N/A	N/A
IL-10	Upregulated. Precise role unclear.	ENKTL and CAEBV	(31)	N/A	N/A
IFNGR1	Upregulated. Binding of IFN-γ activates JAK-STAT pathway.	ENKTL and CAEBV	(31)	N/A	N/A
INHBA	Upregulated. Promotes survival and inhibits apoptosis of EBV infected T-cells.	ENKTL and CAEBV	(31)	N/A	N/A
CDK2, HSPCA	Upregulated. Promotes proliferation and survival of cancer cells.	ENKTL and CAEBV	(34)	N/A	N/A
BIRC1, IL-1A, TNFRS10D	Upregulated, inhibits apoptosis.	ENKTL and CAEBV	(34)	N/A	N/A
Survivin	Upregulated. Inhibits apoptosis.	ENKTL, Systemic EBV+T-cell Lymphoma and CAEBV	(17); (26)	Survivin inhibition *in vitro* induced apoptosis, suggesting potential therapeutic role.	(17); (8)
P53	Upregulated (e.g., by mutation). Inhibits apoptosis.	ENKTL, Systemic EBV+T-cell Lymphoma and CAEBV	(17); (36)	N/A	N/A
ATR	Deregulation (e.g., deletion) resulting in abnormal DNA damage response.	ENKTL	(38)	N/A	N/A
PD-L1	Upregulated. Involved in immune evasion.	ENKTL and EBV+PTCL NOS	(6); (8)	Patients with relapsed ENKTL showed response to pembrolizumab, an antibody against PD1. No data yet on EBV+PTCL NOS	(69)
CD38	Upregulated. Exact role unknown but associated with poorer prognosis.	ENKTL	(40); (8)	Good *in vitro* efficacy of daratumumab and one case report documenting complete response.	(44); (71)
VEGF	Upregulated. Promotes tumor vascularization and growth.	ENKTL	(49); (8)	Potential therapeutic target.	(49)
EBV lytic genes (BHRF1, BKRF3, BZLF1)	Upregulated. Potential pathogenic role in ENKTL and CAEBV. BHRF1 may have anti-apoptotic role due to sequence homolog to human BCL-2.	ENKTL and CAEBV	(34)	N/A	N/A
PDGFRα	Upregulated. Mediates migration, proliferation, and cell survival.	ENKTL	(8)	Potential therapeutic target for tyrosine kinase inhibitors.	(15)
NOTCH	Upregulated in ENKTL, involved in developmental processes and cancer.	ENKTL	(15)	Potential therapeutic target for NOTCH inhibitors.	(54)

(Continued)

TABLE 1 | Continued

Transcriptomic abnormality	Role in lymphoma biology	Subtypes of EBV+TNKLPD	References	Clinical significance for therapeutics	References
miR-150 miR-101 miR-26a miR-26b miR-28-5 miR-363 miR-146	Downregulated of miRNAs in ENKTL. Targets of these miRNAs include genes in critical pathways such as p53, MAPK and EZH2	ENKTL	(57)	N/A	N/A
miR-21 miR-155	Upregulated and have a pro-oncogenic function	ENKTL	(59)	N/A	N/A
miR-146a	Downregulated, associated with poor prognosis	ENKTL	(60)	N/A	N/A
miR-221	Upregulated, associated with poor prognosis.	ENKTL	(61)	N/A	N/A
C1QC FGL232 PSTPIP233 FCGR1A (CD64A) FCGR1B (CD64B)	Dysregulation, suggesting a relatively hyperactive phagocytosis and monocyte-mediated antibody-dependent cellular cytotoxicity (ADCC) in CAEBV.	CAEBV	(46)	N/A	N/A
GBP1	Upregulated. Contributes to vascular dysfunction in chronic inflammation.	CAEBV	(31); (50)	N/A	N/A
TNFAIP6	Upregulated, Multiple roles in chronic inflammation and tissue remodeling.	CAEBV	(50)	N/A	N/A
IL-10 TGF-β IFN-γ	Higher levels expressed in T cells in CAEBV. May be a viral evasion mechanism in CAEBV.	CAEBV	(51)	N/A	N/A
LMP1	LMP1 expressed in CAEBV and promotes proliferation.	CAEBV	(52)	N/A	N/A
miR-BART 1-5p miR-BART 2-5p miR-BART 5 miR-BART 22	Upregulated.	CAEBV	(62)	N/A	N/A
miR-BART 2-5p miR-BART 13 miR-BART 15	Upregulated in patients with active compared to inactive disease.	CAEBV	(56); (62)	N/A	N/A

ENKTL, Extranodal NK Tcell lymphoma; EBV, Epstein Barr Virus; CAEBV, Chronic Active EBV; PTCL NOS, Peripheral T-cell lymphoma not otherwise specified; ANKL, Aggressive NK cell leukemia; N/A, No available data to support a therapeutic role at present.

IMPACT OF TRANSCRIPTOMIC ABNORMALITIES ON DISEASE CLASSIFICATION AND REFINING DIAGNOSIS

In addition to characterizing deregulated oncogenic pathways, GEP studies have provided new perspectives on the molecular biology, ontogeny, and classification of ENKTL. The GEP of ENKTL is distinct from PTCL NOS and shows higher expression of genes associated with NK cell lineage (14, 15). In addition, ENKTL demonstrated a similar molecular signature to a subset of gamma-delta (γδ) T-cell lymphomas that are non-hepatosplenic in presentation and akin to a subset of ENKTL derived from γδ T-cells (16). Interestingly, these "molecularly defined" γδ T-cell lymphomas had similar clinical outcomes to ENKTL (14, 16).

EBV-PTCL is an EBV-associated T/NK cell lymphoma with primary nodal disease presentation and shows molecular and clinical features distinct from ENKTL, including older age, lack of nasal involvement and CD8-positive/CD56-negative phenotype (6). Gene set enrichment analysis revealed significant enrichment for hallmark gene sets, such as MTORC1_SIGNALING, IL6_JAK_STAT3_SIGNALING as well as several gene sets related to cell cycle and genomic instability, including G2M_CHECKPOINT, E2F_TARGETS, MYC_TARGETS, and APOPTOSIS. Current data support the WHO proposal to classify this disease separately from ENKTL and this distinction is clinically important as EBV-PTCL is significantly more aggressive than ENKTL and should be managed differently (6).

Since there is significant overlap in the clinicopathologic features of ENKTL and EBV-TNKLPD-childhood, studies have also been conducted to compare the molecular signature of these entities. GEP results indicated a high degree of similarity between EBV-TNKLPD-childhood and ENKTL, with overexpression of p53, survivin, and EZH2 (26). Notably, there is a distinctive enrichment of stem cell-related genes in EBV-TNKLPD-childhood compared

to ENKTL (26). The discovery of potential cancer stem cell phenotype in EBV-TNKLPD-childhood has potential therapeutic implications and may explain why conventional chemotherapy, without hematopoietic stem cell transplant, is often unsuccessful in the treatment of the disease in childhood (63, 64).

IMPACT OF TRANSCRIPTOMIC ABNORMALITIES ON DISEASE PROGNOSTICATION

The challenge in EBV-TNKLPD-childhood remains to identify morphological or clinical markers to predict outcome. While age, liver dysfunction, and treatment with transplantation play a role in prognosis (65), criteria such as presence of systemic symptoms, T-cell clonality, amount of EBV-positive cells, and/or density of the infiltrate do not help in predicting disease progression (66). In this regard, Ng et al. compared the molecular signature between 2 groups of EBV-TNKLPD-childhood with different outcomes and identified overexpression of cyclinE2 gene and protein to be significantly associated with poor outcome (67).

The prognostication of ENKTL is largely based on clinical features (68). Transcriptomics abnormalities have not made a significant contribution to the risk stratification of ENKTL although there is a suggestion that specific miRNAs (miR-146a and miR-221) may have a prognostic impact (60, 61).

IMPACT OF TRANSCRIPTOMIC ABNORMALITIES ON THERAPY

Transcriptomics aberrancies in EBV-TNKLPD have provided insight into potential therapeutic targets. Among the most promising is immune checkpoint inhibition. Overexpression of PD-L1 mRNA and protein has been demonstrated in ENKTL and EBV-PTCL (6, 8) and patients with relapsed ENKTL have shown remarkable response to pembrolizumab, an antibody against PD1 (69). However, there is a lack of correlation between clinical response to pembrolizumab with PD-L1 expression in tumor cells. Further research is therefore required to understand the mechanism of action and to identify new predictive biomarkers for checkpoint inhibition.

The anti-CD38 monoclonal antibody, daratumumab, is approved for multiple myeloma (70). Recent *in vitro* studies revealed that daratumumab may also have efficacy against ENKTL (44). This hypothesis was supported clinically by the dramatic response of a patient with refractory ENKTL to daratumumab monotherapy (71). Targeting LMP1/2 using cytotoxic T-lymphocytes has also revealed encouraging results in EBV-associated lymphomas. While these data have shown initial promise, further study is required to evaluate the mechanism of action and clinical efficacy of these agents (40).

The JAK-STAT pathway is another potentially useful therapeutic target with JAK3 and STAT3 inhibitors showing *in vitro* activity against ENKTL (25, 72). JAK inhibitors are currently being evaluated against ENKTL in a phase 2 clinical trial (NCT02974647) (73). The potential for JAK inhibition to target the INF-γ pathway in CAEBV is also an attractive therapeutic option requiring further study (31). Proteasome inhibitors to target NF-kB have been evaluated in clinical trials in combination with chemotherapy and additional evaluation is necessary to assess their efficacy in ENKTL (74, 75). Other potential therapeutic targets based on preliminary *in vitro* data on ENKTL include PDGFRa, VEGF, AURKA, NOTCH, CDK2, MYC, EZH2, and survivin (7, 8, 15–17, 22, 23).

CONCLUSIONS AND FUTURE DIRECTIONS

Understanding gene and miRNA transcriptomic abnormalities in EBV TNKLPD has improved our understanding of the molecular biology of this group of tumors, with an impact on disease classification, prognosis, and treatment. The key abnormalities associated with each entity are summarized in **Table 1** and an overview of the major deregulated pathways is represented in **Figure 1**. Further characterization of the molecular signatures of these tumors, especially those occurring in childhood, will help direct functional studies on the disease pathogenesis and decipher the role of EBV while stimulating insights into development of new treatment strategies for these patients.

AUTHOR CONTRIBUTIONS

S-BN and W-JC conceptualized the review. SdM and S-BN wrote the manuscript. JT prepared the figure and table. AJ and W-JC critically reviewed and edited the manuscript, figure, and table.

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CASE REPORT
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Epstein-Barr Virus-Associated γδ T-Cell Lymphoproliferative Disorder Associated With Hypomorphic *IL2RG* Mutation

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Chronic active Epstein-Barr virus (EBV) infection (CAEBV) is an EBV-associated lymphoproliferative disease characterized by repeated or sustainable infectious mononucleosis (IM)-like symptoms. EBV is usually detected in B cells in patients who have IM or Burkitt's lymphoma and even in patients with X-linked lymphoproliferative syndrome, which is confirmed to have vulnerability to EBV infection. In contrast, EBV infects T cells ($CD4^+$ T, $CD8^+$ T, and γδT) or NK cells mono- or oligoclonally in CAEBV patients. It is known that the CAEBV phenotypes differ depending on which cells are infected with EBV. CAEBV is postulated to be associated with a genetic immunological abnormality, although its cause remains undefined. Here we describe a case of EBV-related γδT-cell proliferation with underlying hypomorphic *IL2RG* mutation. The immunological phenotype consisted of γδT-cell proliferation in the peripheral blood. A presence of EBV-infected B cells and γδT cells mimicked γδT-cell-type CAEBV. Although the patient had normal expression of CD132 (common γ chain), the phosphorylation of STAT was partially defective, indicating impaired activation of the downstream signal of the JAK/STAT pathway. Although the patient was not diagnosed as having CAEBV, this observation shows that CAEBV might be associated with immunological abnormality.

Keywords: chronic active Epstein-Barr virus infection, γδT-cell, common γ chain, *IL2RG*, JAK/STAT pathway

INTRODUCTION

Epstein-Barr virus (EBV) infection is a very common disease that is found in >90% of all adults with a lifelong occurrence. EBV infections commonly occur asymptomatically in infants and young children, but some individuals present infectious mononucleosis (IM), which typically manifests as fever, pharyngitis with petechiae, exudative pharyngitis, lymphadenopathy, hepatosplenomegaly,

and atypical lymphocytosis. EBV is usually detected in B cells from patients who have IM or Burkitt's lymphoma. Even in X-linked lymphoproliferative syndrome (XLP), which is a primary immunodeficiency disease (PID) characterized by vulnerability to EBV infection, B cells are similarly infected with EBV. On the other hand, in most patients with chronic active EBV infection (CAEBV), which is characterized by repeated or sustainable IM-like symptoms, the virus is detected in T cells (mainly in $CD4^+$ T cells, and less in $CD8^+$ T cells and γδT cells) or NK cells (1, 2). Hypersensitivity to mosquito bites and elevated levels of serum IgE are observed in patients with NK cell-type CAEBV (3). In contrast, in Europe and the United States, which are known to have fewer cases than Asian countries, CAEBV patients are likely to show B-cell-type infection, B-cell depletion and hypogammaglobulinemia (4). This is suggested to be due to differences in the genetic background or environmental factors, and the pathological condition may differ depending on such differences in those infected with EBV; however, the pathophysiology of this condition remains unclear.

Severe combined immunodeficiency (SCID) is a severe form of PIDs, and is defined as a combined functional disorder of both T cells and B cells, which finally results in cell-mediated and humoral immunodeficiency (5). X-linked SCID, which is a common γ chain (γc) deficiency, is the most common phenotype. As next-generation sequencing (NGS) becomes a more common diagnostic tool, the numbers of inherited immune defects might rise even further. Immunodeficiency and autoinflammatory diseases might be found to be atypical phenotypes of SCID caused by hypomorphic *IL2RG* mutation. Here, we report on a Japanese adult with recurrent respiratory infection and EBV-associated leiomyoma during childhood, who developed recurrent infection in his adolescence. The patient was diagnosed as having CAEBV-like EBV-associated γδT-cell lymphoproliferation, and was finally revealed to have *IL2RG* mutation.

RESULTS

Case Presentation

The patient was a 21-years-old Japanese male with no family history suggestive of immunodeficiency. He was born to non-consanguineous Japanese parents. He had experienced recurrent respiratory infections since childhood. At the age of 6 years, he was hospitalized with EBV-associated leiomyoma in his right bronchus, and complement deficiency (C2 and C9), low T-cell count, and reduced responses to phytohemagglutinin (PHA) and concanavalin A (ConA) were also found (6). PID of unknown cause was suspected and Trimethoprim-Sulfamethoxazole (TMP-SMX) was started. He developed Yersinia enteritis at the age of 8 and pleurisy at the age of 9. After that, he did not experience severe infection for 10 years, even after discontinuing TMP-SMX at the age of 12. Chronic cough, purpura, edema, and pain of the lower limbs appeared at the age of 19. A skin biopsy was performed, which led to a diagnosis of leukocytic fragmentative vasculitis; however, immunosuppressive therapy was postponed due to his past medical history of immunodeficiency. At the age of 21, he was hospitalized with invasive *Haemophilus influenzae* infection, which had been stabilized following adequate antimicrobial therapy, and he also suffered from recurrent pneumonia caused by multiple pathogens. Extensive immunological evaluations showed dysgammaglobulinemia, with reduced IgG (608 mg/L) and IgG2 (109 mg/dL), elevated IgA (692 mg/dL), normal IgM (62 mg/dL), reduced IgE (<3 IU/mL), and reduced CH50 levels (16 U/mL) (**Supplementary Table 1**), along with reduced lymphocyte proliferation (PHA 6,700 cpm and ConA 4,460 cpm). Lymphocyte subpopulation analysis showed reduced T cells, a paucity of B cells, and an increase of NK cells (**Table 1**). In $CD3^+$ T cells, a markedly increased number of γδT cells was observed, and T cells were skewed to the memory phenotype, especially central memory T cells. The kappa-deleting recombination excision circles level was low but detectable, while the T-cell receptor excision circles level was undetectable. The patient exhibited normal production of specific antibodies against varicella zoster virus (VZV), mumps, rubella, and measles.

Virological Examination

Virus DNA quantitative tests revealed the presence of EBV in peripheral blood mononuclear cells (PBMCs) and plasma (9.0×10^2 copies/μgDNA and 4.3×10^2 copies/mL, respectively), and cytomegalovirus (CMV) was also detected in plasma (4.5×10^3 copies/mL). EBV was detected not only in $CD19^+$ B cells (2.1×10^4 copies/μgDNA) but also in γδT cells (2.1×10^2 copies/μgDNA). Interestingly, RT-PCR analysis demonstrated that EBV in B cells was positive for EBNA1, EBNA2, LMP1, LMP2A, and LMP2B transcripts, whereas EBV in γδT cells was positive for EBNA1, LMP1, and LMP2A, but negative for EBNA2 and LMP2B transcripts. These findings indicated that EBV in B cells showed latency III infection; however, EBV in γδT cells showed latency II. Chronological data of EBV-related antibodies were shown in **Supplementary Table 2**.

Genetic Findings

Whole-exome sequencing (WES) identified a hemizygous mutation in *IL2RG* c.982C > T (p. R328*) in the patient. This mutation was confirmed by Sanger sequencing (**Figure 1A**). The mother was the heterozygous carrier of this variant. WES also revealed a homozygous mutation in C9 c.346C > T (p. R116*), indicating the cause of his complement deficiency.

Immunological Findings

The mutation was present in exon 8 of *IL2RG*, which corresponds to the intracellular domain of the γc chain (**Figure 1B**). Flow cytometric examination using an antibody recognizing the extracellular domain of the CD132 molecule was positive (**Figure 1C**). However, the phosphorylation of STAT3, STAT5, and STAT6 after cytokine stimulation was partially defective (**Figure 1D**). In the patient, proliferative capacity was slightly decreased in both $CD4^+$ and $CD8^+$ T cells, and markedly decreased in $CD4^-CD8^-$ cells which correspond to γδT cells (**Figures 2A,B**). The function of NK cells was normal as revealed by assessing the expression of CD107a (**Figure 2C**). EBV-specific $CD8^+$ T cells were detectable as well as CMV-specific $CD8^+$ T cells (**Supplementary Figure 1**). Southern blot analysis of

TABLE 1 | Lymphocytes profile of the patient at 21 years of age.

Lymphocyte profile	% (/μL)	Reference value in adults
T CELL LINEAGES		
T cells ($CD3^+$/Lymphocytes)	58.1 (1,258)	67.8 ± 5.4 (718–2,630)
Th cells ($CD4^+/CD3^+$)	13.5 (170)	59.9 ± 9.9 (407–1,550)
Tc cells ($CD8^+/CD3^+$)	16.0 (201)	34.1 ± 8.7 (210–1,140)
$CD4^+/CD8^+$	0.84	0.8–3.0
Naïve Th cells ($CD45RA^+$ $CCR7^+/CD3^+CD4^+$)	1.9	32.3 ± 24.0
$CD4^+$ T_{CM} ($CD45RA^-$ $CCR7^+/CD3^+CD4^+$)	92.2	30.3 ± 18.7
$CD4^+$ T_{EM} ($CD45RA^-$ $CCR7^-/CD3^+CD4^+$)	4.13	25.3 ± 16.1
$CD4^+$ T_{EMRA} ($CD45RA^+$ $CCR7^-/CD3^+CD4^+$)	1.75	12.1 ± 20.2
Naïve Tc cells ($CD45RA^+$ $CCR7^+/CD3^+CD8^+$)	13	40.1 ± 35.5
$CD8^+$ T_{CM} ($CD45RA^-$ $CCR7^+/CD3^+CD8^+$)	71.9	20.8 ± 25.3
$CD8^+$ T_{EM} ($CD45RA^-$ $CCR7^-/CD3^+CD8^+$)	7.2	19.7 ± 20.3
$CD8^+$ T_{EMRA} ($CD45RA^+$ $CCR7^-/CD3^+CD8^+$)	7.9	19.2 ± 25.8
αβT cells ($TCR\alpha\beta^+TCR\gamma\delta^-/CD3^+$)	28.1	89.6 ± 4.8
γδT cells ($TCR\alpha\beta^-TCR\gamma\delta^+/CD3^+$)	71.6	5.2 ± 4.2
Double negative T cells ($CD4^-$ $CD8^-/CD3^+TCR\alpha\beta^+$)	0.83	0.77 ± 0.35
Regulatory T cells ($CD25^+IL7R^-/CD3^+CD4^+$)	9.16	3.11 ± 1.02
Follicular helper T cells ($CD45RO^+CXCR5^+/CD3^+CD4^+$)	3.06	7.02 ± 3.43
Invariant natural killer T cells ($Vb11^+Va24^+/CD3^+$)	0.027	0.018 ± 0.012
B CELL LINEAGES		
B cells ($CD19^+$/Lymphocytes)	2.01 (44)	12.2 ± 4.4 (110–627)
Transitional B cells ($CD24^+$ $CD38^+/CD19^+$)	2.2	8.1 ± 6.5
Memory B cells ($CD27^+/CD19^+$)	45.6	18.5 ± 8.2
IgM memory B cells ($CD27^+$ $IgM^+/CD19^+$)	7.47	11.2 ± 4.0
Switched memory B cells ($CD27^+$ $IgD^-/CD19^+$)	36.9	13.2 ± 7.2
IgG memory B cells ($CD27^+$ $IgG^+/CD19^+$)	5.43	2.4 ± 1.4
IgA memory B cells ($CD27^+$ $IgA^+/CD19^+$)	11.9	3.3 ± 2.8
$CD21^+$ B cells ($CD20^+/CD19^+$)	79.7	14.3 ± 5.6
Plasmablasts ($CD38^+$ $IgM^-/CD19^+$)	27.6	3.2 ± 2.3
NK CELL LINEAGE		
NK cells ($CD16^+$ $CD56^+$/Lymphocytes)	33.8 (732)	13.4 ± 4.1 (82–760)

Values above than the normal range are shown in red, and values below than the normal range are shown in blue. Th, helper T; Tc, cytotoxic T; T_{CM}, central memory T; T_{EM}, effector memory T; T_{EMRA}, $CD45RA^+$ effector memory T.

the TCRβ chain showed extra bands in the patient, indicating that the TCRβ chain was rearranged (**Figure 2D**). The TCR repertoire profile showed oligoclonal expansions of γ-expressing clonotypes (**Figure 2E**). These findings indicated that expanded EBV-infected γδT cells might have impaired immunological function and play a pivotal role in the pathogenesis of the disease.

Clinical Course After Diagnosis and Immunological Examination

A few months after the diagnosis, the patient presented with high fever, whole body rash with small blisters, and EBV (6.8×10^3 copies/μgDNA) and VZV (1.7×10^4 copies/μgDNA) viremia. The symptoms disappeared after the initiation of oral valacyclovir (VACV) for 5 days, but EBV and VZV were persistently positive in blood. Three weeks after the VACV treatment, the patient was admitted to hospital with the symptoms of high fever, cough, abdominal pain, and purpura, edema, and pain of the lower limbs. Intravenous antibiotic, acyclovir, and intravenous immunoglobulin treatment were not effective. Rituximab was also used to diminish the EBV infection in B cells, but it did not help to resolve the clinical symptoms. CMV and HHV-7 became positive along with EBV and VZV a week after admission, and the antiviral drug was switched to ganciclovir. Methylprednisolone pulse (15 mg/kg/day × 3 days) treatment was performed against hypercytokinemia [neopterin: 52 nmol/L (<5), IL-18: 3,260 pg/mL (<500), IL-6: 104 pg/mL (<5), sTNF-RI: 2,020 pg/mL (484–1,407), and sTNF-RII: 5,800 pg/mL (829–2,262)]. These treatments successfully resolved the symptoms and all four viruses became negative.

At the age of 21, the patient underwent a bone marrow transplantation from an HLA-matched unrelated donor (total nucleated cell dose 3.2×10^8 cells/kg) with fludarabine at 180 mg/m^2, melphalan at 140 mg/m^2, etoposide at 450 mg/m^2, and 3 Gy of total body irradiation. Graft vs. host disease (GvHD) prophylaxis with tacrolimus and short-term methotrexate were given. Although the patient achieved prompt neutrophil engraftment on day +17, acute GvHD (Grade 1: skin 2, liver 0, gut 0) developed. Additional therapy with prednisolone controlled the GvHD. Complete donor chimerism of PBMCs was demonstrated at day +21 and that of bone marrow mononuclear cells at day +29. EBV could not be detected from γδT cells, other types of T cells, B cells, NK cells, or blood plasma at day +85.

DISCUSSION

The protein encoded by *IL2RG* is an important signaling component of many cytokine receptors, including those of IL-1, −4, −7, −9, −15, and −21, and is thus referred to as γc (7). Mutations in *IL2RG* cause signal abnormality of these cytokines and the development of $T^-B^+NK^-$ SCID. In the present case, although numbers of T cells and NK cells were relatively well-maintained, most of the T cells were γδ T cells lacking much of an ability to proliferate. Immunological assessment showed that phosphorylation of STAT3, STAT5, and STAT6 was partially reduced but not completely diminished. These findings suggested that this mutation (p. R328*) was hypomorphic.

The patient was also associated with C2 an C9 deficiency, and homozygous nonsense mutation in the *C9* was identified. C9 deficiency is the most common complement deficiency in

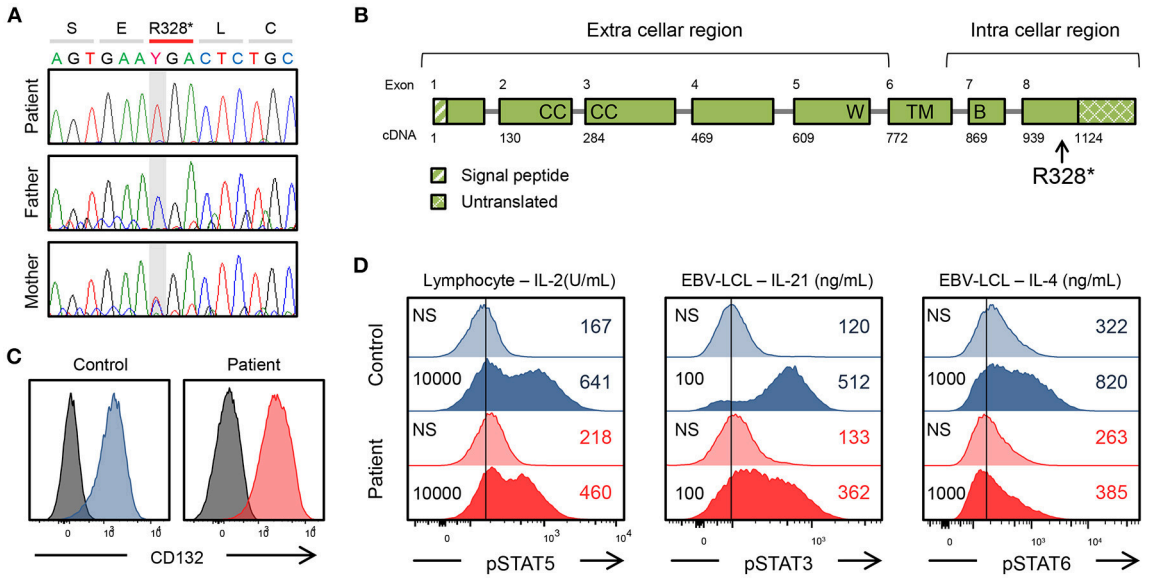

FIGURE 1 | Genetic and immunological studies 1. **(A)** *IL2RG* mutation of the patient's family and *in vitro* analysis of the *IL2RG* mutant. **(B)** Gene structure of IL2RG. p.R328* is located in intracellular region. W, WSEWS box; B, Box1-Box2 domain; CC, conserved cysteine; TM, transmembrane. **(C)** Surface CD132 expression in lymphocytes. **(D)** Flow cytometric analysis of pSTAT5, pSTAT3, and pSTAT6. Histogram in lymphocytes or EBV-lymphoblastoid cell line. The number on the left is the amount of cytokine. NS, non-stimulation.

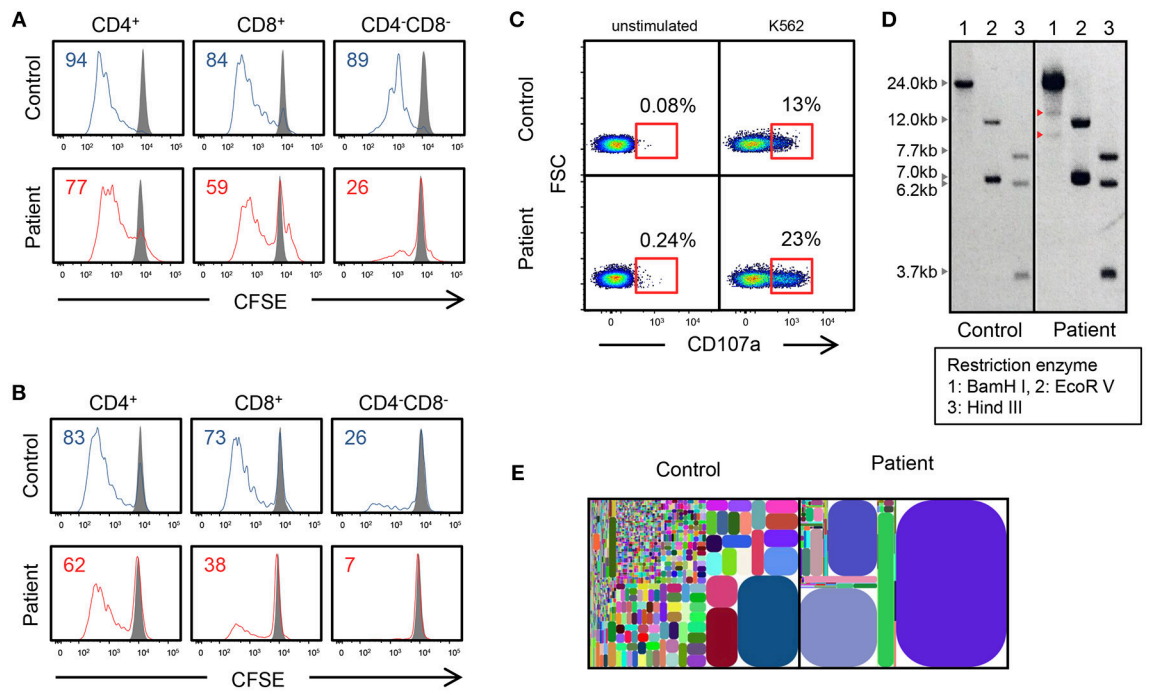

FIGURE 2 | Genetic and immunological studies 2. **(A,B)** Proliferation assay of $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ cells using CSFE after PHA and IL-2 stimulation and anti-CD3/CD28 stimulation. **(C)** CD107a expression of $CD3^-CD56^+$ NK cells, which were cultured with K562 target cells. Cells are gated on $CD3^-CD56^+$ cells. **(D)** Southern blot analysis of TCRβ chain. The patient shows extra bands (red arrowhead). **(E)** T-cell γ receptor repertoire.

Japan, but is very rare in western countries. The incidence of C9 deficiency was estimated to be 0.086–0.12% in Japan (8–10). Autoimmune, renal and infectious diseases were observed in some patients with C9 deficiency. The patient suffered from leukocytic fragmentative vasculitis, which might be associated with C9 deficiency.

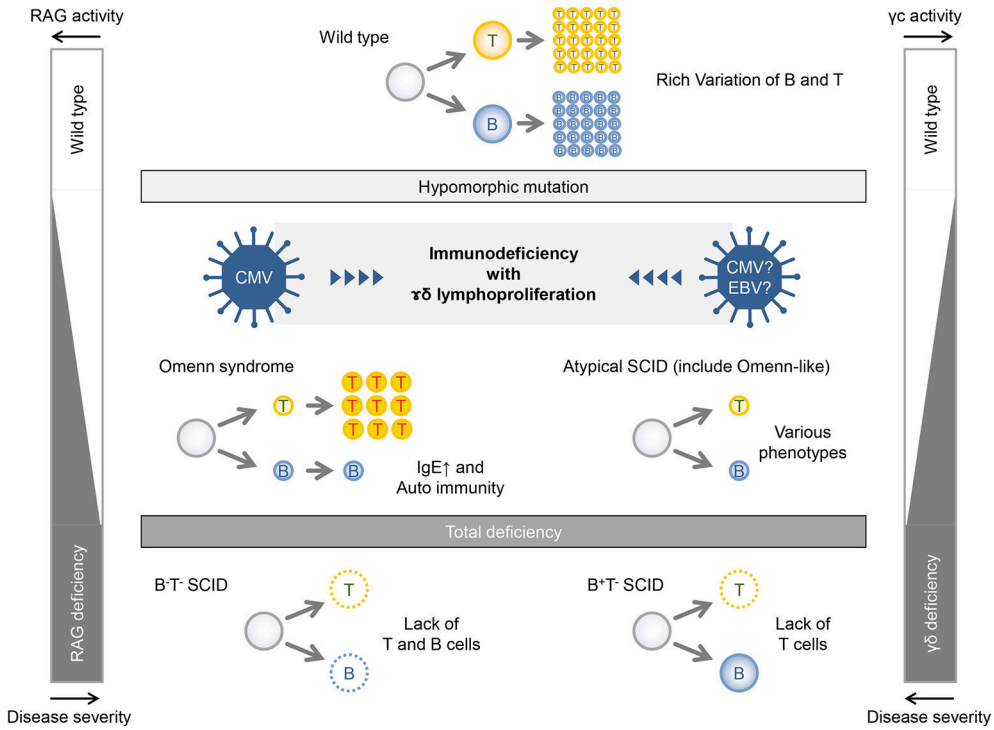

FIGURE 3 | Relationship between gene activities and disease severities in patients with RAG deficiency and γc deficiency.

The EBV latent infection type is classified into four types depending on the EBV genes expressed: latency I, latency II, latency III, and latency 0. Latency I is seen in Burkitt's lymphoma or nasopharyngeal carcinoma, latency II in Hodgkin lymphoma or nasal NK/T lymphoma, and latency III in opportunistic lymphoma with HIV infection and PIDs. In CAEBV, EBV infection shows latency II (11). Latency II infection in γδT cells might be compatible with CAEBV and other malignancies, and latency III infection in B cells might be compatible with PIDs.

The patient had EBV-associated leiomyoma at the age of 6 (6). EBV-positive smooth muscle tumor (SMT) is an extremely rare entity, and it is observed in patients infected with human immunodeficiency virus or undergoing immunosuppressive treatment after organ transplantation. In addition, SMT is observed in pediatric patients with PIDs including SCID (12).

Recombinase activating gene (RAG)1 and RAG2 are involved in V(D)J recombination of immunoglobulin and T-cell receptor (13). Patients with complete loss-of-function mutations of *RAG1/2* genes show complete lack of T and B cells ($T^-B^-NK^+$ SCID). On the other hand, in patients with remaining activity of RAG1/2 caused by hypomorphic mutation, B and T cells are somewhat differentiated, although they lose their diversity. This leads to the failure of immune tolerance, abnormal proliferation and activation, cytokine production biased toward Th2, and inappropriate IgE production by B-cell clones. Patients who have these conditions present with Omenn syndrome at birth (**Figure 3**, left panel). Patients with less hypomorphic RAG1/2 deficiency were reported to have CMV infection and γδT-cell proliferation (14, 15). In addition to the instability of immunity due to genetic abnormalities, environmental factors, such as viral infection might lead to γδT-cell proliferation. Likewise, patients with hypomorphic *IL2RG* mutation also present with an Omenn-like phenotype, while complete loss-of-function mutation in the *IL2RG* gene is linked to X-linked SCID ($T^-B^+NK^-$ SCID) (**Figure 3**, right panel). Partial activity of the *IL2RG* gene makes the immunity fragile and may facilitate the infection of herpesviridae viruses, such as CMV and EBV and may feature a characteristic pathological condition of γδT-cell proliferation as well as in the case of hypomorphic RAG1/2 deficiency with CMV infection and γδT-cell proliferation. Recently, the same mutation was noted in another patient with SCID; however, the phenotypic data for that case were not reported (16). Accordingly, this is the first description of the effect of this *IL2RG* mutation.

CONCLUDING REMARKS

The patient developed EBV-associated γδ T-cell lymphoproliferative disorder, which is virologically similar with γδ T-cell type CAEBV. The patient also presented atypical γc deficiency with hypomorphic *IL2RG* mutation. Although the diagnosis of CAEBV is made without underlying diseases including PIDs, the disease is supposed to be associated with immunological deficit. A few cases of CAEBV is associated with *PRF1* and *STXBP2* mutations (17, 18). Although the pathology of CAEBV remains unknown, the experience of this case suggests that immune abnormality is deeply involved in its onset. The

accumulation of such cases should promote our understanding of the pathophysiology of CAEBV and related illness.

ETHICS STATEMENT

This study was conducted in accordance with the Helsinki Declaration and approved by the Ethics Committee of Tokyo Medical and Dental University and written and informed parental consent was obtained for publication of this case report.

AUTHOR CONTRIBUTIONS

HK conceived the study. KT and HK wrote the manuscript. KT, AH, TO, and TY performed the immunological and genetic studies. K-II performed EBV studies. AH, TK, KEI, MY, AS, MI, HT, and HK were involved in the clinical care of the patient. TS and MR performed the whole exome sequencing. MT, KOI, SO, CK, and TM provided critical discussion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2019.00015/full#supplementary-material

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CASE REPORT
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Systemic Epstein–Barr Virus-Positive T/NK Lymphoproliferative Diseases With *SH2D1A/XIAP* Hypomorphic Gene Variants

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X-linked lymphoproliferative disease (XLP) is one of the X-linked primary immunodeficiency diseases (PIDs) with defective immune response to Epstein–Barr virus (EBV) infection. Chronic active EBV infection (CAEBV) and EBV-hemophagocytic lymphohistiocytosis (HLH) are recognized as systemic EBV-positive T-cell and natural killer (NK)-cell lymphoproliferative diseases (LPDs) arising from the clonal proliferations of EBV-infected T cells and NK cells. A high incidence of CAEBV in East Asia implies the unknown genetic predisposition. In patients with XLP, EBV-infected cells are generally B cells. No mutation of *SH2D1A/XIAP* genes has ever been identified in patients with systemic EBV-positive T-cell and NK-cell LPD. We report herewith a male case of NK-cell type CAEBV with *SH2D1A* hypomorphic mutation (c.7G > T, p.Ala3Ser), two male cases of CAEBV/EBV-HLH with *XIAP* hypomorphic variant (c.1045_1047delGAG, p.Glu349del), and another female case of $CD4^+$CAEBV with the same *XIAP* variant. The female underwent bone marrow transplantation from an HLA-matched sister with the *XIAP* variant and obtained a complete donor chimerism and a cure of laryngeal LPD lesion, but then suffered from donor-derived $CD4^+$ T cell EBV-LPD. These observations demonstrated that *SH2D1A* and *XIAP* genes are critical for the complete regulation of EBV-positive T/NK cell LPD. X-linked lymphoproliferative disease (XLP) is one of the X-linked primary immunodeficiency diseases (PIDs) reported to have a defective immune response to Epstein–Barr virus (EBV) infection. Mutations in *SH2D1A* and *XIAP* genes cause XLP. Systemic EBV-positive T-cell and natural killer (NK)-cell lymphoproliferative diseases (LPDs) consist of three major types: EBV-positive hemophagocytic lymphohistiocytosis (HLH), chronic active EBV infection (CAEBV), and EBV-positive T-cell/NK-cell lymphoma. CAEBV is recognized as a poor prognostic disease of EBV-associated T-cell and NK-cell LPD arising from the clonal proliferation of EBV-infected T cells ($CD4^+$, $CD8^+$, and $TCR\gamma\delta^+$) and/or NK cells. The majority of cases with CAEBV were reported from East Asia and South America. In Caucasian patients with CAEBV disease, the target of infection is exclusively B cells. These imply a genetic predisposition to EBV-positive T/NK cell LPD according to ethnicity.

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In reported cases with XLP, EBV-infected cells are B cells. On the other hand, no mutation of *SH2D1A*/*XIAP* genes have been determined in patients with T/NK-cell-type (Asian type) CAEBV. We here describe, for the first time, four case series of CAEBV/EBV-HLH patients who carried the hypomorphic variants of XLP-related genes. These cases included a male patient with CAEBV carrying *SH2D1A* hypomorphic mutation (c.7G > T, p.Ala3Ser) and two male patients with CAEBV/EBV-HLH carrying the *XIAP* hypomorphic variant (c.1045_1047delGAG, p.Glu349del), along with another female patient with CAEBV carrying the same *XIAP* variant. The female case underwent bone marrow transplantation from a healthy HLA-matched sister having the same *XIAP* variant. Although a complete donor chimerism was achieved with the resolution of laryngeal LPD lesions, systemic donor-derived $CD4^+$ T-cell EBV-LPD developed during the control phase of intractable graft- vs. -host-disease. These observations demonstrated that *SH2D1A* and *XIAP* genes are critical for the complete regulation of systemic EBV-positive T/NK-cell LPD.

Keywords: Epstein–Barr virus, chronic active EBV infection, lymphoproliferative disease, hemophagocytic lymphohistiocytosis, SAP, XIAP, X-linked lymphoproliferative disease

INTRODUCTION

Epstein–Barr virus (EBV) infects preferentially human B lymphocytes and epithelial cells. The majority of subjects are asymptomatic after a primary infection with EBV, and a part of them suffer from acute infectious mononucleosis (IM). EBV-positive T-cell and natural killer (NK)-cell lymphoproliferative diseases (LPDs) are classified into three major types: EBV-positive hemophagocytic lymphohistiocytosis (HLH), chronic active EBV infection (CAEBV), and EBV-positive T-cell/NK-cell lymphoma (1). CAEBV is a rare persistent active mononucleosis syndrome presenting with fever, liver dysfunction, cytopenias, and hepatoplenomegaly. The patients occasionally progress to the lethal course of HLH, LPD, and lymphoma. CAEBV is currently recognized as EBV-associated T/NK-cell LPD arising from the clonal proliferations of EBV-infected T cells ($CD4^+$, $CD8^+$, and $TCR\gamma\delta^+$) and/or NK cells. The majority of cases were reported from East Asia and South America. The reported cases of chronic EBV disease in the United States were exclusively B-cell-type (Caucasian type) CAEBV (2). These may account for the genetic predisposition to T/NK-cell-type (Asian type) CAEBV (3). Recently, Okuno et al. (4) have reported that somatic mutations of infected cells and gene mutations of EBV were concurrently involved in the development of T/NK cell type (Asian type) CAEBV, which were considered to be similar to malignant lymphoma. Allogenic hematopoietic stem cell plantations (HSTs) are needed for curing of progressive CAEBV (5). On the other hand, there is little information on the prolonged survival of patients with indolent CAEBV.

X-linked lymphoproliferative disease (XLP) is one of the X-linked primary immunodeficiency diseases (PIDs) with defective immune response to EBV infection (6). The manifestations of XLP are typified by EBV-driven fatal IM and/or HLH, regenerative anemia, dysgammaglobulinemia, and B-cell lymphoma. In cases of XLP-related EBV-HLH, EBV-infected cells are predominantly B cells (7, 8). Currently, two causative genes have been determined for XLP1 and XLP2: SLAM-associated protein (SAP) deficiency due to *SH2D1A* gene mutation called XLP1 and XIAP (X-linked inhibitor of apoptosis) deficiency due to *XIAP* gene (previously termed *BIRC4*) mutation called XLP2. SAP is composed almost exclusively of an Src homology 2 (SH2) domain (9). SAP expressed in T cells, NK cells, and natural killer T (NKT) cells enhances immune response binding to signaling lymphocyte activation molecule (SLAM) families. In XLP1 patients, decreased cytotoxic activities in $CD8^+$ T cells and NK cells are associated with the developing risk of HLH. In addition, the lack of invariant NKT (iNKT) cells leads to a nonexcludability of EBV-infected B cells and subsequent development of fatal IM or B-cell lymphoma. XIAP is a member of the inhibitor of apoptosis protein (IAP) family containing three baculovirus IAP repeat (BIR) domains and one really interesting new gene (RING) domain. XIAP regulates apoptotic cell death with the inhibition of procaspase 9 by BIR3 domain and the inhibition of caspase 3 and 7 by BIR2 domain (10). The RING domain ubiquitylates receptor-interacting serine/threonine-protein kinase 2 and recruits linear ubiquitin chain assembly complex (LUBAC) to nucleotide-binding oligomerization domain 2 (NOD2). LUBAC activity is required for efficient NF-κB activation and secretion of proinflammatory cytokines after NOD2 stimulation (11). Increased activation-induced cell death (AICD) in T cells and a decreased number of iNKT cells are found in XIAP-deficient patients (12). XLP2 patients with a mutation in the RING domain exhibit interference with ubiquitin ligase activity (11). The symptoms start usually in early childhood with recurrent infection, with HLH (frequently recurrent and of a more indolent course than seen in other primary HLH diseases) associated with chronic EBV disease, splenomegaly, and chronic colitis. In contrast to XLP1, B-cell lymphomas have not been reported in XLP2 patients (13).

We herein describe four novel cases of systemic EBV-related T/NK-LPD having the hypomorphic variants of *SH2D1A*/*XIAP* and discuss their association.

METHODS

Genetic Analysis

Genomic DNA was extracted from peripheral blood and/or biopsied samples of the lesion obtained from patients according to the standard method, after informed consent was obtained from the individuals or parents. Mutation analysis of the genes responsible for familial HLH (*PRF1*, *UNC13D*, *STX11*, and S*TXBP2*) and XLP or XLP-like (*SH2D1A*, *XIAP*, *ITK*, and *CD27*) was performed by a PCR-assisted DNA sequencing method with a capillary DNA sequencer (ABI3130/3730, Applied Biosystems, Foster City, CA, USA) in all cases, and the whole exome sequencing, using a short-read next-generation DNA sequencer (MiSeq, Illumina, San Diego, CA, USA) as described previously (14) in case 1 and case 4.

EBV Analysis

Analysis of EBV gene expression by real time-PCR was carried out as previously described (15). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Lymphosepar I (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). Then, $CD19^+$, $CD4^+$, $CD8^+$, and $CD56^+$ cells were serially removed from PBMCs using the IMag Cell Separation System (BD Biosciences, Franklin Lakes, NJ, USA). DNA extraction was performed for each fraction, and quantification of EBV-DNA was performed with real-time quantitative PCR based on the TaqMan system (Applied Biosystems, Foster City, CA, USA).

CLINICAL CASE REPORTS

Case 1: Male Patient With NK-Cell-Type CAEBV

An 8-years-old boy presented with high fever, photosensitivity, and hypersensitivity to mosquito bites and then received the diagnosis of NK-cell-type CAEBV. These manifestations have gradually relieved until 12 years of age. The comprehensive genetic analysis of peripheral blood-derived DNA revealed one reported pathological mutation of *SH2D1A* gene hemizygously (c. 7G > T, p.Ala3Ser) (16, 17). During the following 13 years, he has continued to have photosensitivity alone. Repeated laboratory tests have shown unremarkable titers of anti-EBV antibodies indicating past infection and low titer of EBV genome copies in peripheral blood (7.3×10^2/ml), with no any evidence of cytopenia, dysgammagulobulinemia, or elevation in soluble interleukin (IL)-2 receptor.

Case 2: Male Patient With NK/B-Cell-Type CAEBV

A 2-years-old boy had suffered from intermittent fever, diarrhea, and hypersensitivity to mosquito bites. An EBV genome load was high in $CD19^+$ B cells (5.6×10^3 copies/μgDNA) and slightly positive levels in $CD16^+$ NK cells (8.1×10^1 copies/μgDNA). The comprehensive genetic analysis of peripheral blood-derived DNA determined a reported hemizygous variant of *XIAP* gene (c.1045_1047delGAG, p.Glu349del) (7, 8). NK cell activity was 18 %lysis (reference range; 18–40). After the diagnosis of chronic EBV^+B-LPD, four courses of anti-CD20 antibody (Rituxan®, Chugai Pharmaceutical Co., LTD., Tokyo, Japan) therapies led to a complete disappearance of the EBV genome in circulation and an improvement in hypersensitivity to mosquito bites. Six months after rituximab therapies, a reappearance of B cells in the peripheral blood without the detection of EBV genome indicated the eradication of EBV-B-LPD. However, EBV genome level was again positive (1.5×10^3 copies/μgDNA of whole peripheral blood) 10 months after rituximab therapy, but there were no symptoms or abnormal data, including immunoglobulin levels, in the follow-up screening tests.

Case 3: Male Patient With $CD8^+$ T-Cell-Type EBV-HLH

A 1-year-old boy developed fever, skin eruptions, and hepatoplenomegaly with pancytopenia, hyperferritinemia (5,181 ng/ml), and elevated soluble IL-2 receptor (6,797 U/ml). Anti-EBV antibodies indicated a primary infection of EBV. High EBV loads in peripheral blood and $CD8^+$ T cells of the patient (1×10^5 copies/ml and 1×10^6 copies/μgDNA, respectively) led to the diagnosis of EBV-HLH. NK-cell activity was 30 %lysis in normal (reference range; 18–40). Additional two courses of etoposide injection (100 mg/m^2) were needed to control the relapsing HLH after the immunomodulation therapy using high-dose intravenous immunoglobulin, oral cyclosporine, and prednisolone. Circulating levels of EBV genome came to be undetectable after the immunochemotherapy. The comprehensive genetic analysis of peripheral blood-derived DNA determined a hemizygous variant of the *XIAP* gene (c.1045_1047delGAG, p.Glu349del). He is alive and well, without sequelae or dysgammaglobulinemia at 7 years of age. The numbers of $CD19^+IgD^-CD27^+$ switched memory B cells and $CD4^+CD45RA^-CXCR5^+$ follicular helper T cells were not decreased (data not shown).

Case 4: Female Patient With NK/$CD4^+$ T-Cell-Type CAEBV

A 24-years-old woman was hospitalized because of dyspnea and hoarseness. The patient had received the diagnosis of NK-cell and $CD4^+$ T-cell-type CAEBV because of recurrent fever and hypersensitivity to mosquito bites at age 14 years. Histopathological and molecular studies of the cutaneous lesion

Abbreviations: AICD, activation-induced cell death; BIR, baculovirus IAP repeat; BMT, bone marrow transplantation; CAEBV, chronic active Epstein–Barr virus infection; EBV, Epstein–Barr virus; FDG-PET, fluorodeoxyglucose-positron emission tomography; HLA, human leukocyte antigens; HST, hematopoietic stem cell transplantation; HLH, hemophagocytic lymphohistiocytosis; IL, interleukin; LPD, lymphoproliferative disease; LUBAC, recruits linear ubiquitin chain assembly complex; NK, natural killer; NOD2, nucleotide-binding oligomerization domain 2; PID, primary immunodeficiency disease; RING, really interesting new gene; SAP, SLAM-associated protein; SH2, Src homology 2; SNP, single nucleotide polymorphism; XIAP, X-linked inhibitor of apoptosis; XLP, X-linked lymphoproliferative disease.

indicated clonal proliferation of EBV-infected cells. Thereafter, clinical resolution and declining levels of EBV load in circulation had allowed no treatment and observation. After admission, an urgent tracheostomy prevented airway obstruction by the laryngeal mass (**Figure 1A**). She was then transferred to our hospital for further management. Fluorodeoxyglucose-positron emission tomography (FDG-PET) showed increased levels of uptake in the stomach and terminal ileum as well as the laryngeal lesion (**Figure 1B**). Circulating EBV DNA was at undetectable levels. However, histopathological and molecular analysis of the laryngeal lesions demonstrated a proliferation of EBER-positive $CD4^+$ cells and increased copy number of EBV-DNA ($2–4 \times 10^3$ copies/μgDNA). The comprehensive genetic analysis of peripheral blood-derived DNA identified a heterozygous variant of *XIAP* gene (c.1045_1047delGAG, p.Glu349del) alone. A histocompatible sister aged 20 years carried the same *XIAP* variant. The anti-EBV antibody titers and undetectable EBV DNA in circulation verified a past infection of EBV in the healthy sister. The gene expression analysis indicated no skewing inactivation of X chromosome among DNA samples obtained from the bone marrow cells, PBMCs, and laryngeal tumor of the patient as well as PBMC of the sister (data not shown). After four courses of combined chemotherapies with cyclophosphamide, pirarubicin, vincristine, steroid, and etoposide (CHOP-VP), the patient underwent bone marrow transplantation from the sister. The laryngeal lesion disappeared after a compete donor chimerism was achieved (**Figure 1C**). However, systemic but not local proliferation of EBV-infected donor-derived $CD4^+$ T cells (1×10^4 copies/ml of whole peripheral blood and 3×10^3 copies/μgDNA of $CD4^+$T cells, respectively) developed 2 months posttransplantation (**Figure 1D**). Discontinuation of immunosuppresants and donor lymphocyte infusions effectively controlled the posttransplantation LPD, but she died of uncontrollable severe graft-vs.-host-disease with Candida sepsis.

None of the four patients had a positive family history suggesting PID and/or chronic EBV disease. The clinical profile and treatment course of these patients are summarized in **Table 1**.

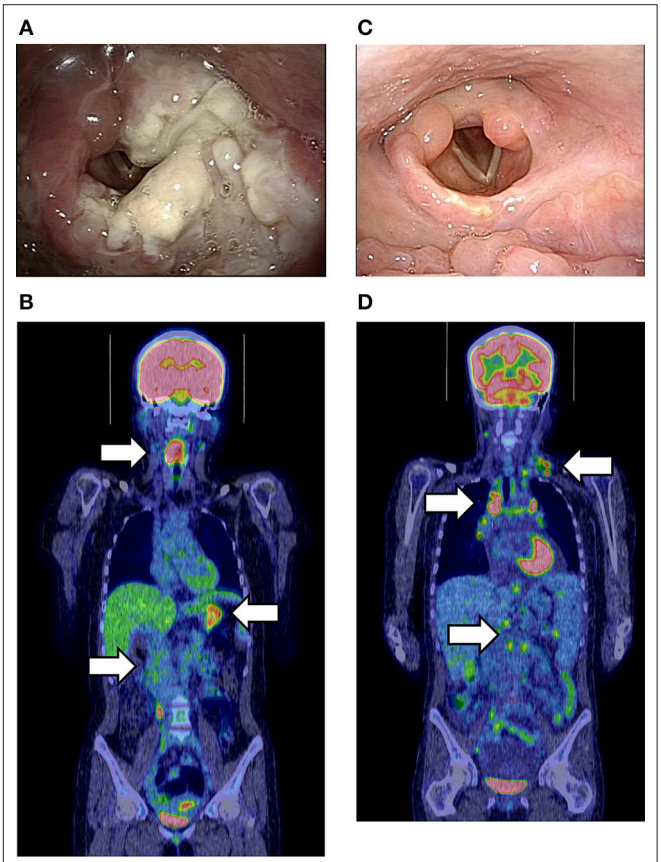

FIGURE 1 | (A) Endoscopic findings of the laryngeal lesion of $CD4^+$T-cell lymphoproliferative disease (LPD) lesion in case 4 prior to cancer chemotherapy. **(B)** Fluorodeoxyglucose-positron emission tomography (FDG-PET) at the onset of LPD. White arrows show the increased FDG uptake in the larynx, stomach, and terminal ileum. The maximum standardized uptake value (SUVmax) was 11.8. **(C)** Improvement of the laryngeal LPD lesion after bone marrow transplantation (BMT). **(D)** FDG-PET at the onset of donor-derived CD4+T-LPD after BMT. White arrows show the increased FDG uptake in multiple lymph nodes without laryngeal lesion. The SUVmax was 9.7.

DISCUSSION

We report the first case series of CAEBV/EBV-HLH patients who carried the hypomorphic mutation of XLP-related genes. B cells are the major target of EBV infection in patients with XLP. No mutation in *SH2D1A*/*XIAP* genes has been reported in patients with CAEBV (4, 18). Patient 1 with SAP Ala3Ser and patients 2, 3, and 4 with XIAP Glu349del presented with the phenotype of NK^+CAEBV, B^+/NK^+CAEBV, $CD8^+$EBV-HLH, and $NK^+/CD4^+$CAEBV, respectively. EBV preferentially infects B cells *via* CD21 and also infects T cells or NK cells at a lower frequency during the acute phase of viremia (3). The absolute number of EBV-infected T/NK cells would be too small to have an advantage for proliferating in healthy individuals. On the other hand, SAP/XIAP variant carriers may have a modest ability to control the proliferation of EBV-infected T/NK cells.

Low levels of circulating EBV DNA continue to be detected in NK cells, but no symptoms other than photosensitivity have continued in patient 1 for 17 years. The *SH2D1A* gene variant (c.7G > T, p.Ala3Ser) is located at the N-terminal of the SAP protein, outside of the SH2 domain, with residual protein functions. This variant has no deleterious effect on the function depending on SH2 domain. A healthy elderly male carrier with c.7G > T *SH2D1A* variant indicates that the variant does not always develop XLP or lead to fatal outcomes for patients (17). However, one male patient with the same p.Ala3Ser variant demonstrated a marked reduction in SAP expression in $CD8^+$ T cells (2.7%, reference range: 21.6–90.8%) and died at the age of 40 years with EBV infection and HLH (16). Another male patient with the hemizygous *SH2D1A* variant (c.7G > T) and heterozygous missense *PRF1* mutation (c.127C > A, p.Leu43Met) reportedly suffered from severe HLH and required HST, and the other female with the heterozygous *SH2D1A* variant (c.7G > T) had a lethal HLH (19). The *SH2D1A* c.7G > T may

TABLE 1 | Summary of the patients with *SH2D1A/XIAP* mutations who developed EBV-infected T/NK cell LPD.

Case	Sex	Gene mutation	Diagnosis	Age at onset (years)	Age at treatment (years)	EBV-infected cell type	Treatment	Follow-up period (years)	Outcome
1	Male	*SH2D1A*: c.7G>T (hemizygous)	CAEBV	8	Not yet	NK cells	none	17	Alive
2	Male	*XIAP*: c.1045_1047delGAG (hemizygous)	CAEBV	2	2	B cells and NK cells	RTX (four courses)	2	Alive
3	Male	*XIAP*: c.1045_1047delGAG (hemizygous)	EBV-HLH	1	1	$CD8^+$T cells	VP-16, PSL, IVIG	7	Alive
4	Female	*XIAP*: c.1045_1047delGAG (heterozygous)	CAEBV	14	24	$CD4^+$T cells	Chemotherapy* (four courses), rBMT	12	Deceased

CAEBV, chronic active Epstein-Barr virus; EBV, Epstein-Barr virus; EBV-HLH, EBV-associated hemophagocytic lymphohistiocytosis; IVIG, intravenous immunoglobulin; LPD, lymphoproliferative disorder; PSL, prednisolone; rBMT, related donor bone marrow transplantation; RTX, rituximab.

**Chemotherapy: etoposide 100–150 mg/m^2 × 3 days, cyclophosphamide 750 mg/m^2 × 1 day, vincristine 1.5 mg/m^2 × 1 day, pirarubicin 25 mg/m^2 × 2 days, PSL 1 mg/kg × 3 days.*

be pathogenic in cases with late onset or indolent expression of disease.

The XIAP Glu349del (rs199683465) was reportedly detected in 3.5% of healthy Japanese people. The variant may be a single nucleotide polymorphism exerting the founder effect in Japanese people (20). Although the XIAP protein expression was normal in the Glu349del variant, the numbers of $CD19^+IgD^-CD27^+$ switched memory B cells and $CD4^+CD45RA^-CXCR5^+$ follicular helper T cells were decreased and immunoglobulin production was reduced *in vitro*. Immunoglobulin-related gene expression was also decreased in the variant carriers. On the other hand, they did not exhibit increased AICD because Glu349 is distant from the BIR2 or BIR3 domains. It remains unclear how the Glu349del mutation affects the NOD pathways (20).

Male patient 2, with persistent EBV infection in B/NK cells accompanied by hypersensitivity to mosquito bites, was diagnosed as having CAEBV. Rituximab therapy temporarily cleared EBV genome and all signs and symptoms. Although the EBV copy number was increased again, no symptoms recurred. Persistent EBV infection leads to the diagnosis of XLP2 without dysgammaglobulinemia or HLH. Patient 3 is the first Japanese individual who is a Glu349del carrier who developed EBV-HLH. No HLH developed in the three reported Japanese XLP2 patients with Glu349del (20). On the other hand, a 1-year-old Chinese male with XIAP Glu349del has been recently reported to present with HLH (21). Patient 3 suffered from severe $CD8^+$EBV-HLH requiring chemotherapy, which did not recur after the first resolution. Unlike other *XIAP* mutations, the Glu349del variant would not lead to uncontrollable or relapsing HLH.

The major concern is the intractable course of patient 4 posttransplantation. How did a heterozygous *XIAP* variant affect the female patient? The *XIAP* variant is a genetic polymorphism found in 3.5% of healthy Japanese individuals. The healthy sister of patient 4 had experienced a primary infection of EBV. Therefore, heterozygous variant carriers are less likely to have acute fatal IM and/or chronic EBV diseases. X-linked recessive PID develops in females with X chromosome skewing (22, 23), but no skewing expression of *XIAP* was determined in the PBMC or LPD lesion of patient 4. EBV DNA levels did not increase in the peripheral blood even at the time of developing LPD after 10 years from the first CAEBV diagnosis. Additional factors such as somatic mutations and EBV genome mutations might contribute to the evolution of laryngeal tumor (4). Furthermore, as shown from her developing posttransplanted donor-derived EBV-related $CD4^+$T-LPD, unknown host genetic predispositions other than *XIAP* or immunocompromised state of suppressing EBV-specific CTL activity might be involved in the onset of CAEBV (24). Somatic mutations of EBV-infected donor $CD4^+$ T cells might also affect the developing posttransplantation EBV-T-LPD (4). Genetic backgrounds, including hypomorphic variants, may need to be considered in the selection of HST donors for the cure of systemic EBV-positive T/NK cell LPD.

CONCLUSIONS

We reported the first four case series of CAEBV/EBV-HLH with *SH2D1A*/*XIAP* gene variants. PID-related genetic predispositions to EBV infection should be considered for the treatment of EBV-T/NK cell LPD.

ETHICS STATEMENT

This study was carried out in accordance with the Declaration of Helsinki and the recommendations of the institutional review board of Kyushu University. The protocol was approved by the institutional review board of Kyushu University (#531-01). The subjects (or their parents) gave written informed consent about the study and the publication of this report.

AUTHOR CONTRIBUTIONS

MI and SO were the principal investigators, taking primary responsibility for the paper. AS, KE, MS, and YA performed the clinical management with helpful discussion regarding the

completion of the work. KI completed the EBV analysis. MS and HY completed the genetic analysis.

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Cytologic Analysis of Epstein-Barr Virus-Associated T/Natural Killer-Cell Lymphoproliferative Diseases

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Rapid, precise diagnosis of Epstein-Barr virus-associated T lymphocyte or natural killer cell lymphoproliferative diseases is clinically important to prevent disease progression and avoid fatal outcomes for patients. In addition to detecting increased copy numbers of Epstein-Barr virus, identification of the lymphocyte subpopulation targeted by the virus infection is crucial to reaching the final diagnosis. However, these procedures are laborious and require large amounts of sample. In contrast, flowcytometric analysis may provide crucial information for initial screening of diseases using only small amounts of sample and involves little labor. In addition to the increase of a particular subpopulation, selective HLA-DR expression indicates selective activation and expansion of a virus-infected clone. Presence of a characteristic HLA-DR^{high} CD5$^{dim/negative}$ fraction within CD8^{+} T lymphocytes indicates a possible diagnosis of Epstein-Barr virus-associateds hemophagocytic lymphohistiocytosis. One should note, however, that cases with familial hemophagocytic lymphohistiocytosis may exhibit a similar abnormal fraction within CD8^{+} T lymphocytes. These T cells are oligoclonally expanded reactive T cells expanding in response to B cells infected with Epstein-Barr virus.

Keywords: epstein-barr virus, EBV-associated hemophagocytic lymphohistiocytosis, chronic active EBV infection, hydroa vacciniforme, hypersensitivity to mosquito bite, flowcytometry

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INTRODUCTION

Epstein-Barr virus (EBV) is one of the most ubiquitous infectious viruses, with >90% of the human beings infected worldwide (1, 2). Primary EBV infection occasionally results in infectious mononucleosis in children and young adults. In the majority of individuals infected with EBV, latent infection is established for life without any specific clinical manifestation. In primary EBV infection, B cells are the cellular targets of EBV and latent EBV infection persists for life in B cells, as well as in nasopharyngeal cells. For this reason, these cells play an important role as a reservoir for EBV (3). In hosts with normal immune function, EBV latently infects B cells without any pathological disorders, but EBV is also known to play significant roles in the pathogenesis of various non-hematological and hematological diseases. EBV rarely causes two clinically distinct and unusual infections: the acute form of fulminant life-threatening diseases with hemophagocytic lymphohistiocytosis (HLH); and chronic persistent infection associated with systemic organ involvement and malignant transformation at the late stage (4, 5). These

two diseases are clinically defined as EBV-associated HLH (EBV-HLH) and chronic active EBV infection (CAEBV), respectively. In these two diseases, ectopic EBV infection in lymphocyte subpopulations other than B lymphocyte has been demonstrated and plays significant roles in the pathogenesis of these diseases (6). As the major targets of EBV infection are T cells and natural killer (NK) cells, these pathologies are collectively called EBV-associated T/NK-cell lymphoproliferative diseases (LPD). Although asymptomatic primary EBV infection or acute infectious mononucleosis usually require no specific treatment, early diagnosis, and therapeutic interventions are critical for patients with EBV-HLH or CAEBV. In this article, I briefly review the clinical characteristics of the two important EBV-associated T/NK-cell LPDs. Next, the particular utility of cytologic analysis in the diagnosis of these pathologies is frequently overlooked, but the clinically significant illnesses are discussed.

EBV-ASSOCIATED HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

HLH is an acute systemic inflammatory illness characterized by macrophage activation, hemophagocytosis in the bone marrow, pancytopenia, and hepatosplenomegaly, all of which reflect intense inflammatory cytokine production *in vivo* (7). HLH has multiple primary causes, including infections, collagen vascular diseases, malignancies and some metabolic diseases. Primary immunodeficiency diseases affecting NK function and killer T-cell functions are also the targets of HLH. EBV is the most common triggering agent of HLH in Japan, particularly among children 1–15 years old (8). Because immunochemotherapy with etoposide and corticosteroids can be lifesaving for patients with EBV-HLH, early correct diagnosis is of paramount importance (9, 10). Left unrecognized, these patients may experience rapidly progressive cytokinemia and deterioration of multiple organ functions. Although the HLH-2004 protocol has been shown to be helpful in establishing the diagnosis of HLH, some findings in these criteria only occur late in the disease course, and therapeutic intervention is thus often delayed if clinicians wait until the criteria are satisfied (11, 12). Data on valuable diagnostic parameters, including EBV copy numbers, profiles of inflammatory cytokines, NK cell function, and mutation analysis of the genes related to genetic HLH, are only available from specialized laboratories. In addition, it is difficult to distinguish EBV-HLH from IM by serological tests for EBV and routine immunophenotypic analysis of lymphocyte subsets. Patients with IM sometimes exhibit marked T cell activation and cytokine production to regulate EBV-infected B cells. Therefore, infectious mononucleosis may share some typical clinical features with EBV-HLH, such as cytopenia, hypercytokinemia, and hemophagocytosis, even though IM involves a benign self-limited episode and usually does not require any specific treatment. Diagnosing EBV-HLH in the early stage thus remains difficult.

In acute infectious mononucleosis, B cells are the targets of EBV infection and T cells are activated to control the expansion of these EBV-infected B cells. In contrast, EBV ectopically infects $CD8^+$ T cells without producing a sufficient number of EBV-specific cytotoxic T cells in EBV-HLH (6). For this reason, therapeutic intervention differs critically for these two distinct categories of EBV-associated lymphoproliferation. Controlling hypercytokinemia is a sufficient goal in acute infectious mononucleosis. In contrast, control of abnormal expansion of EBV-infected $CD8^+$ T clones is mandatory in addition to cytokine regulation in EBV-HLH. Finding targets of EBV infection during the acute episode of EBV-HLH is technically difficult when the peripheral lymphocyte count is very limited and the patients are generally young. A novel rapid and easy diagnostic approach to detect clonal expansion of EBV-infected $CD8^+$ T cells is thus highly desirable.

CHRONIC ACTIVE EBV INFECTION

CAEBV is characterized by prolonged or intermittent IM-like symptoms, such as fever, general malaise, liver dysfunction, lymph node swelling, and hepatosplenomegaly. In CAEBV, EBV infects lymphocytes other than B lymphocytes (13). Proof of ectopic EBV infection is very important in the precise diagnosis of CAEBV. We do not know at present if common pathogenetic mechanisms underlie the onset of each CAEBV case. However, it is very important that these cases share common clinical features, such as ectopic infection by EBV of a single clone of NK cells or T lymphocytes, and appearance during the course of illness of an acute episode of HLH or malignant transformation of the EBV-infected cells at later stages. Diagnostic criteria for CAEBV include the following according to the guideline proposed in 2005 (14):

- Persistent or recurrent IM-like symptom;
- Unusual pattern of anti-EBV antibodies with raised anti-VCA and anti-EA, and/or detection of increased EBV genomes in affected tissues, including peripheral blood; and
- Chronic illness that cannot be explained by other known disease processes at diagnosis.

The cardinal features of the above criteria are the detection of EBV in cells other than B lymphocytes (NK cells, $CD4^+$ T cells, $CD8^+$ T cells or TCRγδ T cells), and confirmation of increased EBV genome in the peripheral blood or tissue specimens. CAEBV consists of several different clinical phenotypes (15). Distinct clinical profiles for each phenotype seem to reflect the differences in the targets of EBV infection in each disease. Two well-characterized CAEBV and related illnesses with distinct skin manifestations are hypersensitivity to mosquito bite (HMB) and hydroa vacciniforme (HV).

HMB is known for its distinct clinical features, including intense local skin reaction to mosquito bite, which includes early inflammatory vesicle formation soon after mosquito bites, followed by development of a large erythematous lesion with central necrosis or crust (16). The tissue damage is so intense that the vesicles are often associated with intravesicular hemorrhage. Dermal inflammation is generally very deep, resulting in ulceration and necrosis with thick crust and scar formation (17). Patients often show old scars from mosquito bites of

the previous seasons. The severe skin lesions are associated with systemic symptoms, including fever and general malaise. Liver dysfunction is generally observed. For severe cases in later years, patients with HMB may show signs of HLH and hypercytokinemia. Virtually all patients with HMB show high titers of specific immunoglobulin (Ig) E against mosquito antigens and have antigen-specific $CD4^+$ T cells. Peripheral blood basophils and T lymphocytes from patients therefore show positive reactions in response to mosquito antigens *in vitro* (18, 19).

Because the clinical characteristics of HMB are relatively easy to recognize, diagnosis can be made early after onset, once the physician can recall this particular illness from the typical symptoms. However, some conditions show a similar clinical presentation after mosquito bites. Some patients who have specific IgE antibody to mosquito antigen may show immediate, intense skin reaction after mosquito bites. These skin lesions do not persist for long and the lesion will heal without forming local necrosis or scar formation (20). The patients never present with fever, malaise, or liver dysfunction. Another subset of patients who show intense reaction to mosquito bites present with systemic symptoms including fever and liver dysfunction. Again, the skin lesions are not severe and go away within several days without scar formation. Clinical diagnosis of HMB is thus not very difficult, but requires demonstration of an increase in EBV copy number in peripheral blood and ectopic EBV infection of NK cells, and rarely other non-B lymphocytes. Flowcytometric (FCM) analysis offers rapid screening to detect increased, activated NK cells in these patients.

HV is another unique EBV-associated LPD, in which the patient shows characteristic skin lesions on ultraviolet-exposed areas, including the ear lobes, dorsal surfaces of the hands, and cheeks. HV can be divided into a benign classic type and more aggressive systemic-type illnesses (21, 22). The skin lesions often become worse in seasons with increased UV exposure, typically May through September. Some patients show acute aggravation of skin lesions after going skiing in winter because of the intense reflection of UV from the snow-covered ski area. Similar to HMB, the skin lesions in HV are deep, with vesicles that are often hemorrhagic, leaving scars after healing. In contrast to HMB patients, many patients with HV do not show systemic symptoms even when the skin symptoms become aggravated. Patients with HV on rare occasions progress to show systemic symptoms and may be categorized under the diagnosis of CAEBV. In virtually all cases, monoclonal expansion of EBV is identified (23). As many patients with HMB show ectopic EBV infection of NK cells, patients with HV show activation and expansion of EBV-infected γδ T cells. Fractions of γδ T cells in peripheral blood are increased and are selectively activated, again offering tools for early diagnosis of the disease (24).

According to the data analyzed in Japan, age at onset >9 years old and activation of EBV-associated genes at the local skin lesions are regarded as poor prognostic factors (25). Furthermore, cases of benign classic-type illness progressing to aggressive systemic type illness have been reported (26). Monitoring the activity of EBV-infected T-cell clones by FCM and pathological analysis is therefore important.

FLOWCYTOMETRIC ANALYSIS OF CIRCULATING LYMPHOCYTES

FCM analysis of peripheral blood lymphocytes offers useful diagnostic information in some EBV-related diseases, and provides many advantages. FCM is a rapid assay requiring <1 h to complete. This analysis can be performed in any standard laboratory, and does not require a large amount of sample. Similar to other laboratory examinations, the assay can be repeated many times. For these reasons, FCM should be used as the first screening examination when EBV-associated T/NK-cell LPD is suspected. FCM examination consists of multiple steps.

First, we routinely examine the distributions of lymphocyte subpopulations as a screening assay to see if a particular subpopulation of lymphocytes is abnormally expanded. In many cases of EBV-associated T/NK-cell LPD, EBV-infected lymphocytes are increased in number. Simple analysis of the lymphocyte subpopulation will thus reveal abnormally increased NK cells, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes or TCRγδ T cells. However, the increase in percentages of a particular lymphocyte subpopulation may vary depending on the clinical course, and further examination of the activation status of each lymphocyte subpopulation is therefore warranted.

In the next step, the activation status of each lymphocyte subpopulation is evaluated using expression of HLA-DR as an indicator of cell activation (**Figure 1**). Because clinical symptoms, including skin lesions, often suggest the clinical diagnosis and the target of EBV-infection, identifying selective activation of a particular lymphocyte subpopulation is particularly important when the responsible subpopulation is not increased to a significant level.

TCRγδ T cells are usually increased well above average levels in patients with HV (**Figure 1A**) (23). Furthermore, these cells are highly activated as suggested by the increased levels of HLA-DR expression. Activation is selective and no other lymphocyte subpopulations show increased HLA-DR in these cases. In contrast, NK cells are increased and highly activated in HMB (**Figure 1B**).

The third step is for cases with T-cell proliferation. We examine the distribution of TCR Vβ repertoire usages by FCM to identify any clonal expansion of either $CD4^+$ or $CD8^+$ T cells. In some cases, clonal expansion of EBV-infected T cells is recognized by commercially available monoclonal antibody (27). We can follow-up patients using the particular TCR Vβ as a sensitive marker for the presence of a sizable residual clone after treatment starts.

Finally, we look for a particular fraction of $CD8^+$ T cells when EBV-HLH is suspected (**Figure 2**). For example, in acute IM, significant activation and expansion of $CD8^+$ T cells is detectable. Increases in $CD3^+$ T cells expressing high levels of HLA-DR and $CD45RO^+$ memory $CD8^+$ T cells are the characteristic findings in acute IM (28). It is important to note that the FCM

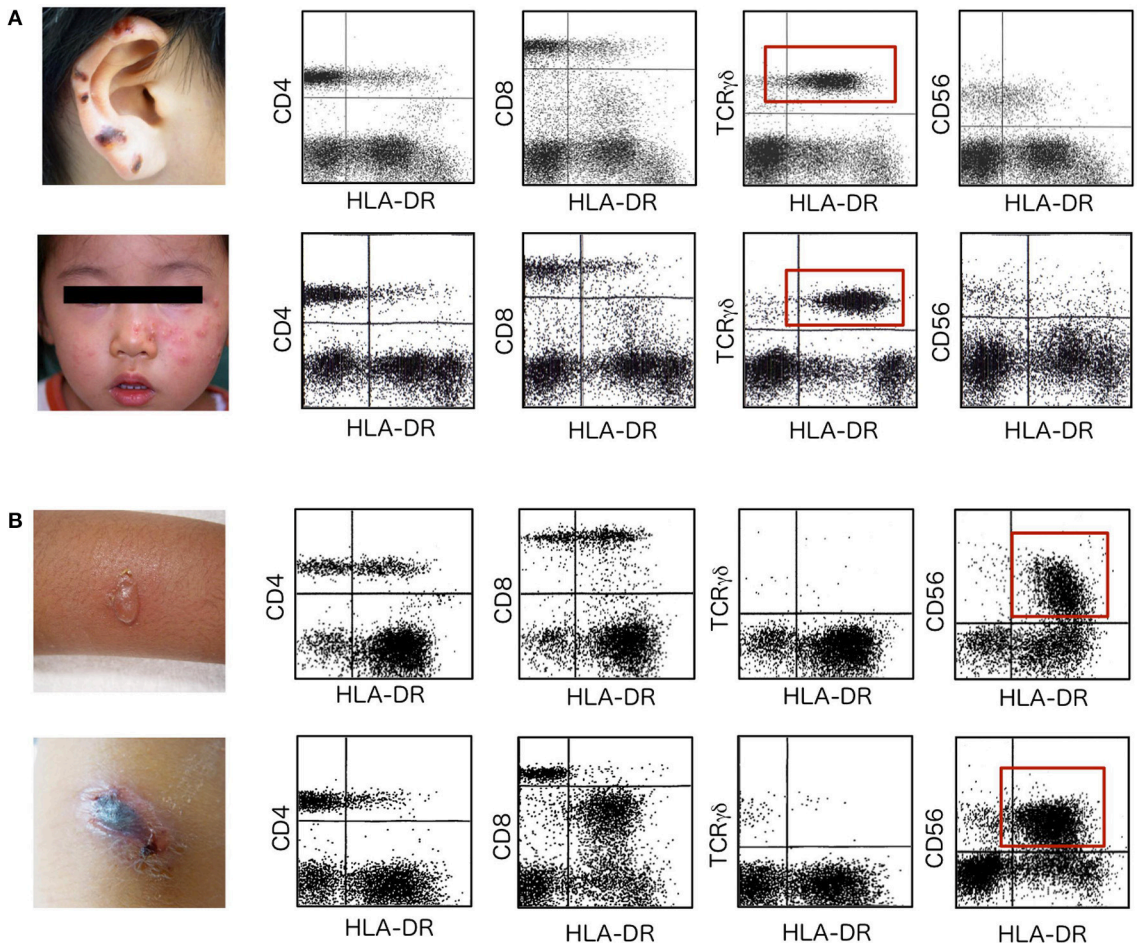

FIGURE 1 | HLA-DR expression on lymphocyte subpopulations. Flowcytometric analysis of HLA-DR expression was performed to detect selective expansion and activation of a particular lymphocyte subpopulation in patients with HV and HMB. **(A)** Upper data are from a 12-year-old boy with hemorrhagic vesicles that started to appear 2 years earlier on sun-exposed areas of skin, including the earlobes. Lower data are from a 2-year-old girl who presented with clusters of erythematous vesicles on bilateral cheeks. In these two patients with HV, TCRγδ T cells are increased in number and express HLA-DR to significant levels (red squares). Other lymphocyte subpopulations, including $CD4^+$ T cells, $CD8^+$ T cells and $CD56^+$ NK cells, express little HLA-DR on the cell surface. Skin lesions are frequently observed on UV-exposed areas including the ear lobes, cheek of the face and dorsal surfaces of the hands. **(B)** Upper data are from an 8-year-old girl who had a 5-year history of repeated episodes of vesicle formation and fever after mosquito bites. Lower data are from a 10-year-old boy who started to experience intense skin lesions with hemorrhagic vesicles with fever and general malaise 1 year before the first visit. In patients with HMB, $CD56^+$ NK cells are increased and levels of HLA-DR expression are extremely increased (red squares).

findings of acute infectious mononucleosis and EBV-HLH are indistinguishable if only T-cell activation and increase in memory $CD8^+$ T cells are analyzed. In the following paragraphs, examples of such analyses are shown.

In a series of studies comparing lymphocyte phenotypes in acute infectious mononucleosis and EBV-HLH, we found characteristic features observed only in EBV-HLH (**Figure 2**) (29, 30). Without exception, certain fractions among $CD8^+$ T cells show lost or diminished expression of CD5 on the cell surface. These $CD5^{dim/negative}$ $CD8^+$ T cells express particularly significant levels of HLA-DR, forming distinct clusters of $CD5^{dim/negative}$ $HLA\text{-}DR^{high}$ cells among $CD8^+$ T cells (**Figure 2A**, EBV-HLH, red square). These abnormal cell fractions can be detected at the earliest stage of EBV-HLH and offer a useful diagnostic clue. They are never detected in cases of acute IM, however severe the case is. In EBV-HLH, EBV infects a single clone of $CD8^+$ T cells, leading to massive expansion and activation of the infected clone. Vigorous production of inflammatory cytokines, including interferon γ, by these activated cells is responsible for the significant clinical symptoms seen in EBV-HLH (31). Because the abnormal $CD5^{dim/negative}$ $HLA\text{-}DR^{high}$ $CD8^+$ T cells are derived from a single clone, the clonal origin of these cells can be identified by analyzing expression profiles of TCR Vβ using FCM (**Figure 2B**, EBV-HLH). If the particular clone uses TCR Vβ detectable by commercially available antibody, selective expansion of cells with a particular TCR Vβ can be identified as $CD5^{dim/negative}$ $HLA\text{-}DR^{high}$ $CD8^+$ T cells.

Percentages of $CD5^{dim/negative}$ $HLA\text{-}DR^{high}$ $CD8^+$ T cells correlate relatively well with serum ferritin levels and EBV

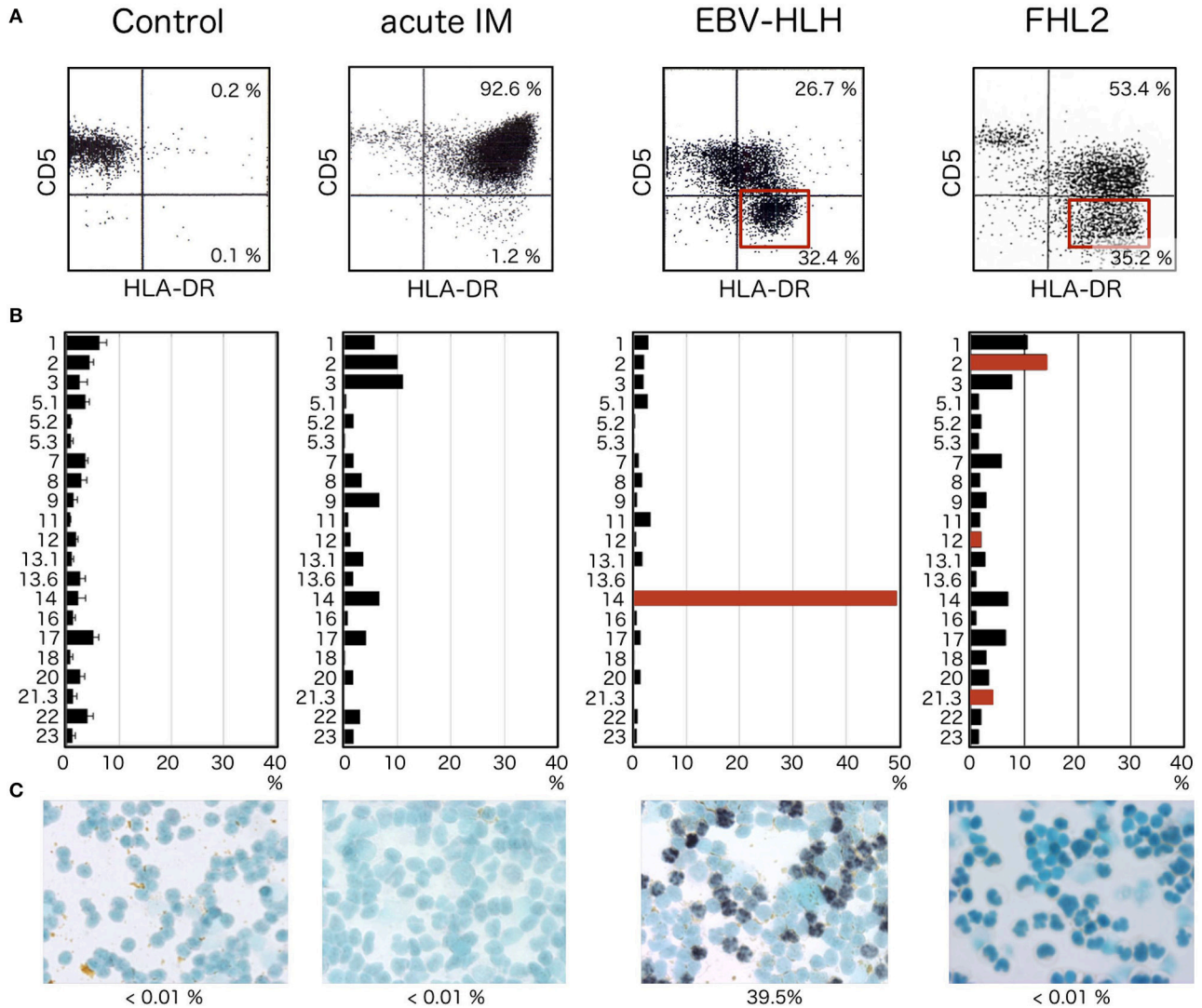

FIGURE 2 | HLA-DR and CD5 expression in patients with acute HLH. **(A)** Expressions of CD5 and HLA-DR simultaneously examined by 3-color flowcytometry. Within the lymphocyte region, $CD8^+$ cells are further gated and analyzed for expression of CD5 and HLA-DR. $CD5^{dim/negative}$ $HLA\text{-}DR^{high}$ $CD8^+$ T cells are not detected in controls. Although $CD8^+$ T cells express high levels of HLA-DR in acute infectious mononucleosis, most of these cells express normal, or only slightly decreased, levels of CD5. In contrast, significant fractions of $CD5^{dim/negative}$ $HLA\text{-}DR^{high}$ cells are seen within $CD8^+$ T cells from EBV-HLH patients (red square). In cases with FHL, in which intense activation of oligoclonal T cells occurs as a response to EBV infection of B cells, significant reduction of CD5 is seen among $CD8^+$ T cells (red square). **(B)** TCR Vβ distribution analyzed by FCM using commercially available monoclonal antibodies against different Vβ. Selective expansion of a single clone of $CD8^+$ T cells is identified by a significant increase in T cells with a specific Vβ (red bar), whereas cells expressing other types of Vβ are universally suppressed. In patients with FHL2, several clones with different Vβs are activated with diminished expression of CD5 (red bars). **(C)** Only $CD8^+$ T cells from EBV-HLH show EBER-1-positive cells within sorted $CD8^+$ T-cell fractions. $CD8^+$ T cells from controls or patients with acute infectious mononucleosis or FHL2 do not show EBER-1 positivity.

copy numbers in peripheral blood. Detection of this particular cell fraction is thus useful not only for early diagnosis of EBV-HLH, but also valuable for easy, repeatable evaluation of the clinical responses to therapy and prediction of the prognosis. Percentages of the abnormal cell fraction reduce rapidly in parallel with response to appropriate therapy, improvement of clinical symptoms and normalization of laboratory data, including ferritin and soluble interleukin-2 receptor.

Although downregulation of CD5 antigen within the $CD8^+$ T-cell fraction is a characteristic finding in cases of acute EBV-HLH and facilitates early diagnosis, the finding is not necessarily specific to EBV-HLH. FCM analysis alone is insufficient to make a definitive diagnosis unless the $CD5^{dim/negative}$ fraction is determined to be of a single clone by TCR Vβ repertoire analysis. We have encountered multiple cases of familial hemophagocytic lymphohistiocytosis (FHL), in which $HLA\text{-}DR^{high}$ $CD5^{dim/negative}$ fractions can be identified within $CD8^+$

T cells (**Figure 2A**, FHL2, red square). The target of EBV infection is the B cell in patients with FHL, but activation and expansion of oligoclonal $CD8^+$ T cells are associated with downregulation of CD5 for unknown reason (32, 33). Confirming that the target of EBV infection is $CD8^+$ T cells and not B cells is therefore important whenever patients with an increased EBV copy number and HLH phenotype show oligoclonal expansion of HLA-DR^{high} $CD5^{dim/negative}$ cells (**Figure 2B**). $CD8^+$ T cells are the target of EBV infection only in cases with EBV-HLH (**Figure 2C**). No $CD8^+$ T cell is infected with EBV in acute IM or FHL cases.

Since our publications of the first article on EBV-HLH and CD5 expression (29, 30), we have experienced more than 50 cases of acute EBV-HLH, later confirmed by EBV clonality and the ectopic EBV infection to $CD8^+$ T cells. Without exception, we could identify the unique $CD5^{dim/negative}$ HLA-DR^{high} $CD8^+$ T cells in these cases. The percentages of this population within $CD8^+$ T cells ranged from 5 to 90%. It is important to note that one should focus on large cells to detect the abnormal cells and bone marrow may serve as a better source for the analysis (unpublished data). Clonal expansion of $CD8^+$ T cells may not be identified by available monoclonal antibodies. Our experience is that in 50–60% of the cases, the abnormal expansion of $CD8^+$ T cells with particular TCR Vβ repertoire can be identified. When clonal expansion is not confirmed by flow cytometry, it is necessary to separate lymphocyte subpopulations and identify the target of EBV infection by either EBER-1 *in situ* hybridization or real time PCR. Whenever EBV infection was found in B cells, and not in $CD8^+$ T cells, one should suspect FHL and genetic analysis should be required.

CONCLUSION

Once suspected, EBV-associated T/NK-cell LPD is not difficult to diagnose and appropriate treatment can be initiated with successful control of the disease process. However, large numbers of patients remain undiagnosed or lose their life without suspicion of the disease. It is the responsibility of the physician to suspect the possibility of EBV-associated T/NK-cell LPD at their early stages. FCM analysis at different levels offers rapid and often precise diagnostic information to reach the final diagnosis of EBV-HLH or CAEBV. In combination with more tedious procedures for the identification of EBV-infected lymphocyte subpopulations, FCM data are useful in the initial process of diagnosing EBV-associated T/NK-cell LPD.

AUTHOR CONTRIBUTIONS

AY obtained and analyzed the data, and organized the final structure of the manuscript.

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REVIEW
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Hematopoietic Stem Cell Transplantation for the Treatment of Epstein-Barr Virus-Associated T- or NK-Cell Lymphoproliferative Diseases and Associated Disorders

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Chronic active Epstein-Barr virus infection (CAEBV) is a prototype of EBV-associated T- and/or NK-cell (EBV$^+$ T/NK-cell) lymphoproliferative disorders. Most subtypes of these are lethal. We established a unified treatment strategy composed of step 1 (immunochemotherapy: steroids, cyclosporine A, and etoposide), step 2 (multi-drug block chemotherapy), and step 3 (allogeneic hematopoietic stem cell transplantation; HSCT) for CAEBV and its related diseases. Allogeneic HSCT is the only cure for CAEBV with few exceptions. Primary-EBV infection-associated hemophagocytic lymphohistiocytosis (primary-EBV HLH) is also an EBV$^+$ T/NK-cell lymphoproliferation. The nature of EBV$^+$ T/NK cells in CAEBV and those in primary-EBV HLH differ. In primary-EBV HLH, most patients need step 1 only and some require step 2 for the successful induction of apoptosis in EBV-infected T cells; however, some exceptional patients require HSCT. We herein present our single institutional experience of CAEBV and primary-EBV HLH, together with that of post-transplant EBV$^+$ T/NK-cell lymphoproliferative disease. We also discuss some practical points on HCST with a review of the literature.

Keywords: CAEBV, EBV, HLH, LPD, PTLD, HSCT

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INTRODUCTION

Epstein-Barr virus (EBV), a B-cell lymphotropic virus, was revealed to have the potential to infect T and NK cells and cause lymphoproliferative diseases (LPDs) in 1988 and 1989 (1–4). Since then, as the prototype of these LPDs, various efforts have been made to diagnose and treat chronic active EBV infection (CAEBV) and investigate its etiology and pathophysiology. The natural clinical course of these LPDs with only supportive care is lethal with some exceptional disease subtypes. Allogeneic hematopoietic stem cell transplantation (HSCT) has been the most reliable radical treatment. However, it has a mortality rate of ∼10%, and a severe morbidity rate of another 10%. It is challenging to select an adequate treatment before HSCT, identify which patients need HCT, and establish which type of HSCT is appropriate. We herein present our current strategy, which has been developed by an institutional review, and discussed it with a review of the literature.

The current classification of EBV-associated T- and/or NK-cell (EBV^+ T/NK-cell) lymphoproliferative disorders (5, 6) and their requirement for HSCT are listed in **Table 1**. The treatment is the same between CAEBV and its related diseases, i.e., hypersensitivity to mosquito bites (HMB) and severe-type hydroa vacciniforme (sHV). The main targets of our current literature are CAEBV, its related diseases, and primary-EBV infection-associated hemophagocytic lymphohistiocytosis (primary-EBV HLH); however, EBV^+ T/NK-cell lymphomas or leukemia and post-transplant EBV^+ T/NK-cell lymphoproliferative disease (T/NK-LPD) are also referred. This retrospective study was approved by the Research Ethics Committee of Osaka Women's and Children's Hospital (the name has been changed: previously, Osaka Medical Center and Research Institute for Maternal and Child Health).

OVERVIEW OF CAEBV

Background

CAEBV is a chronic, but progressive and lethal disease with persistent/recurrent infectious mononucleosis (IM)-like symptoms (7). Kawa et al. developed a treatment strategy for CAEBV based on their early etiological and pathophysiological findings (3, 4). In their early studies, IL-2 transiently controlled the symptoms of patients with CAEBV; however, this effect was only observed in a limited number of patients (8). Steroid monotherapy, or later with cyclosporine A (CsA), resulted in the remission of symptoms in most patients, and etoposide (Etp) was effective for CAEBV-associated HLH. However, without radical treatment, all patients died of disease progression: uncontrollable HLH flare followed by distributive shock or multi-organ failure, affected-cell infiltration resulting in organ failure, or disease progression to a refractory malignant lymphoma/leukemia (9–11).

Based on the idea that CAEBV is not a simple infection of EBV, but an EBV-associated neoplasm, they administered and developed multi-drug block chemotherapy comprised of anti-cancer drugs, and finally allogeneic HSCT with revolutionary success (12). These ideas and treatment concepts were gradually accepted and are now widely employed, and CAEBV is partially cited in the WHO classification in 2008 and revised classification in 2017.

TABLE 1 | EBV-associated T/NK-cell lymphoproliferative disorders.

Category	Disease	Requirement for HSCT
1. Acute/transient	• Primary-EBV infection-associated hemophago-cytic lymphohistiocytosis (Primary-EBV HLH)	Some
	• Classical hydroa vacciniforme (cHV)	None
2. Chronic/progressive	• Chronic active EBV infection (CAEBV)	Nearly all
	• Hypersensitivity to mosquito bites (HMB) (Mosquito bite allergy; MBA)	
	• Severe-type hydroa vacciniforme (sHV)	
3. Malignant	• Aggressive NK-cell leukemia (ANKL)	Mostly
	• Extranodal NK/T-cell lymphoma, nasal type (ENKTL)	
	• Hepatosplenic T-cell lymphoma	
	• Peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS)	
4. Others	• Post-transplant EBV-associated T/NK-cell lymphoproliferative disease (EBV^+ T/NK-PTLD)	Uncertain

CAEBV-Related Diseases

In HMB and sHV, systemic IM-like symptoms are induced with a topical skin reaction by mosquito bites and sunlight, respectively. HV is a skin disease induced by sunlight, which generally regresses spontaneously within several years (13, 14). However, some patients with HV exhibit disease progression, in which systemic manifestations are also induced by sunlight, and die with a CAEBV-like clinical course. The former is nomenclated as classical HV (cHV), and the latter as sHV.

Patients with HMB manifest a cutaneous ulcer, with a systemic reaction as the disease progresses, induced by a mosquito sting. In contrast to HV, all patients with HMB follow a CAEBV-like lethal clinical course (15–17). HV and HMB were revealed to be EBV^+ T/NK-LPDs, and sHV and HMB are regarded as CAEBV-related diseases and treated using the same strategy as that for CAEBV.

TREATMENT OF CAEBV: A SINGLE INSTITUTIONAL REVIEW

Unified Treatment Strategy

The unified treatment strategy for CAEBV (with and without HLH), sHV, and HMB is shown in **Figure 1** [F]. The initial treatment is immunochemotherapy (step 1). At the onset of HLH, it is sometimes difficult to make a differential diagnosis between primary-EBV HLH and HLH as a manifestation of CAEBV, other types of EBV^+ T/NK-LPDs, or even severe IM (a kind of acute/transient EBV^+ B-LPDs). Etp was omitted for patients without symptoms of HLH. In CAEBV and its related diseases, for a radical treatment, most patients are moved to multi-drug block chemotherapy (step 2) within 1–2 weeks.

Patients received 2–3 courses of chemotherapy on average in step 2. During the interval of chemotherapy, lower doses of prednisolone (PSL) and CsA were maintained, particularly in patients with a higher burden of residual disease. First-line chemotherapy is modified CHOP (**Figure 1**). Second-line chemotherapy is ESCAP. However, patients with complications may undergo the Capizzi regimen instead: high-dose cytosine arabinoside (HDCA) 3 g/m^2 4 times (every 12 h), L-asparaginase (L-Asp) 10,000 units/m^2 once (4 h post-CA), and PSL 30 mg/m^2/d (days 1 and 2). The interval is typically 3–4 months between the initiation of treatment and allogeneic HSCT (step 3).

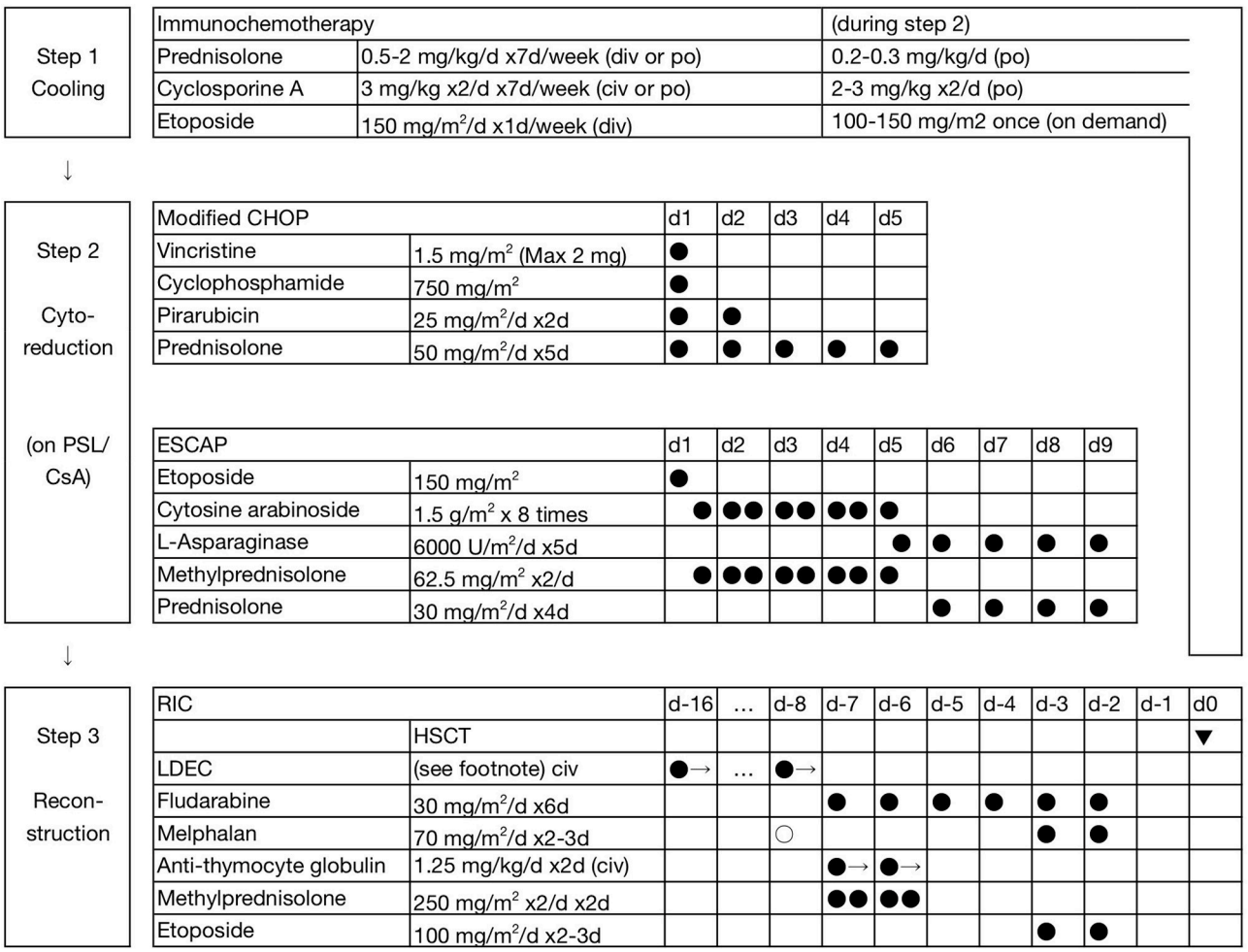

FIGURE 1 | Three-step strategy for the treatment of EBV + T/NK-LPDs. In step 1 (cooling), the initial dosage of prednisolone (PSL) is 1–2 mg/kg/d for children and 0.5–1 mg/kg/d for adults. Etoposide (Etp; 150 mg/m^2 or 5 mg/kg, weekly) is omitted in patients without symptoms of HLH. In step 2 (multi-drug chemotherapy), lower doses of PSL and cyclosporine A (CsA) were maintained, particularly for patients with a higher burden of residual disease. LDEC: low-dose Etp 30 mg/m^2/d and cytosine arabinoside 20 mg/m^2/d are continuously administered for 24 h for 1.5 (0.5–2) weeks before the initiation of RIC. Closed circles indicate fixed administration, and open circles indicate optional administration. One precedent dose of melphalan 70 mg/m^2/d is added (total 210 mg/m^2/d) for children and adolescents at a high risk of rejection, and is replaced by systemic irradiation of 3 Gy with gonadal blockade in adults.

Significance of Multi-Drug Chemotherapy

We published our single center experience of 77 patients with CAEBV and its related diseases, HMB and sHV, along the 3-step strategy (6). The EBV-DNA load in whole peripheral blood (PB) is a practical and useful marker for residual disease in CAEBV and its related diseases (HMB and sHV). Effective chemotherapy is defined as a reduction of $\leq$1/10, measured with quantitative PCR, by each course of chemotherapy. In 6 out of 77 patients, the EBV-DNA load was reduced to the minimum detection limit (200 copies/mL) or less by several courses of chemotherapy. Two patients underwent successful HSCT under their parents' and physician's choice, another 2 patients exhibited early elevations in the EBV load and successfully underwent HSCT, and the remaining 2 patients (CAEBV 1 and HMB 1) received one additional course of effective chemotherapy and have been in continuous complete remission (CR) without HSCT (6, 18).

The necessity for and significance of multi-drug chemotherapy (step 2) is under debate. In our strategy, multi-drug chemotherapy is important as a preparation for subsequent allogeneic HSCT. It provides (1) a safe bridge to HSCT by suppressing disease activity, (2) a high rate of engraftment of donor cells at HSCT, and (3) a low relapse rate after HSCT (6). The majority of patients with CAEBV died of disease within observation periods unless they received radical treatment. Some physicians consider there to be exceptional cases without disease progression and organ failure. We hypothesize that exceptional cases may be present in the subgroup of patients achieving complete molecular CR by multi-drug chemotherapy.

Allogeneic HSCT

In our institute, 75 patients had the intention to receive HSCT. Of these, 12 patients progressed to an emergency medical condition. Therefore, 63 patients who underwent planned transplantation state were involved in the following analysis. Reduced-intensity conditioning (RIC) was superior to myeloablative conditioning (MAC) (19), and revised 3-year overall survival rates (3y-OS) were reported to be 90.7 ± 4.0% ($n = 54$) and 66.7 ± 15.7% ($n = 9$), respectively ($p < 0.05$) (6). Furthermore, the incidence of late sequelae after RIC, such as gonadal dysfunction, may be lower than that after MAC (20). The current RIC regimen (standard RIC) includes a total melphalan (LPAM) dose of 140 mg/m^2, as shown in **Figure 1**.

Cord blood transplantation (CBT) and bone marrow transplantation (BMT) are excellent sources of HSCT, and 3y-OS were 93.3 ± 6.4 and 92.9 ± 6.9%, respectively ($p = 0.87$); however, the incidence of engraftment failure was higher in CBT (21). RIC for CBT was successfully augmented thereafter, and no engraftment failure has since been observed ($n > 10$) (6). The current augmentation of RIC for CBT is LPAM 70 mg/m^2 on day-8 in children and adolescents, and systemic irradiation with 3 Gy with gonadal blockade in adults if a recipient has received only 2 or 3 courses of chemotherapy before CBT.

HSCT FOR CAEBV IN VARIOUS SITUATIONS

Adult-Onset CAEBV

CAEBV is now recognized to occur not only in children and adolescents, but also in adults at any age. Half of the children (including adolescents) with CAEBV died in 5 years, and most of them died in 10–15 years without radical treatment (10). Two studies reported that adult-onset CAEBV progresses rapidly, and most of patients died within 5 years (22, 23). In our series, 3y-OS was equivalent between adults (≥ 20 years of age at onset) and children (71.4 ± 12.1, 76.6 ± 5.3%, respectively; $p = 0.61$) (6). Therefore, we concluded that our 3-step strategy is also applicable to adults. Arai et al. reported similar findings (OS 61.5%) for adult-onset CAEBV (24).

Emergent HSCT

In our series, in contrast to the promising findings of planned HSCT ($n = 63$; 3y-OS 87.3 ± 4.2%), most patients with advanced/uncontrollable disease ($n = 12$), including 8 who managed to undergo emergent HSCT, were not rescued (3y-OS 16.7 ± 10.8%) (6). Our findings were consistent with those by Arai et al. who reported that OS was 100% after HSCT for inactive disease, but was 0% after HSCT for active disease (24).

Patients with liver-transaminase elevations or hyperferritinemia were restored by HSCT, i.e., remedial HSCT (2 out of 5 survived). However, HSCT did not save patients (compassionate HSCT, 0 out of 3 survived) with severe jaundice (liver failure), anuria (renal failure), or tracheal intubation (due to distributive shock after HLH flare); these difficult cases were attributed to disease progression and not to chemotherapy or age (6). Therefore, we consider that initiating treatment earlier to complete HSCT in advance leads to higher survival, although HLH flare or disease progression may occur at any time, even under treatment.

PRIMARY-EBV HLH

Background

Primary-EBV HLH is a secondary HLH following a primary EBV infection; secondary means that it occurs in children (and occasionally in adolescents and young adults) without known immunodeficiencies, including familial HLH (FHL). It has a lethal potential for HLH flare followed by multi-organ failure without an adequate treatment. These more severe manifestations of primary-EBV HLH than those of other infection-induced HLH may be attributed to primary-EBV HLH not being simple infection-induced HLH, but LPD-associated HLH based on EBV$^+$ T/NK-cell proliferation (typically CD8$^+$ T cells). The EBV infection of T cells in primary-EBV HLH has also been reported in non-Asian children (25).

The majority of patients with primary-EBV HLH are simply cured with immunochemotherapy (steroids, CsA, and Etp), such as the FHL-oriented protocol (HLH-94 or HLH-2004) or our step 1 (26, 27). Remission was achieved by immunochemotherapy in 86–90%, and recurrence was observed in 8–17% (28, 29). Notably, eight out of the 9 patients (89%) who did not achieve remission during the initial steroid treatment/CsA/Etp died (29). Therefore, allogeneic HSCT is required for patients refractory to immunochemotherapy. In prospective studies including a small ratio of patients with a congenital gene mutation responsible for HLH (2–8%), HSCT was administered to 15–23% of patients (27, 28). OS was 76–90% (28, 30), and varied between 53 and 86% in patients who underwent HSCT (27, 28, 31, 32).

In prospective studies, patients with severe/persistent or recurrent disease moved to HSCT without multi-drug chemotherapy (33). In contrast, in practical situations, ∼50% of patients were treated with multi-drug chemotherapy before HSCT (32). However, few studies have been published from the viewpoint of the effectiveness of multi-drug chemotherapy. In one study on 20 patients, 13 achieved remission after steroids with or without CsA and/or Etp (34). Thereafter, 4 out of the remaining 7 patients achieved remission by multi-drug chemotherapy (mostly CHOP) and continued to be disease-free without HSCT.

Prolonged high doses of steroids/CsA/Etp may lead to a severe immunodeficiency, and result in the occurrence of another complication, immunodeficiency-associated B-LPD (35). If immunodeficiency-associated B-LPD can be diagnosed early, rituximab may be effective. A systematic review and meta-analysis showed that survival ratios were 68 and 80% in the immunochemotherapy (without HSCT) group and HSCT group, respectively, and concluded that both approaches equally contributed to decreasing mortality (36); however, disease severity may differ in each group.

A Single Institutional Review

We treated 23 patients with primary-EBV HLH between 2000 and 2017 since the measurement of the EBV-DNA load with quantitative PCR became available (paper under preparation). In

our institutional review, a bias was expected to exist in disease severity; our present study may include a higher ratio of severe patients than others because, as an example, 50% of severely affected patients were referred from areas outside our prefecture.

Patients with primary-EBV HLH follow the unified treatment strategy shown in the section on CAEBV (**Figure 1**). Most patients with severe HLH required pulsed high-dose methylprednisolone (HD-mPSL; 0.5 g/m^2/d for 3 days) and Etp (150 mg/m^2/d or 5 mg/kg/d one day per week). In contrast, the delayed initiation of and/or dose reductions in CsA were allowed for the patient conditions. Patients start the tapering of PSL within 1–2 weeks if they achieve the remission of symptoms, whereas those refractory to step 1 move on to step 2.

Treatment responses varied; 9 patients needed only step 1 (group A), 6 needed steps 1 and 2, but did not require HSCT (group B), and the remaining 8 needed steps 1, 2, and 3 (group C). OS also varied; all patients (15/15) are alive and well in groups A and B, in contrast to only 3/8 (38%) in group C. In group A, 2 patients showed a complete response to PSL and CsA without Etp, and 7 received Etp (mainly once or twice). Patients refractory to or dependent on immunochemotherapy (step 1) were administered multi-drug chemotherapy (in groups B and C). First-line chemotherapy was modified CHOP, and second-line chemotherapy contained HDCA and/or L-Asp (**Figure 1**).

Together with groups B and C, 6 out of 14 patients successfully avoided allogeneic HSCT. Patients with resistant disease against multi-drug chemotherapy were regarded as absolute candidates for allogeneic HSCT. One severe patient omitted multi-drug chemotherapy for urgent HSCT. Among the 8 patients in group C, 5 had early deaths (mainly due to HLH), 1 relapsed 12 months after RIC followed by BMT (RIC-BMT), but was successfully rescued by MAC-CBT, and 2 maintained CR after HSCT.

Absolute Eligibility for HSCT in Primary-EBV HLH

In our series, group B included 3 patients who were referred to our institute requiring further treatment than steroids/CsA/Etp, i.e., allogeneic HSCT, but successfully substituted multi-drug chemotherapy for HSCT. As a corollary, patients in group C were absolutely eligible for HSCT. Similar to CAEBV, one of the roles of multi-drug chemotherapy is to identify who definitely needs HSCT and who does not among patients with primary-EBV HLH.

Group C may form a distinct disease subset. In some patients, disease activity progressed after a significant reduction in the EBV load or even after achieving complete donor chimerism by HSCT. These patients in group C had no mutation in the known genes responsible for FHL or other primary HLH. They possibly had other unknown gene mutations in T/NK cells and/or non-T/NK cells that predisposed them to the induction/maintenance of progressive HLH (37–39).

OTHER TYPES OF EBV$^+$ T/NK-LPDS

Post-transplant EBV$^+$ T/NK-LPD

Typical post-transplant LPD (PTLD) has been described as EBV-associated and B-cell type LPD, occurring before 12 months (particularly in the third month) after HSCT or at any time after organ transplantation (40, 41). Typical PTLD is susceptible to rituximab. In contrast, T/NK-PTLD is a dismal complication after transplantation (OS was ∼20%) (42). However, EBV$^+$ T/NK-PTLD had a better prognosis than EBV$^-$ T/NK-PTLD (OS of ∼38 and 13%, respectively) (42).

As one atypical PTLD, we have had 3 recipients with late-onset EBV$^+$ T/NK-PTLD including one patient reported previously (patient 1) (18). All 3 patients responded to HDCA- and/or L-Asp-containing chemotherapy, and are in continuous CR without HSCT (paper under preparation). Although this atypical PTLD belongs to the variety of EBV$^+$ T/NK-LPDs, a patient subset may exist that responds to chemotherapy and may be cured without HSCT.

Malignant EBV$^+$ T/NK-LPDs

Our patient number was limited in this subset. In the literature, more than half of patients with localized extranodal NK/T-cell lymphoma of the nasal type (ENKTL) were successfully treated with local irradiation and chemotherapy (2y-OS 78%) (43). Malignant T/NK-cell lymphoma/leukemia is susceptible to HDCA and/or L-Asp, such as the SMILE regimen (44–47). However, the SMILE regimen was highly toxic in elderly patients or those with organ dysfunctions (48). Patients with advanced ENKTL or aggressive NK-cell leukemia (ANKL) are treated with the SMILE regimen and HSCT (46, 47); however, OS is still dismal, particularly in ANKL (1y-OS <30%).

DIFFERENCE BETWEEN PRIMARY-EBV HLH AND CAEBV

Proapoptotic Potential in EBV-Infected Cells of Primary-EBV HLH

In primary-EBV HLH, treatment is tapered alongside the remission of symptoms. Residual disease of primary-EBV HLH, molecularly assessed by the EBV-DNA load with quantitative PCR, may be significantly reduced with successful treatment; however, molecular CR (EBV < 200 copies/mL in PB) and the complete clearance of EBV-infected T/NK cells (typically CD8$^+$ T cells) were not mandatory at the end of the treatment, and minimal residual disease (MRD) disappeared within a half or a couple of years. This was in contrast to CAEBV, for which a positive result for MRD without HSCT resulted in overt relapse.

Investigators failed to infect peripheral blood T/NK cells from healthy volunteers (PB-T/NK cells) with EBV *in vitro*, except in one study (49). Furthermore, although EBV managed to infect NK-cell lines and PB-T/NK cells, most were lost due to apoptosis within 72 h (49). In two studies using clinical specimens, EBV-infected T cells and NK cells were detected in 0–50 and 50–100% of patients with IM, i.e., initial EBV infection, respectively (50, 51), but are rarely maintained and merely result in T/NK-LPD. These findings are consistent with our previous observations, and provided a rationale for our recent treatment.

Different Nature Between Primary-EBV HLH and CAEBV

Our observations and the findings described above prompted us to speculate that the nature of EBV-infected T/NK cells in primary-EBV HLH and CAEBV completely differs (**Figure 2**). In primary-EBV HLH, EBV-infected T/NK cells are activated due to primary EBV infection, and maintain an antiapoptotic predisposition due to a specific internal environment, such as a high concentration of cytokines during early EBV infection. Therefore, the purpose of treatment is to induce apoptosis in affected and activated cells, as well as to suppress the T-cell cytokine storm and monocyte/macrophage hyperinflammation. In contrast, EBV-infected T/NK cells in CAEBV have acquired a self-expanding nature during years of evading eradication (**Figure 2**), and the purpose of treatment is complete cell death (total cell killing).

Spectrum Between CAEBV and Malignant T/NK-LPDs

CAEBV shares some common features with ANKL and ENKTL, revealing a spectrum between CAEBV and malignant EBV-associated T/NK-LPDs (52, 53). Difficulties are associated

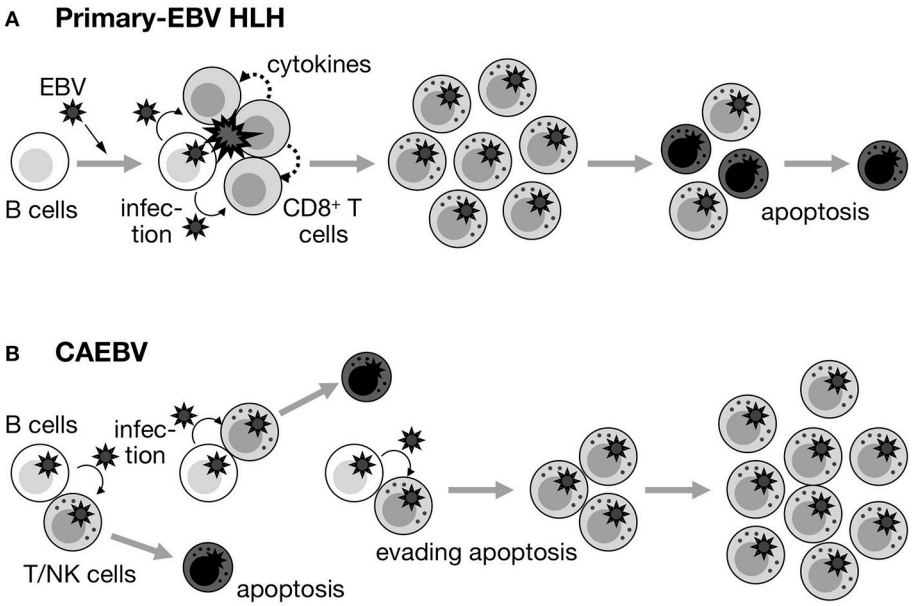

FIGURE 2 | Fate of EBV$^+$ T/NK cells in primary-EBV HLH and CAEBV. **(A)** Primary-EBV HLH. EBV-infected T/NK cells may transiently proliferate under specific conditions (primary EBV infection and large amounts of cytokines). However, they maintain a proapoptotic nature, and may be induced to enter apoptosis by themselves, steroids/CsA, and/or anti-cancer drugs. **(B)** CAEBV. Although EBV occasionally infects T/NK cells, EBV-infected T/NK cells inherently have pro-apoptotic effects and repeatedly appear and disappear. However, some of these cells acquire a self-maintaining and self-expanding predisposition over the course of years and contribute to the development of disease.

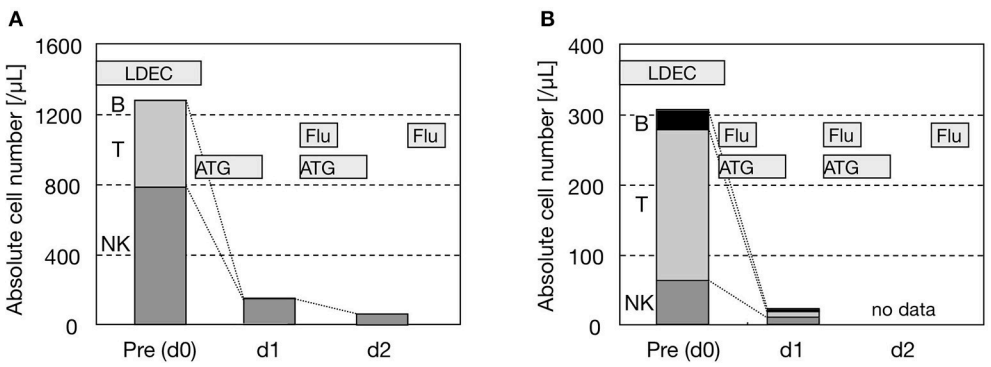

FIGURE 3 | Reductions in NK cells by ATG. **(A)** UPN605. **(B)** UPN612.Both were diagnosed as HMB of the NK-cell type. The absolute number of each lymphocyte subset in PB was counted on days 0, 1, and 2 before and after the administration of ATG as the initiation of RIC. Other drugs were also shown. The lymphocyte subset was analyzed by three-color flow cytometry (FCM) using fluorescein isothiocyanate-, phycoerythrin/rhodamine-, and phycoerythrin-cyanine 5-conjugated antibodies (Beckman Coulter Inc., USA). FCM was performed with EPICS XL® (Coulter Inc., USA). B, T, and NK cells were defined as CD20 + CD19 + CD45 + cells, CD3 + CD45 + cells, and CD3 - CD56 + CD45 + cells, respectively. LDEC, low-dose etoposide and cytosine arabinoside; Flu, fludarabine.

with making a differential "subdiagnosis" between CAEBV and malignant EBV$^+$ T/NK lymphoma/leukemia (ANKL and ENKTL) in some cases. However, their distinction may be practically less important and the diagnosis of "EBV-associated T/NK-LPD" may be sufficient for a severely ill emergent patient because both need similar therapeutic approaches that include allogeneic HSCT (5). In contrast, EBV$^+$ T/NK cells in PTLD, arising under some immunodeficient conditions, particularly after transplantation, may reserve the potential for apoptosis and susceptibility to chemotherapy.

OTHER CONSIDERATIONS FOR HSCT

Alloimmunity as the Main Effector Against CAEBV After HSCT

The relapse rate after planned HSCT among patients with CAEBV was as low as <5%, although most of their conditioning regimens were RIC rather than MAC (6). The relapse rate did not increase even after the transplantation of CB, which is naïve to EBV (21). In one study, cytotoxic lymphocytes (CTLs) against EBV were induced after allogeneic HSCT in two patients with CAEBV (54). However, CTLs against EBV were not properly induced and EBV DNAemia was often observed after HSCT (21), which may correlate with progression to EBV$^+$ B-PTLD, but not with the relapse of CAEBV. In contrast, mixed chimerism or autologous recovery correlated with the relapse of CAEBV (6). These findings prompted us to speculate that alloimmunity is the main effector against EBV$^+$ T/NK cells after HSCT, and that the success of allogeneic HSCT over CAEBV mostly depends on allo-reactive CTLs against recipient cells (21).

This is not the case for malignant EBV$^+$ T/NK-LPDs. The incidence of recurrence is high, even after allogeneic HSCT, particularly in patients with ANKL. CTL against EBV may have some benefit for these patients as maintenance therapy after HSCT (55).

Effects of Anti-thymocyte Globulin on EBV$^+$ T/NK-LPD

The purpose of our early administration of low-dose rabbit anti-thymocyte globulin (ATG; Thymoglobulin®, Sanofi, France; 1.25 mg/kg/d on d-7 and d-6) is to reduce recipient T-cell immunity and enforce donor-cell engraftment, and, in addition, reduce EBV-infected T/NK-cell numbers for better disease control.

Sanacore et al. suggested that the early administration of ATG at 4.5 mg/kg 2 weeks before HSCT resulted in a sub-therapeutic ATG concentration (< 1 μg/mL in serum) on day 0 of HSCT; therefore, it only depleted host T cells selectively and enhanced donor-cell engraftment (56). Their pharmacokinetics suggest that the concentration of ATG may be lowered to a sub-therapeutic level on the day of HSCT (day 0), even if ATG is administered at 2.5 mg/kg 1 week before HSCT (i.e., early dosing regimen). Penack et al. reported that although Lymphoglobulin® (equine anti-thymocyte globulin) was less effective for NK-cell depletion, ATG induced apoptosis and necrosis in NK and T cells (57). However, the effects of ATG on NK cells currently remain unknown in clinical settings.

We measured the absolute number of each lymphocyte subset in PB during RIC (**Figure 3**). In addition to the absolute number of T cells, that of NK cells was also diminished by ATG: <1/5 in 1 day and <1/10 in 2 days after the initiation of ATG. Therefore, although NK cells were counted as a whole without the distinction of EBV-infected and EBV-uninfected NK cells, we regarded ATG to be effective for the disease control of EBV$^+$ NK-LPD as well as EBV$^+$ T-LPD. As a result, if ATG is administered to patients with a high burden of residual disease, conditioning-induced (possibly ATG-induced) HLH needs to be carefully considered (21). ATG actually induces apoptosis and necrosis in EBV$^+$ T/NK cells in clinical settings. However, the cytoreductive effects of ATG on EBV$^+$ T/NK cells may be transient. When the complete engraftment of donor cells fails (mixed chimerism or autologous recovery), the EBV load may increase again with hematopoietic recovery after HSCT (6).

FUTURE DIRECTIONS

Despite the evolution of treatment strategies against EBV$^+$ T/NK-LPDs, the prognosis of patients with progressive/refractory disease remains poor. Better methods in step 2 are desired. JAK 1/2 inhibitors may exert prophylactic and therapeutic effects on HLH (58). The SMILE regimen has been used in some hospitals. The novel combination of romidepsin and pralatrexate was found to be effective for T-cell lymphoma (59). Furthermore, bortezomib and ganciclovir have been suggested to reduce the disease burden (60). Cell therapy, such as CTLs against EBV (as acquired immunity) or HLA-haplotype incompatible lymphocytes (for alloimmunity), may also be effective to some extent (55, 61, 62). We consider better disease control with these modalities before and after HSCT to contribute to more promising prognoses.

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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Challenges in Managing EBV-Associated T- and NK-Cell Lymphoproliferative Diseases

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Epstein-Barr virus (EBV) infects >90% of adults worldwide and is closely linked to multiple B-cell malignancies, including Burkitt lymphoma, diffuse large B-cell lymphoma, Hodgkin lymphoma, and post-transplant lymphoproliferative disorder (PTLD) (1). Epstein-Barr virus also infects T-cells and natural killer (NK) cells causing EBV-associated T- and NK-cell (EBV-T/NK) malignancies, including extranodal NK/T-cell lymphomas, nasal type (ENKL), aggressive NK-cell leukemia, and lymphoproliferative diseases (LPDs). These EBV-associated T/NK-cell tumors have basically neoplastic properties with clonal proliferation and organ infiltration (2).

Chronic active EBV infection (CAEBV), an EBV-T/NK LPD, is a potential life-threatening illness in children and young adults, characterized by the clonal proliferation of EBV-infected lymphocytes (3, 4). The T/NK-cell type of this disease is more frequent in East Asians and some Native American populations in Western countries. CAEBV patients from the United States more often have EBV in B- or T-cells (3, 5). Patients with CAEBV often progress to overt lymphoma or leukemia. Although concurrent chemoradiotherapy along with non-anthracycline-based chemotherapy has improved the survival of patients with these EBV-T/NK malignancies, the survival outcome remains poor because of relapse or treatment-related mortality (6). The only curative treatment is stem-cell transplantation, albeit the incidence of transplantation-related complications is high (7, 8). To improve the treatment of EBV-T/NK malignancies, novel approaches using molecular targets have been attempted (**Table 1**).

Immune checkpoint blockade with monoclonal antibodies directed at the inhibitory immune receptors, programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1), has emerged as a successful treatment approach for patients with advanced cancers. Since EBV-infected lymphoma cells upregulate PD-L1 (19), these molecules are, therefore, the target of the antitumor effect. Pembrolizumab, the humanized anti-PD-1 monoclonal antibody, is effective for relapsed/refractory ENKL (9), suggesting that checkpoint inhibitors have a promising effect in the treatment of relapsed disease.

In addition to checkpoint inhibitors, some antibodies and inhibitors are also treated as potential molecular therapeutic targets in the developmental and preclinical stages. Kanazawa et al. showed that CC chemokine receptor 4 (CCR4) was expressed on most EBV-infected T/NK-cell lines and a humanized anti-CCR4 monoclonal antibody, mogamulizumab, inhibited the growth of EBV-positive NK-cell lymphomas in a murine xenograft model (10). Another challenge is targeting histone deacetylase (HDAC). The HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and romidepsin, have been approved by the United States Food and Drug Administration and their efficacies in non-Hodgkin lymphoma, acute myeloid leukemia, cutaneous T-cell lymphoma, and relapsed and refractory peripheral T-cell lymphoma have been confirmed by clinical trials (20–22). SAHA suppressed tumor progression and metastasis in a murine xenograft model, although there were no significant differences observed between EBV- positive and EBV-negative cell lines (11). However, a pilot study using romidepsin for the treatment of relapsed/refractory ENKL patients in Korea was discontinued due to serious adverse events. As

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TABLE 1 | Recent findings of targeted therapies for EBV-T/NK LPDs.

Class	Drug	Target	Note	References
Monoclonal antibody	Pembrolizmab	PD-1	Five of 7 relapsed/refractory ENKL patients in at least clinical complete responses (CRs)	(9)
	Mogamulizumab	CCR4	Growth inhibition of EBV-associated T/NK-cell lymphoma in murine xenograft model	(10)
HDAC inhibitor	SAHA	HDAC	Tumor growth suppression in murine xenograft model	(11)
	Romidepsin	HDAC	Discontinued due to the EBV reactivation-associated adverse events	(12)
Hsp90 inhibitor	BIIB021	LMP1	Suppression of EBV-positive NK-cell growth in murine xenograft model	(13)
mTOR inhibitor	Rapamycin, CCI-779	Akt/mTOR pathway	Inhibition of EBV-associated T/NK-cell lymphoma growth in NOG mice	(14)
JAK inhibitor	Ruxolitinib	JAK1, JAK2	Suppression of inflammatory cytokines production in CAEBV patient-derived cells	(15)
	Tofacitinib	JAK3	Tumor growth inhibition in EBV-associated T-cell lymphoma in NOG mice	(16)
Proteasome inhibitor	Bortezomib	Ubiquitin-proteasome system	Suppression of EBV-associated tumor growth in murine xenograft model	(17, 18)

romidepsin treatment caused EBV reactivation, patients developed fever and elevated liver enzyme and bilirubin levels immediately after their first dose of romidepsin (12). These results suggest that the further accumulation of evidence in the preclinical stage is required for safer application of drug candidates in clinical trials.

The EBV-encoded latent membrane protein 1 (LMP1) is a major oncogene that activates the nuclear factor kappa B (NF-κB), c-Jun N-terminal kinase (JNK), and phosphatidylinositol 3-kinase (PI3K) signaling pathways, thereby, promoting the cell growth and inhibiting apoptosis (23). LMP1 is expressed in EBV-infected T/NK-cells. Screening a library of small-molecule inhibitors identified heat shock protein 90 (Hsp90) inhibitors as suppressors of LMP1 expression (24). In EBV-positive cells, the synthetic Hsp90 inhibitor BIIB021 suppressed the LMP1 expression and that of its downstream signaling proteins NF-κB, JNK, and Akt. The BIIB021 inhibited the growth of established EBV-positive NK-cells in NOD/Shi-*scid*/IL-2Rγ^{null} (NOG) mice (13). Moreover, constitutive PI3K/Akt/mTOR activation is critically involved in EBV-associated B-cell lymphoma (25, 26). Kawada et al. demonstrated that intraperitoneal treatment with an mTOR inhibitor significantly inhibited the growth of EBV-associated NK-cell lymphomas in a murine xenograft model and decreased the EBV load in peripheral blood, while T-cell lines were more sensitive to the mTOR inhibitors, but there were no significant differences between EBV-positive and EBV-negative cell lines (14). A series of studies of the JAK-STAT axis in EBV-T/NK LPDs provided new insight into its development. The STAT3 was activated in T/NK-cells in six of seven patients with CAEBV, promoting survival and cytokine production (15). Indeed, the selective JAK3 inhibitor, tofacitinib, significantly inhibited the growth of established tumors in NOG mice (16). We have already demonstrated the antitumor activity of the proteasome inhibitor bortezomib on EBV-associated lymphoma cells (17, 18). Therefore, combining these agents is a promising strategy to improve the treatment of EBV-T/NK lymphomas.

A fundamental question regarding the etiology of EBV-T/NK LPDs remains. The precise mechanism of T/NK-cell tumorigenesis remains to be elucidated because EBV-T/NK tumors are rare, and the generation and handling of EBV-positive T/NK cells are more difficult than with B-cells. To elucidate the genetic background related to these rare tumors, next-generation sequencing (NGS), including whole-genome sequencing and whole-exome sequencing, is a powerful, unbiased approach. Mutations of DDX3X, TP53, BCOR1, and STAT3 have been found in Chinese (27) and Japanese (28) patients with ENKL, although the mutation rates differed between these cohorts. Li et al. showed that genetic variation at *HLA-DPB1* is a strong contributor to extranodal NK/T-cell lymphoma (29). These findings highlight a pathogenic link between genetic variation and EBV-associated neoplastic proliferation. However, the possibility that specific EBV strains or variants have a higher tendency to develop T/NK-cell tumors cannot be eliminated now. Notably, Kimura's group also revealed that the EBV genome in CAEBV patients harbored frequent intragenic deletions (Dr. Kimura, personal communication). The genetic data generated from NGS-based approaches are required for their subsequent validation as definitively disease causing. Therefore, patient registries and biospecimen repositories are needed to accelerate bridging research from the developmental and preclinical stages to a clinical setting. In Japan, a nationwide registry of EBV-T/NK LPDs has been started (currently only Japanese, https://www.med.nagoya-u.ac.jp/virus/caebv/). We hope that this registry will grow and be linked to international registries to improve the efficacy and quality of the treatment of EBV-associated tumors.

As Abraham Lincoln, the 16th president of the United States, once said, "I will prepare and someday my chance will come."

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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