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Takumi Kumai[(1)], Hiroya Kobayashi[(2)], Yasuaki Harabuchi[(1)(2)]

(1) Department of Otolaryngology, Head and Neck Surgery, Asahikawa Medical University

(2) Department of Pathology, Asahikawa Medical University

Midorigaoka-East 2-1-1-1, Asahikawa 078-8510, JAPAN

Phone: +81-166-68-2554, Fax: +81-166-68-2559

TK: t-kumai@asahikawa-med.ac.jp

HK: hiroya@asahikawa-med.ac.jp

YH: hyasu@asahikawa-med.ac.jp

Summary

Extranodal natural killer/T-cell lymphoma, nasal type (NKTL) is a rare but highly aggressive Epstein-Barr virus-related malignancy, which mainly occurs in nasopharygeal and nasal/paranasal areas. In addition to its high prevalence in Asian, Central American and South American populations, its incidence rate has been gradually increasing in Western countries. The current mainstay of treatment is a combination of multiple chemotherapies and irradiation. Although chemoradiotherapy can cure NKTL, it often causes severe and fatal adverse events. Because a growing body of evidence suggests that immunotherapy is effective against hematological malignancies, this treatment could provide an alternative to chemoradiotherapy for treatment of NKTL. In this review, we focus on how recent findings could be used to develop efficient immunotherapies against NKTL.

Keywords: Natural killer/T-cell lymphoma, immunotherapy, c-Met, CCR4, Epstein-Barr virus, mutation, peptide vaccine, chemokine

1. Introduction

Until the late 20th century, a fatal destructive tumor that develops around the nasopharyngeal and nasal/paranasal areas, sometimes penetrating the palate or the cranial dura mater, resulting in death was called lethal midline granuloma[1]. Study of cell surface markers in immunity has shown that expression of CD56, a marker of natural killer cells (NK cells) and some γδ T-cells, can be used to define this disease as extranodal natural killer/T-cell lymphoma, nasal type (NKTL)[2]. In recent years, the epidemiology of NKTL has undergone a gradual shift. Originally, NKTL was endemic to East Asia, Central and South America and was extremely rare in Europe and the United States[3]. However, the incidence of NKTL has increased during the last several decades in countries where it was previously not epidemic. Duval *et al* reported that the annual incidence rate of sinonasal extranodal NKTL increased by nine percent from 2000 to 2011 in the United States[4]. Thus, NKTL is no longer a local disease, but is becoming a common malignancy, and physicians should take into consideration in this era of globalization.

Although the prognosis for NKTL is extremely poor, a combination of radiotherapy and chemotherapy is effective to treat NKTL patients. Several chemotherapy regimens, such as SMILE (steroid, methotrexate, ifosfamide, L-asparaginase, and etoposide) and MPVIC-P (methotrexate, peplomycin, etoposide, ifosfamide, and carboplatin) have been used to treat NKTL patients and have shown favorable clinical responses[5, 6]. However, chemotherapy causes severe adverse effects, including deaths from serious infections[6], and radiotherapy can cause radiation injury of the brain because of NKTL localization. Therefore, a novel strategy to treat NKTL that lacks severe adverse effects is required.

In 1990, we first reported that Epstein-Barr virus (EBV) DNA and latent membrane protein (LMP) are expressed in NKTL biopsy samples[7]. Moreover, high levels of anti-EBV antibodies were detected in serum samples from NKTL patients, suggesting that EBV is associated with this disease. A growing body of evidence demonstrates that EBV plays a critical role in NKTL progression by modulating the tumor micromilieu, indicating that NKTL is an EBV-related

malignancy[8, 9]. Because EBV is an exogenous antigen in humans and CD8 T-cells or NK cells can recognize and destroy virus-infected cells[10], immunotherapy is an ideal strategy to treat EBV-related malignancies. For example, high lymphocyte infiltration is found in EBV-positive gastric cancer and is associated with significantly increased cancer-related survival of patients, compared to that in their EBV-negative counterparts[11]. Autologous EBV-specific cytotoxic T-cells have been reported to prevent EBV-related lymphoproliferative disorder in transplant patients[12]. In addition, we previously showed that antigen-specific CD4 T-cells could kill NKTL cells[13]. Given the evidences indicating that mediators of antitumor immunity can be effective against NKTL, there is an urgent need to develop appropriate immunotherapy treatments for NKTL patients. In this review, we will summarize recent findings on NKTL and discuss how to establish potent immunotherapy against NKTL.

2. The mechanism of immune evasion by NKTL

Cytokines

Because NKTL cell lines require exogenous IL-2 to survive *in vitro*[14], one would speculate that the NKTL microenvironment *in vivo* might contain sufficient IL-2 from other cells to support NKTL proliferation. Since lymphocytes are the major producer of IL-2, the histopathologic findings for NKTL revealed diffuse infiltration of lymphocytes with broad coagulative necrosis in tumor tissues[15]. In spite of the presence of lymphocytes and IL-2 in the microenvironment, which seems desirable for antitumor immunity, endogenous T-cell reposes are not able to reject NKTL. What are the factors that allow NKTL to avoid immune surveillance? First, cytokine competition may occur in the NKTL microenvironment. Because NKTL consumes cytokines, such as IL-2 and IL-15, that play a critical role in protecting CD8 T-cells from apoptosis in the contraction phase, tumor-reactive T-cells would not survive if insufficient cytokines were present surrounding the NKTL. Meanwhile, it should be noted that the direct injection of IL-2, which has been applied for treatment of solid tumors, is not a suitable method to treat NKTL because of its role in NKTL proliferation. The possibility of a shortage of cytokines near the tumor prompts us to consider

using adjuvants that can target APC to derive cytokines as well as TCR and costimulatory signals directly to T-cells.

In addition to competition with favorable cytokines, NKTL releases cytokines that are unfavorable to antitumor cells. NKTL produces IL-10, which enhances its proliferation through the upregulation of IL-2 receptor expression[16]. Because IL-10 robustly suppresses CD8 responses, NKTL would directly inhibit T-cells via IL-10 production. Recently, we found that NKTL cells produced chemokine (C-C motif) ligand (CCL) 22[17]. This cytokine has been reported to activate regulatory T-cells (Treg) that inhibit antitumor immune responses[18]; therefore, the production of CCL22 by NKTL supports immune evasion by tumors. Likewise, NKTL expresses cyclooxygenase-2 (COX-2)[19], a derivative of which (prostaglandin E2) attenuates antitumor helper T-cell responses[20], indicating that COX-2 inhibitor is a candidate for immune adjuvants. Collectively, in addition to cytokine competition, NKTL suppresses immune cells by production of immunosuppressive cytokines, chemokines, and prostaglandin.

Monocytes and macrophages

To clarify immune regulation by NKTL, it is vital to understand the precise source of the cytokines that feed NKTL. Our group has demonstrated that monocytes but not granulocytes contribute to NKTL proliferation in a membrane-bound IL-15-dependent manner[21]. Monocytes co-localized with lymphoma cells in NKTL clinical samples. NKTL cells produce interferon-γ inducible protein 10 (IP-10) to attract monocytes to the tumor micromilieu and interestingly, the level of IP-10 in serum samples from NKTL patients significantly decreased after chemoradiotherapy[22]. Similar to monocytes, macrophages can activate NK cells by trans-presentation of IL-15[23]. IL-10 as well as IP-10 were produced by NKTL and induced the conversion of monocytes into macrophages instead of DCs[24]. Although macrophages have the ability to engulf tumor cells, CD47 expression on tumors acts as a “don’t eat me” signal to diminish this ability[25]. In addition, macrophages block CD8 responses by suppressing IL-12 production from DC[26]. Thus, both monocytes and macrophages might be responsible for NKTL proliferation.

Immune checkpoints

Once programmed death-ligand 1 (PD-L1) molecules on tumor cells or APCs bind to T-cells through programmed cell death-1 (PD-1), T-cells become functionally tolerogenic. Chen *et al* have reported that PD-L1 was expressed in 67% of NKTL samples[27]. Because IL-10 is known to induce PD-L1 expression on macrophages, NKTL utilizes the PD-L1/PD-1 pathway via two distinct mechanisms to suppress T-cells: direct inhibition by PD-L1 expression on NKTL cells and indirect inhibition by induction of PD-L1 expression on macrophages via IL-10 production.

3. Peptide Vaccine

Epitope selection

CD8 T-cells play an essential role in antitumor immunotherapy by directly killing tumor cells. In addition to CD8 T-cells, innumerable reports have indicated that CD4 T-cells, which were originally considered to aid cytotoxic CD8 T-cells via cytokines and costimulatory signaling also have a direct cytotoxic activity against tumors[28]. To stimulate T-cells, a complex comprising major histocompatibility

complex (MHC), antigens and T-cell receptors (TCR) is needed in addition to costimulatory molecules (signal 2) and cytokines (signal 3). Antigen-presenting cells (APCs) present antigens to CD4 or CD8 T-cell receptors via on MHC molecules. Antigens have a specific amino acid sequence (mostly 9-mer for CD8 and 15-mer for CD4 T-cells), known as the epitope, which binds tightly to MHC Class I or Class II molecules. The classical function of CD8 T-cells is to recognize intracellular-synthesized proteins, such as virus-derived antigens presented by APCs. Another function of CD8 T-cells is to react with exogenous antigens, which are captured and presented (cross-presentation) by professional APCs, known as dendritic cells (DCs)[29]. CD4 T-cells also recognize exogenous antigens, which are pruned in early endosomes for loading on MHC Class II molecules in APCs. Although tumor cells are not appropriate APCs to initiate immune responses, because of their low expression of costimulatory molecules and their lack of cytokines, T-cells can react with tumors, once stimulated by the appropriate APCs with antigens such as chemically synthesized epitope peptides. Synthetic peptides have several advantages over

whole tumor lysate or protein, such as reproducibility, cost-effectiveness and simplicity of manufacturing. The autologous lymphoblastoid cell line (LCL) has been reported to activate EBV-specific CD8 T-cells for treating EBV-related lymphoproliferative disorder; however, it is difficult to use LCL as a source of antigens to treat NKTL because of differences in expression of EBV-related immunogenic proteins (LCL: EBV latency type III infection, NKTL: EBV latency type II infection)[12, 30].

To develop a CD8 peptide vaccine for treatment of NKTL, the first step is to determine the frequency of each MHC allele in NKTL patients for preferential epitope selection. However, limited data is available for MHC alleles in NKTL. One report has been published showing that the frequency of HLA-A*0201, one of the most globally prevalent HLA-A alleles, was significantly lower in NKTL patients than that in the healthy population[31] suggesting that HLA-A*0201 provides protective immunity against NKTL. However, the frequency of the HLA-A2 allele in nasopharyngeal cell carcinoma, another EBV latency type II infection-related malignancy, remains a point of controversy[32]. Because each

HLA allele presents different epitopes, additional research is needed to elucidate whether HLA alleles can determine susceptibility to NKTL.

The most crucial step in developing a peptide vaccine is to identify and verify epitopes that elicit anti-NKTL T-cell responses. Because insufficient data is available on the frequency of HLA alleles in NKTL, the epitope peptide should be designed to bind to common HLA alleles. The advantage of treating NKTL with a peptide vaccine is that this tumor expresses EBV proteins, which are exogenous antigens that can induce strong immune responses. Indeed, EBV lytic cycle antigens have been reported to induce robust CD4 T-cell responses in EBV-infected donors[33]. However, subdominant components of anti-EBV T cell responses are elicited against EBV lytic cycle antigens such as BNRF1[34]. Because latency type II EBV causes NKTL, which expresses the LMP-1, LMP-2 and EBNA-1 proteins but not the lytic cycle antigens, EBNA-2-6 or ZEBRA[5], identification of epitopes from LMP-1, LMP-2 or EBNA-1 is required to elicit anti-NKTL T-cell responses. We previously identified an LMP-1-derived CD4 epitope that could elicit LMP-1-specific CD4 T-cell responses by using

computer-based algorithms[13]. This $LMP1_{159-175}$ epitope could bind to many types of HLA-DR (HLA-DR9, -DR15, and -DR53), indicating that $LMP1_{159-175}$ is a promiscuous epitope. Notably, $LMP1_{159-175}$-specific CD4 T cells directly killed NKTL cells, suggesting that an LMP1-targeted peptide vaccine is a promising strategy to treat NKTL patients. Hislop *et al* have summarized the amino acid sequences of other CD4 and CD8 epitopes derived from EBV latent proteins including LMP1, LMP2 and EBNA1[35].

Tumor-associated antigens (TAA) are the proteins that are expressed abundantly in tumors compared to that in normal tissues. Because high-avidity self-reactive T-cells are eliminated in the thymus, TAA-reactive T-cells might not react with normal tissues but only with tumors that express high levels of TAA. For example, epidermal growth factor receptor (EGFR) is a TAA that is highly expressed in head and neck cancer and only slightly expressed in normal tissues such as the colon[36]. We have shown that EGFR-reactive T-cells are present in head and neck cancer patients without any clinical symptoms of autoimmune diseases, supporting the notion that TAA-reactive T-cells mainly

recognize tumors and not healthy tissues[37]. Thus, TAA-derived epitopes are candidates for peptide vaccines. In NKTL, aurora kinase A and enhancer of zeste homolog 2 (EZH2) are upregulated and play a role in anti-apoptosis and cell cycle acceleration[38, 39]. We previously reported that CD4 epitopes from aurora kinase A and EZH2 (Aurora-$A_{161\text{-}175}$, Aurora-$A_{233\text{-}247,}$ and $EZH2_{95\text{-}109}$) that could induce antitumor CD4 T-cell responses, suggesting the utility of these peptides for development of peptide vaccines for NKTL treatment[40, 41]. Similarly, c-Met is a tyrosine kinase receptor that is expressed in many types of tumors and that participates in inducing cancer stem-like cells and tumor viability. Our group recently found that c-Met was expressed in NKTL clinical samples and that hepatocyte growth factor/c-Met signaling was partially responsible for NKTL proliferation[14]. Moreover, using novel c-Met CD4 epitopes (c-$Met_{278\text{-}292}$, c-$Met_{817\text{-}831,}$ and c-$Met_{1244\text{-}1258}$), we established c-Met-specific CD4 T-cell lines that could directly kill NKTL cells. The IL-9 receptor is another antigen that can be a target of NKTL peptide vaccines. We have shown that the IL-9 receptor is expressed in NKTL cells and that IL-9/IL-9 receptor signaling contributes to

NKTL survival in an autocrine manner[8]. Although the IL-9 receptor is expressed on healthy immune cells (e.g., eosinophil), we found that precursor CD4 T cells that specifically react with the IL-9 receptor-derived epitope were present in healthy donors[42]. These IL-9 receptor$_{168\text{-}182}$-specific CD4 T cells showed cytotoxicity against malignant tumors expressing the IL-9 receptor. Thus, TAAs such as aurora kinase A, EZH2, c-Met, and IL-9 receptor are potential candidates for development of an NKTL peptide vaccine.

Malignant tumors often show mutations. Several studies have reported that the mutated peptides could be suitable targets of a peptide vaccine because peptides that contain mutated amino acid sequences are not expressed in normal tissues and therefore function as foreign antigens[43]. Thus, mutated CD4 or CD8 epitopes may elicit anti-NKTL T cell responses via their high immunogenicity. Mutations in Fas and p53 are common in NKTL (Fas: 50-60%, p53: 20-60%)[5, 44-46]. Interestingly, both are apoptosis-related proteins, indicating that these mutations render NKTL more resistant to apoptosis. We analyzed potential mutation epitopes of the p53 protein that showed a better

HLA binding score than their wild-type counterparts (Table 1). Although these mutation peptides would strongly bind to HLA, each patient had a separate mutation indicating that there is no hot spots mutation that can be utilized as an off-the-shelf peptide vaccine. Thus, these mutation peptides might be targets for tailor-made vaccine in NKTL treatment. Because wild-type $p53_{139-147}$ has been reported to induce p53-reactive CD8 responses[47], it would be valuable to investigate the mutated $p53_{139-147}$Q144L peptide as a heteroclitic peptide to determine whether this mutation stimulates wild-type p53-reactive CD8 T-cells more strongly than its wild-type counterpart, based on its HLA binding score. Thus, EBV-derived antigens, TAA or mutated antigens may provide sources of epitopes for development of peptide vaccine for NKTL treatment.

Adjuvants

Although peptide vaccines are a promising approach for inducing antitumor responses, TCR signaling alone is thought to generate peripheral immune tolerance or ignorance to antigens[48]. To overcome this tolerance using peptide vaccines, adjuvants are indispensable. The adjuvant most widely used in mice

and humans is incomplete Freund's adjuvant (IFA), which consists of non-metabolizable oil and a surfactant. After mixing with a peptide epitope, IFA forms a water-in-oil emulsion and slowly releases the antigen over more than 30 days[48]. Although the ability of IFA plus antigen to elicit humoral immune responses has been established[49], its ability to stimulate CD8 T-cells and Th1 cells is not yet evident. Indeed, the IFA-peptide combination has been reported to induce immune tolerance and subsequent tumor growth[50]. Hailemichael *et al* clearly demonstrated that T-cells became apoptotic after accumulation in IFA deposits and did not migrate into the tumor bed[51]. Moreover, clinical studies with IFA (Montanide) and TAA peptides resulted in undesirable outcomes[52], and preclinical and clinical data indicate that IFA is probably a suboptimal adjuvant for tumor peptide vaccines. Based on the understanding of pattern recognition receptors, many toll-like receptors (TLR) and STING ligands are thought to be attractive adjuvants for peptide vaccines. For example, use f the STING ligand c-di-GMP with a peptide enhances antigen-specific CD8 T-cell responses, followed by tumor regression[53], and TLR 5 and TLR 7/8 stimulation

upregulates proliferation and Th1 cytokine production by CD4 T-cells[54]. However, because NKTL cells are thought to originate from NK cells or γδ T-cells[5], application of TLR ligands as adjuvants is not as straightforward as in solid tumors, because TLR signaling could stimulate NK cells[55]. However, TLR 2 triggering has been reported to induce EBV lytic protein, which is more highly immunogenic than latent protein, indicating that the TLR 2 ligand can play a role not only in stimulating immune cells but also in augmenting the immunogenicity of NKTL[56]. In addition to TLR 2 ligands, valproic acid increases the susceptibility of NKTL to T-cells because of its ability to increase EBV lytic protein expression in EBV-positive tumors[57]. Although costimulatory molecules such as CD40, OX40, and 4-1BB are attractive adjuvants for peptide vaccines against solid tumors, caution should be taken in using them for treatment of NKTL because these costimulatory molecules can also stimulate NK cells[58]. c-Met inhibitors could be used to upregulate the efficacy of peptide vaccines. We previously described that c-Met inhibition attenuated production of TGF-β, a representative immunosuppressive cytokine, in NKTL cells[14].

Considering that antibody enhances cross-presentation by capturing antibody-tumor conjugates by APCs through Fc lesion, c-Met antibody would be better than c-Met tyrosine kinase inhibitor for the combination of peptide vaccine.

4. Genetically modified cells

The limitations of peptide vaccines include the low frequency of antigen-specific T-cells and tolerogenic APCs or T-cells in the tumor microenvironment, which can be partially overcome by use of appropriate adjuvants, as discussed above. Recent advances in bioengineering have permitted the development of artificially modified immune cells for use in the field of tumor immunology. Although this procedure is more complicated and expensive than development of synthetic peptides, transduction of the entire protein sequence of the antigen into APCs can circumvent the necessity to explore epitope sequences. K562 cells are commonly used as artificial APCs by engineering with virus to express costimulatory molecules and cytokines[59]. In NKTL, the transduction of EBV LMP-1 and LMP-2 vectors to autologous DCs from lymphoma patients has been

reported[60]. These modified DCs induced LMP-1 or LMP-2-specific T-cell responses (both CD4 and CD8) and showed favorable clinical results.

Chimeric antigen receptor (CAR)-T-cells are the primary type of genetically modified cells employed as effector cells. Unlike TCR, CAR consists of an antigen-binding site, commonly derived from monoclonal antibodies and a T-cell signaling domain (e.g, CD3ζ and 4-1BB). CAR-T-cell treatment has demonstrated encouraging results in B-cell and mantle cell lymphoma[61] suggesting that this treatment strategy can be also applied to NKTL patients. Because CD30 is expressed in NKTL[62], CD30-targeted CAR T-cells might be an option for adoptive transfer therapy[63]. The merits of CAR T-cells over peptide vaccines are as follows; this *ex vivo* method can induce a large number of antigen-specific T-cells that are quite rare in nature, with no need for MHC expression on the tumor; and it is relatively easy to generate off-the-shelf drugs because CAR T-cells are not restricted by MHC molecules. Although CAR T-cell therapy has shown some promising clinical results, several concerns remain. Firstly, the target antigens of CAR T-cells are limited to cell surface proteins.

Once tumor downregulate or internalize the target protein, CAR T-cell responses are inhibited. In this respect, peptide vaccines are superior to CAR T-cells because antigen internalization is not a problem in antigen processing and even if the antigen is downregulated, it is relatively easy to use peptides from other antigens as the epitope. Secondly, the functions of CAR and TCR may differ. The responses of CAR T-cells are attenuated after a few days owing to CAR downregulation[64]. Moreover, CAR T-cells show lower cytotoxicity and specificity than native TCR T-cells that recognize the same antigen[65]. To avoid these unfavorable aspects, transduction of EBV LMP2-derived TCR to T-cells has been reported, and it showed an antitumor effect *in vitro* and *in vivo*[66]. Lastly, a crucial concern in use of CAR T-cells is safety. High avidity T-cells, which respond to self-antigens, are usually eliminated in the thymus to prevent autoimmune diseases. Although CAR T-cells can react with any cells that express the target antigen with strong subsequent signaling such as 4-1BB, they can cause unexpected serious adverse effects, including patients' death[67]. Thus, further research is required to establish the efficacy and safety of CAR

T-cells in NKTL treatment.

5. Antibody therapies

Tumor cells express unique molecules on their surface membranes that are targetable for therapeutic antibodies. These antibodies such as cetuximab and rituximab are capable of inducing antibody-dependent cellular cytotoxicity, antibody-dependent complement-mediated cytotoxicity, and antibody-dependent cellular phagocytosis and have shown promising clinical results in both solid and hematological tumor[68, 69]. C-C chemokine receptor 4 (CCR4) is expressed on NKTL and might be a suitable target for the antibody therapy. Because CCR4 is a receptor of CCL22 that activates Treg as discussed above, CCR4-targeted therapy would damage both CCR4-positive NKTL cells and Treg with manageable adverse events[70]. Indeed, we have shown that mogamulizumab, a defucosylated anti-CCR4 antibody, enhances the killing of NKTL cells by normal NK cells via antibody-dependent cell-mediated cytotoxicity[17].

Because NKTL produces immunoregulatory cytokines, neutralizing these

cytokines would be another possible strategy in antibody therapy. NKTL produces IL-10 for its proliferation[16]. Because counteracting IL-10 by using a neutralizing antibody has shown antitumor effects in a melanoma model[71], this treatment would also be an option in NKTL treatment. Although IL-9 is expressed by NKTL and plays a role in tumor survival in an autocrine manner[8], the influence of IL-9 on antitumor immunity remains a point of controversy and further studies are required to apply IL-9 blockade for treatment of NKTL.

In the past decade, one of the most exciting advances in the field of tumor immunology is the identification of immune checkpoints, such as PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Anti-immune checkpoint antibodies have produced a significant improvement in the survival of patients with diverse types of tumors, including lung cancer, colon cancer, renal cancer and melanoma. Because 67% of NKTL samples expressed PD-L1[27], NKTL might also be a target for immune checkpoint blockers.

6. Combination with other therapies

Before immunotherapy can be introduced into current NKTL treatments, it is important to understand whether there are synergistic or whether they interfere with each other. The mainstay of NKTL treatment is a combination of chemotherapy and radiotherapy. Patients treated with our regimen, known as MPVIC-P (methotrexate, peplomycin, etoposide, ifosfamide and carboplatin), achieved a high rate of complete remission [5]. Recent evidence suggests that platinum-containing chemotherapy has immune-modulating effects such as recruitment of effector CD8 T-cells and downregulation of Treg and MDSC[72]. In addition, radiation therapy induces tumor-specific CD8 T-cells via activation of DCs[73]. In spite of these benefits to antitumor immunity, some of the chemical reagents and radiotherapies have detrimental effects on immune cells such as effector T-cells and bone marrow stem cells. Thus, further basic studies are needed to verify the rationale for combining immunotherapy with chemoradiotherapy in NKTL treatment. Allogeneic hematopoietic stem cell transplantation is another option presently used to treat NKTL. This treatment is considered to be a part of immunotherapy because allogeneic cells may reject

tumor cells via a graft versus tumor effect. Because the limited data available on NKTL showed high treatment-related mortality[74], improvement of the protocol or the patient selection criteria is required before this treatment can be applied as a standard procedure.

In experimental treatments, histone deacetylase inhibitors (HDACi) induce apoptosis in NKTL cells[75]. Because HDACis have been reported to induce EBV lytic proteins in EBV-positive tumors[57], the upregulation of antigen expression by these reagents would stimulate EBV-specific T-cells. In addition to their epigenetic effects, HDACi can also induce autophagy, which plays an important role in antigen processing. We previously reported that HDACi pretreatment of tumors augmented subsequent antitumor CD4 T-cell responses by regulating autophagy in tumors[14] suggesting that HDACi modulate antitumor immunity via multiple mechanisms. As discussed above, the immune checkpoint inhibitor PD-L1 is expressed on NKTL and could be a promising target for the therapeutic antibodies[27]. Our group recently showed that the combination of a PD-L1 inhibitor and a TLR ligand resulted in CD8-dependent

tumor regression in a melanoma model[76]. Thus, immune checkpoint inhibitors and other adjuvants are expected to show synergistic effects with peptide vaccines in NKTL immunotherapy.

7. Conclusion

A dynamic paradigm shift in methods for treatment of malignant tumors is currently underway. Immunotherapy had long been considered to be a suboptimal option; however, discovery of cytokines, costimulatory molecules, and immune checkpoints have provided clear evidences that immunotherapy can repress malignancies as effectively as surgery, chemotherapy and radiation therapy. In melanoma patients, anti-PD-1 antibody induced better objective responses than chemotherapy [77]. Although the key elements of antitumor immunity are of hematological origin, some hematologic malignancies are characterized by their sensitivity to immunotherapy. Because autologous CD8 T cells can prevent EBV-associated lymphoproliferative disorders[12], it is not surprising that immune cells reject EBV-related hematologic malignancies,

including NKTL. As discussed above, a growing body of evidence supports the application of immunotherapy to NKTL treatment (Table 2). Indeed, LMP-1-targetd cytotoxic T lymphocytes injection or allogeneic hematopoietic stem cell transplantation have shown some clinical responses in NKTL patients[60, 78], however, the therapeutic significance of this therapy to conventional therapies is totally unknown. Future *in vivo* and clinical studies will reveal whether this therapy will allows us to reach the ultimate goal, a cure for NKTL.

Future perspectives

To translate the basic findings discussed above into a clinical setting, *in vivo* models should be employed to ascertain the potential for unpredictable adverse events and the actual efficiency of immunotherapy in the tumor microenvironment. However, the prerequisite for supplemental IL-2 for survival of NKTL cells has hampered the establishment of an NKTL *in vivo* model. Following advances in genetic engineering, many immunodeficient animals have

been applied as xenograft models of human malignancies. Among them, the NOG mouse, in which NKTL cells can successfully be engrafted without assault by mouse immune cells, shows promise as a model for NKTL [79]. NOG mice possess the combined features of non-obese diabetic (NOD) mice, severe combined immunodeficiency (SCID) mice and IL-2Rγ^{null} mice, i.e., a deficit in complement lytic activity and macrophage function, an absence of typical T and B lymphocyte lineages, and a lack of NK cells. For the same reasons, RAG1^{null} IL-2Rγ^{null} mouse is a potential NKTL xenograft model. While NOG mouse is a promising model, the efficiency of NKTL engraftment was not high maybe because of the lack of IL-2[79]. Thus, the development of reproducible mouse model is still required. Although active immunotherapies, such as c-Met-targeted peptide vaccine, must be tested in animal models to verify their safety and efficacy, CCR4 or PD-1 inhibitors might bypass part of this process, because these drugs have already been applied in treatment of human solid and hematological malignancies[80, 81]. Thus, some immunotherapies would require lengthy investigation, but others have the potential to proceed to clinical trials in

the near future.

Response evaluation criteria such as RESIST, which are routinely used to assess chemotherapy responses, are not ideal to assess tumor responses to immunotherapy because tumors show pseudo-growth after immune cell infiltration[82]. Although no defined benchmark is available for measuring the effectiveness of immunotherapy against hematological malignancies, we have previously shown that serum EBV DNA levels are correlated with NKTL activity[83]. Thus, we conclude that, after developing a suitable immunotherapy for NKTL treatment, the presence of EBV DNA in the serum might provide a useful measure of the responses to this promising therapy.

Executive summary

- The number of patients with natural killer/T-cell lymphoma (NKTL) is increasing and treatment alternative to chemoradiation therapy, which is highly toxic, are needed.
- Because NKTL is a virus-related malignancy, Epstein-Barr virus proteins are

suitable antigens for development of a peptide vaccine against NKTL. Tumor-associated antigens such as c-Met, aurora kinase A, EZH2 or IL-9R are also candidates for peptide vaccine. Mutated p53 peptide shows promise as a neoantigen.

- In peptide vaccines, adjuvants such as TLR ligands are required to induce efficient antitumor responses. HDACi and c-Met inhibitor show promise as adjuvants in NKTL peptide vaccine, because they upregulate antigen expression and suppress immunosuppressive cytokines, respectively.
- Genetically modified cells (APC and CAR- or TCR-transduced T-cells) are new options in NKTL immunotherapy.
- Because NKTL cells express immunosuppressive molecules, targeted inhibition or neutralization of IL-10, CCR4 or COX-2 should be investigated with other immunotherapies.
- Immune checkpoint inhibitors (e.g. PD-L1, CTLA-4) have been applied in a clinical setting and have the potential to cure NKTL patients.
- Further studies in animal models (e.g. NOG mice) are required to translate

in vitro findings into clinical trials.

Conflict of Interests

The authors declare no competing financial interests.

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Table 1

***In silico* analysis of HLA binding score of mutation derived p53 peptide**

The amino acid sequences of mutation derived p53 peptides, which have high HLA binding score than their wild type counterpart are shown. Better binders have low percentile rank. Mutated amino acid is shown in red.

Table 2

Possible immunological targets in NKTL treatment

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Table 1

p53-derived CD8 epitope candidates						
Mutation	Sequence	Wt	Percentile_rank	Mutation	Percentile_rank	HLA restriction
S241A[46]	235-243	NYMCNSSCM	0.75	NYMCNSACM	0.6	HLA-A*24:02
F134L[46]	129-137	ALNKMFCQL	3.2	ALNKMLCQL	2.7	HLA-A*02:01
Q144L[45]	139-147	KTCPVQLWV	4.8	KTCPVLLWV	2.9	HLA-A*02:01

p53-derived CD4 epitope candidates						
Mutation	Sequence	Wt	Percentile_rank	Mutation	Percentile_rank	HLA restriction
C238S[46]	230-244	TTIHYNYMCNSSCMG	1.98	TTIHYNYMSNSSCMG	1.44	HLA-DRB1*04:01
S241A[46]	231-245	TIHYNYMCNSSCMGG	1.9	TIHYNYMCNSACMGG	1.83	HLA-DRB1*04:01
F134L[44]	134-148	FCQLAKTCPVQLWVD	1.12	LCQLAKTCPVQLWVD	1.03	HLA-DRB1*09:01
	131-145	NKMFCQLAKTCPVQL	1.51	NKMLCQLAKTCPVQL	1.48	HLA-DRB1*07:01
G226D[44]	212-226	FRHSVVVPYEPPEVG	2.2	FRHSVVVPYEPPEVD	2.11	HLA-DRB1*09:01
C242R[44]	231-245	TIHYNYMCNSSCMGG	1.9	TIHYNYMCNSSRMGG	0.67	HLA-DRB1*04:01
E258K[44]	248-262	RRPILTIITLEDSSG	7.12	RRPILTIITLKDSSG	1.81	HLA-DRB1*11:01
P142L[45]	129-143	ALNKMFCQLAKTCPV	9.21	ALNKMFCQLAKTCLV	1.43	HLA-DRB1*07:01
P142S[45]	131-145	NKMFCQLAKTCPVQL	1.51	NKMFCQLAKTCSVQL	0.76	HLA-DRB1*07:01
	131-145	NKMFCQLAKTCPVQL	7.36	NKMFCQLAKTCSVQL	2.03	HLA-DRB1*11:01
G245S[45]	231-245	TIHYNYMCNSSCMGG	1.9	TIHYNYMCNSSCMGS	1.89	HLA-DRB1*04:01
I251S[45]	240-254	SSCMGGMNRRPILTI	2.41	SSCMGGMNRRPSLTI	2	HLA-DRB1*11:01

Low percentile_rank = good binder

Table 2

Possible immunological targets in NKTL			
Peptide vaccine	Target antigens	EBV related protein	LMP1[13], LMP2[35], EBNA1[35]
		Tumor associated antigen	Aurora kinase A[38], EZH2[39], c-Met[14], IL-9R[42]
		Mutation antigen	p53 (Table 1)
	Adjuvants	TLR ligands[54], STING ligands[53], Valproic acid[57], Costimulatory molecule agonists, c-Met inhibitor[14]	
Genetically modified cell	APC	LMP-1- and LMP-2-transduced dendritic cells[60]	
	T-cell	CD30-targeted CAR T-cell[63], LMP-2 TCR transduced T-cell[66]	
Targeted therapy	IL-10 neutralizing antibody[71], anti-CCR4 antibody[17], COX-2 inhibitor[19,20]		
Checkpoint inhibitor	PD-1 or PD-L1 inhibitor[27]		
Other modalities	Chemotherapy, radiotherapy, HDAC inhibitor[14,57]		