One method to establish Epstein-Barr virus-associated NK/T cell lymphoma mouse models

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Abstract
Novel nude mice model of human NK/T cell lymphoma were established by subcutaneously injecting two NK/T cell lymphoma cell lines into the right axillary region of mice and successful passages were completed by injecting cell suspension which was obtained through a 70-μm cell strainer. These mice models and corresponding cell clones have been successfully developed for more than 8 generations. The survival rates of both resuscitation and transplantation in NKYS and YT models were 90% and 70% correspondingly. Pathologically, the tumour cells in all passages of the lymphoma-bearing mice and cell lines obtained from tumours were parallel to initial cell lines. Immunologically, the tumour cells expressed the characteristics of the primary and essential NK/T lymphomas. The novel mice models maintained the essential features of human NK/T cell lymphoma, and they would be ideal tools in vivo for further research of human NK/T cell lymphoma.

KEYWORDS
methods to establish mouse models, mouse models, NK/T cell lymphoma

1 | INTRODUCTION

Extranodal natural killer (NK)/T cell lymphoma (ENKTCL) is an aggressive malignancy of putative NK cell origin, with a minority deriving from the T cell lineage. Pathologically, the malignancy occurs in two forms, extranodal NK/T cell lymphoma, nasal type (NNKTC); and aggressive NK cell leukaemia. Although chemotherapy and radiotherapy can improve the disease outcome, the prognosis of NKTC is poor, and no targeted therapy is currently available. The pathogenic mechanisms of NK/T cell lymphoma remain largely unclear, and further investigation may aid in improving the accuracy of lymphoma diagnosis and gene therapy. Meanwhile, lymphoma is one immune-related disease, the microenvironment in vivo matters a lot in the mechanism of lymphoma. Complex biological processes often require in vivo analysis, and many important research advances have been made using mice as a model for the study of various biological systems. Mouse models are irreplaceable tools for the study of carcinogenesis. The mouse shares anatomical, immunological, and genomic similarities with humans and is the most accessible model system. To clarify the molecular pathogenesis of NKTC, we performed establishment of mouse models in NKTC, because animal models enable elucidation of the pathological and physiological mechanisms underlying lymphomas and preclinical testing of new drugs. Meanwhile, lymphoma mouse models with stable...
passages have various advantages over cell lines in vitro and single mouse models that have no passage in lymphoma research in vivo. It is not only an efficient in vivo platform to investigate drug efficacy and resistance studies in various cancers, but likely to be applied to lymphoma therapy. There is a growing need for mouse models that have stable characteristics to mimic in vivo studies of NK/T cell lymphomas. Here we introduce two NK/T cell lymphoma mouse models we have established successfully.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines

The human NK/T cell lymphoma cell lines YT and NKYS were gifts of Dr. John Chen in City of Hope. The origin of cell lines has been confirmed by next-generation sequencing. Every cell line was transplanted with luciferase to get stable cell lines. YT and YT-luciferase were maintained in Roswell Park Memorial Institute 1640 (RPMI1640, Gibco, Beijing, China) supplemented with 10% foetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), NKYS and NKYS-luciferase were maintained in RPMI1640 supplemented with 10% FBS and 100 IU/mL rhIL-2. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

### 2.2 | Nude mice model establishing

Three- to four-week-old female BALB/c (nu/nu) nude mice and NOD/SCID mice were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), weighed approximately 15–20 g, fed in the specific pathogen-free (SPF) barrier system and provided sterile food and water. All procedures were performed under aseptic condition. Before animal experiment, mice were fed adaptively for 1 week in the new circumstances and cyclophosphamide (CTX) for 2 days before cell suspension injection and transplantation. Cells were washed repeatedly in Hank's liquid, resuspended in 0.2 mL RPMI1640 culture media and subcutaneously injected into the right axillary region of mice (NKYS cells in NOD/SCID mice and YT cells in nude mice). The NKYS models were injected with 20 000 IU/kg rhIL-2 once a week intraperitoneally. The general condition and the appearance changes of the lymphoma-bearing mice were observed every day, and the size of tumour was measured in length and width three times a week. As soon as the tumour reached about 2000 mm³ in size or 1.0-2.0 cm at length, or the animal showed distress, the mouse was killed. A part of the tumour tissues was used for continuous transplantation, the remaining part was divided into two parts, one for routine morphologic observation and the other preserved in liquid nitrogen for further use.

### 2.3 | Bioluminescent imaging

To investigate whether the tumour existed in other part of mice, tumour development was quantified by in vivo imaging using an IVIS Lumina III system (PerkinElmer, Waltham, MA, USA). Mice were anaesthetized with isoflurane, injected i.p. with 150 mg/kg body weight Luciferin (PerkinElmer) and imaged 5 minutes after Luciferin injection. Total flux values, which correlate directly with tumour mass, were measured. Images were analysed with Living Image 3.0 software (PerkinElmer).

### 2.4 | Passage

A period of time after cell suspension injection (at least 35 days in NKYS cells and 45 days in YT cells), the tumour-bearing mice (denoted F0) were killed. We took the passage in three ways initially. Inserting the small block from the tumour into the next mice is the first. However, both the tumorigenicity and growth rate were quite low, so lastly we abandoned this method. A part of tissues excised from the tumour were minced and strained through a 70-µm cell strainer (Becton Dickinson, San Jose, CA, USA). One part of the cells obtained through the strainer was injected in the same manner as for the establishment of first mice model. Others were cultured and began to proliferate. In the case of engraftment, 1.5 x 10⁷ to 2 x 10⁷ tumour cells were serially subcutaneously injected into the right axillary region of other mice. Stable mouse models were considered to be established based on the success of over six serial passages of the tumours (F5). Currently, the tissues and cells obtained from F0, F4, and F8 mice were used in the subsequent analyses. Notably, cells were injected with matrigel in the ratio of 1:1.

The cultured cells from every passage tumour have been always maintained. Cyto centrifuge smears of the cells were prepared and stained with May–Giemsa for morphologic evaluation. To confirm that the tumour cells were clonally related to the original tumours, the sequences of the surface markers and EBV-encoded RNA (EBER) in situ hybridization were analysed. All the animal experimental procedures complied with the Regulations regarding Animal Experiments in Zhengzhou University. The cell lines transplanted with luciferase were passaged once or twice to observe the distribution of tumour cells in vivo.

### 2.5 | Time course engraftment analyses after transplantation

To investigate the pattern of engraftment and proliferation of the lymphoma cells in the mouse models, time-lapse engraftment analyses were performed. After subcutaneous injection of 2.0 x 10⁷ tumour cells in the right axillary region of mice, tumour engraftment, and proliferation patterns were analysed every 3 days.

### 2.6 | Histopathology and immunophenotype

For histological examination, an autopsy was performed on each passage of nude mice. The samples were fixed in 10% neural-buffered formalin, embedded in paraffin. Four-micrometre-thick sections were made and stained with haematoxylin and eosin. Morphologic observation included architecture of the tumours and distribution of the tumour cells, the size and shapes of the tumour cells, the number of
mitotic figures and other combined changes including necrosis, angio-
centric invasion and so on. Moreover, we evaluated tumour cells from
the mouse models in terms of characteristics of cell surface antigens
associated with B cell origin and cell adhesion molecules.

2.7 | In situ hybridization

Epstein Barr virus (EBV) encoded EBV-encoded RNA in situ hybridiza-
tion (RISH) was performed to detect RNA transcripts of EBV using
RISH Epstein Barr encoded RNA probe kit (ZSGB-BIO, Beijing, China,
ISH-6022-Y, 170510). Tissue sections were deparaffinized, rehydrated,
and digested with proteinase K enzyme. After pretreatment in
10 mmol L\(^{-1}\) sodium citrate (pH 6.0), sections were hybridized with
digoxigenin labelled RISH EBER probe by incubation at 55°C for
1 hour. Following hybridization, sections were washed with tris buffer
(pH 7.6) and hybridized probe are detected and diaminobenzidene
stained was observed under light microscope. Dark brown staining of the tumour
nucleus was considered as positive for EBER transcripts expression.

2.8 | Flow cytometry (FCM) analysis

To evaluate surface antigens associated with cell adhesion and with the
cell of origin as the normal counterpart of the tumour cells, FCM analy-
ses were performed using the target antigens including CD2, CD3, CD4,
CD5, CD7, CD8, CD45, CD56, CD57, and Ki-67. All antibodies were
obtained from Beckman Coulter (Kraemer Boulevard Brea, CA, USA).
Briefly, trypsinized cells, prepared as a single cell suspension, were sus-
pended in 50 \(\mu\)L aliquots of 0.1% bovine serum albumin-PBS balanced
salt solution (BSA-PBS). Fluorophore-conjugated CD antibodies were
added to the cell suspensions and incubated for 1 hour at room temper-
ature in the dark. Isotype-specific fluorochromated antibodies were
used as negative controls to delineate the negative cell population. The
reaction was stopped by the addition of 1 mL of 0.1% BSA-PBS. The
cells were centrifuged and washed twice suspended in PBS. Flow analy-
sis was done by a BD machine (FACS Canto TM, BD, Piscataway, NJ,
USA) fitted with proper lasers. Events that registered outside this trace
were scored as positive, and 50 000 events were collected for each
sample. The percentage of positive events was determined.

2.9 | Statistical analysis

Two-tailed \(t\) tests of equal variance were used to analyse flow
cytometry data. Bioluminescence was evaluated using unpaired
\(t\) tests. Statistical significance was defined as a \(P\) value of less than
or equal to 0.05. All statistical analyses were done by using Prism 5.

3 | RESULT

3.1 | Time-lapse engraftment analysis of the NK/T
cell lymphoma

We analysed tumour engraftment and proliferation in the mouse
models over a time course. The human NK/T cell lymphoma cell lines
YT and NKYS cells were injected into one 4-week-old female mouse.
The 1st passage of the above tumours was visible 40 and 30 days,
respectively, later after the injection. Subsequent passages were per-
formed with serial tumour suspension injection when tumours grew
up to 1.0-2.0 cm at length. The mouse often showed cachexia in the
late stage. Up to now, the two kinds of lymphomas have been pas-
saged to the 7th and 10th generation, the autopsy findings of the
mouse models are shown in Figure S1. The combined data suggest
that, at least in these models, the tumour cells were not disseminated
(data not shown). Each passage of tumour tissue suspension and/or
cells was injected into the next mice with matrigel. The pieces of
fresh transplanted tumour samples in 7th passage were taken from
the mice, and transplanted directly into the right axillary region of 10
mice respectively. The NKYS and YT transplanted tumours had
appeared in all of the 9 NOD/SCID mice, but YT in all of 7 nude mice.
The growth rate is not different in every passage (data not shown)
and the growth curve in the last passage is shown in Figure S1.

3.2 | Tissue pathologic features

The cells in the process of passage had the same feature with the
original cell lines. In YT and its serial passage mice models tissues,
the normal architecture was effaced, heterogeneous lymphocytes
diffused into the plate, with big volume and abundant cytoplasm.

![FIGURE 1](image.png)

**FIGURE 1** In YT and its serial passage mice models tissues, the normal architecture was effaced, heterogeneous lymphocytes diffused into
the plate, with big volume and abundant cytoplasm. The karyotype in cells is irregular and the chromatin is coarse. apoptotic and necrotic
tumour cells were found
The karyotype in cells is irregular and the chromatin is coarse. apoptotic and necrotic tumour cells were found (Figure 1). The tumour tissues in YT F0, YT F5 and YT F7 were positive for CD56, Granzyme B, Perforin (Figure 2) but negative for TiA1 (Table 1).

Sections of the biopsy from serial NKYS cell mice models showed similar morphological features with YT mouse model tissues (Figure 3). Immunohistochemical staining showed the large atypical cells were positive for CD56, Granzyme B, Perforin, TiA1 (Figure 4 and Table 1).

All characteristics in both kinds of biopsies are consistent with NK/T cell lymphoma. The expression of cell surface markers is summarized in Table 1.

### 3.3 Cell lines features

In the process of passage, the cells of every passage tumour obtained through the strainer were cultured in the same environment with the original cell lines. The cultured cells have continued to proliferate with the same morphology of original cell lines. Cytocentrifuge smears of the cells were prepared and stained with May-Giemsa for morphologic evaluation. The cells can be cryopreserved in cryopreservation-medium (90% FBS, 10% DMSO), stored in liquid nitrogen, thawed again (with a viability of more than 70%) and successfully reconstituted. Flow cytometry analysis was performed on cell suspensions cultured from each passage tumour tissues. YT F0, YT F5, YT F9 cells were CD3−/CD4−/CD8−/CD45+/CD56+/CD57− (Figure 5 and Table 2). Similar to YT cells, the serial NKYS F0, NKYS F4, NKYS F7 cells were CD3−/CD4−/CD7+/CD8−/CD45+/CD56+/CD57−. No difference existed between passages (Figure 6 and Table 2).

### 3.4 EBV infection

EBV was strongly detected in situ hybridization for EBV RNA using the EBER probe in YT, NKYS, and later passage tissues (Figures 7 and 8; Table 1).

### 4 DISCUSSION

In vitro studies have always been a powerful tool to characterize many cancer-associated processes. However, the tumours comprise not only the malignant cells but also a plethora of nonmalignant cells and extracellular matrix-the tumour microenvironment, that’s why numerous aspects of neoplastic growth are difficult to model in vitro alone. In vitro approach has limitations when we extrapolate to the more complex in vivo scenario. In vivo strategies are expected to
incorporate tumour micro environmental clues that cannot be easily modelled in vitro. The latter applies to mechanisms of resistance to therapeutic, including tumour cell intrinsic mechanisms of resistance to low dose metronomic chemotherapy.\textsuperscript{13,14} While in vivo passage is an increasingly applied strategy to improve the modelling of therapeutic resistance.\textsuperscript{15,16} Severe immunodeficient mice (SCID) mice have widely been used to disseminate tumour cells in vivo,\textsuperscript{17} where the cells are engrafted via intravenous injection.\textsuperscript{18,19} These mice have been used to develop a mouse model for human Burkitt lymphoma by using the Daudi cell line or SU-DHL-4 cells.\textsuperscript{20} Improvements in mouse technology have facilitated research and preclinical studies in lymphoma fields, especially the application of NOD.Cg-Prkdc\textsuperscript{scid} Il2rg\textsuperscript{tm1Wjl}\textsuperscript{szJ} (NSG) mice. Mice engrafted with human tumours act as a model for testing various therapeutic drugs for their potency, toxicity, and dosing.\textsuperscript{21} Actually, The development of NSG mice has provided a valuable tool for the development of a NK/T cell lymphoma model, because they lack mature B and T cells and various cytokines such as IL2, 4, 7, 9, 15, and 21, leading to impaired development of NK cells.\textsuperscript{22,23} However, stable passages in lymphoma models have not been reported. It is admitted that NK/T cell lymphoma research has less success and popular in vivo. On one hand, severe immunodeficient mice are limited because they lack mature NFATc3 and CD28, leading to a decreased effect of chemotherapy. On the other hand, stable passages in lymphoma models have not been reported. It is admitted that NK/T cell lymphoma research has less success and popular in vivo.
hand, NK/T cell lymphoma mouse models are hard to be established. The tumours are hard to grow after cells injection because of the stimulation of immune environment in vivo and extra vivo. On the other hand, even if the models are established successfully, they remain not stable in passages, or never have been passaged. So far, only some Japanese established SNK6—which is a NK/T cell lymphoma cell line—mouse model in NOG mice, which is a similar species to NSG.\textsuperscript{24,25} It is known that using NSG and NOG mice to establish models costs too much, meanwhile because of the patent problem, many enthusiastic researchers have not the opportunity to use them. Therefore, if we could find one economic and efficient way to attain the same aim, that will benefit more researchers in the world. Fortunately, we have filled the historical blank with NK/T cell lymphoma mouse models, and in vivo passage makes it possible to screen and evaluate the novel antitumour drugs in NK/T cell lymphoma.

It was reported that there is some discrepancy in different passage of mouse models, for instance, Metildi et al\textsuperscript{26} ever reported that serially passaging in vivo generated more aggressive variants of both human pancreatic cancer cell lines, one of which was Kras wild-type (BXPC-3) and the other Kras mutant, Panc-1, which
displayed faster tumour growth and shortened survival time. To define the characteristics of tumours after passage, we examine the cell surface markers in IHC and Flow cytometry. EBV in ISH. YT cells, known as natural killer (NK) cell lines, resulted from a patient with acute lymphoblastic lymphoma with thymoma.\(^27\) NKYS cells preserved the original characteristics of EBV-associated nasal angio-centic T/NK cell lymphoma.\(^28\) These two cell lines are successfully representative of the typical NK/T cell lymphoma occurring in the world. In our mouse models, there is no significant difference in growth rate, histopathological, and immunohistochemical features among different passages of YT and NKYS models, which largely proves that we have got stable mouse models. More importantly, we invent a method to establish the NK/T mice model. CTX is necessary before injecting and implanting so that the immune system of mice can be inhibited more thoroughly. However, NKYS model is still hard to establish. In terms that NKYS cell line is dependent on IL-2, We hypothesis that the tumour need IL-2 to grow. It is critical that the tumour itself need the same environment with the corresponding cell line. In the future, the mouse models will facilitate the preclinical research of NK/T cell lymphoma.

However, using cell lines as inoculum an IL-2 support does barely reflect “real world” inter-patient heterogeneity of the lymphomas because the subcutaneously injected cells did not disseminate beyond the injection site. That is a world-wild question and there is a long way to go in the research on establishing mouse models that can mimic the real tumour environment in patients.

ACKNOWLEDGEMENTS

MZ, ZL, and XZ were responsible for conception and design. WX, WL, YS, and XL were responsible for collection and assembly of data. WX, YS, XW, GW, and MD and were responsible for data analysis and interpretation. WX and BW drafted the manuscript; all authors reviewed and revised the manuscript, and all authors gave final approval of the manuscript.

CONFLICT OF INTEREST

None.

FIGURE 8  EBV was strongly detected in situ hybridization for EBV RNA using the EBER probe in NKYS and later passage tissues

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Xue W, Li W, Shang Y, et al. One method to establish Epstein-Barr virus-associated NK/T cell lymphoma mouse models. J Cell Mol Med. 2019;23:1509–1516. https://doi.org/10.1111/jcmm.14057