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Review

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Preclinical models in the study of lymph node metastasis

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Abstract: Lymph node metastasis (LNM) is a crucial risk factor influencing an unfavorable prognosis in specific cancers. Fundamental research illuminates our understanding of tumor behavior and identifies valuable therapeutic targets. Nevertheless, the exploration of fundamental theories and the validation of clinical therapies hinge on preclinical experiments. Preclinical models, in this context, serve as the conduit connecting fundamental theories to clinical outcomes. In vivo models established in animals offer a valuable platform for comprehensively observing interactions between tumor cells and organisms. Using various experimental animals, including mice, diverse methods such as carcinogen-induced tumorigenesis, tumor cell line or human tumor transplantation, genetic engineering, and humanization, have been used effectively to craft numerous models for tumor LNM. Carcinogen-induced models simulate the entire process of tumorigenesis and metastasis. Transplantation models, using human tumor cell lines or patient-derived tumors, offer a research platform closely mirroring the histology and clinical behavior of human tumors. Genetically engineered models have been used to delve into the mechanisms of primary tumorigenesis within an intact microenvironment. Humanized models are used to overcome barriers between human and murine immune systems. Beyond mouse models, various other animal models have unique advantages and limitations, all contributing to exploring LNM. This article summaries existing in vitro and animal preclinical models, identifies current bottlenecks in preclinical research, and offers an outlook on forthcoming preclinical models.

Key words: Lymph node metastasis; Preclinical research; Preclinical models; Animal models

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1 Introduction

Cancer has emerged as a leading global cause of mortality, with 90% of cancer-related deaths attributed to tumor metastasis (Dong et al., 2023). Despite the pivotal role of lymph nodes in antitumor immunity, LNM remains an indicator of a poor prognosis across various cancer types, including melanoma, prostate, gastric, breast, and cervical cancers (Ji et al., 2023). The intricate involvement of numerous molecules and signaling pathways in LNM and mechanisms which are yet to be fully clarified have prompted extensive research. Previous studies have identified potential therapeutic targets, leading to the development of drugs (Ji, et al.,

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2023). To enhance patients' quality of life and survival, studies targeting LNM mechanisms and therapeutics have become a research focus, with related clinical trials gradually underway.

Preclinical models serve as an indispensable bridge between basic and clinical research (Mermod et al., 2018). They can be used to demonstrate how biological systems and diseases function in non-human models and provide insights into whether interventions are safe. Unfortunately, despite the practice and necessity of conducting preclinical translational research, many preclinical studies fail to predict the safety and efficacy of clinical trials (Kimmelman and Anderson, 2012). Even after animal studies have shown remarkable results, more than 80% of potential therapies fail when tested in humans, suggesting the urgent need to further understand preclinical models in translational research (Perrin, 2014).

Preclinical models include mainly *in vitro* cell cultures, animal models, organoids, and organoid-chips. *In vitro* cell culture is the simplest preclinical model but does not simulate the natural body environment. Tumors and their metastases do not occur in isolation. The tumor microenvironment (TME) significantly influences tumor growth, invasion, and metastasis, and the "dialogue" between tumor cells and the immune system in TME may determine the patient's prognosis (Fridman et al., 2012). Carcinogen-induced models, transplanted models, genetically engineered models, and humanized models, using animals like mice, rats, rabbits, dogs, and monkeys, can respond holistically to a variety of *ex vivo* and *in vivo* stimuli, compensating for the lack of TME in *in vitro* cultures. Therefore, the holistic nature of animal models undoubtedly provides an appropriate platform for research, bringing scientific studies closer to the human environment (Overgaard et al., 2018). However, the heterogeneity between humans and animals limits the translation of preclinical studies. The new generation of preclinical models represented by organoids and organoids-on-a-chip can simulate the human environment realistically, making them promising models for the future.

In this comprehensive review, we delve into the preclinical models commonly used for lymphatic system research, focusing mainly on LNM (**Fig. 1**). Our analysis encompasses their applications, advantages, and disadvantages, providing readers with a deeper understanding of these diverse models and laying the groundwork for subsequent related research.

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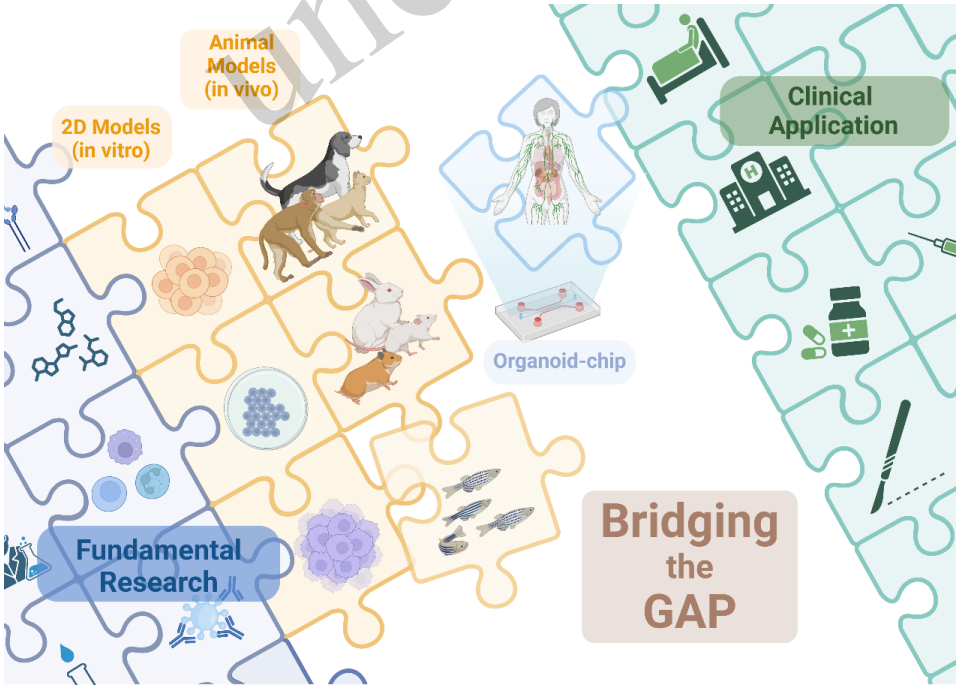

Fig. 1 Bridging the gap between basic research and clinical application. While there is still a gap between animal models and clinical applications, animal models provide the foundation for preclinical research. Subsequent technologies, such as organoid chips, will help fill this gap. Created with BioRender.com.

2 Mouse models

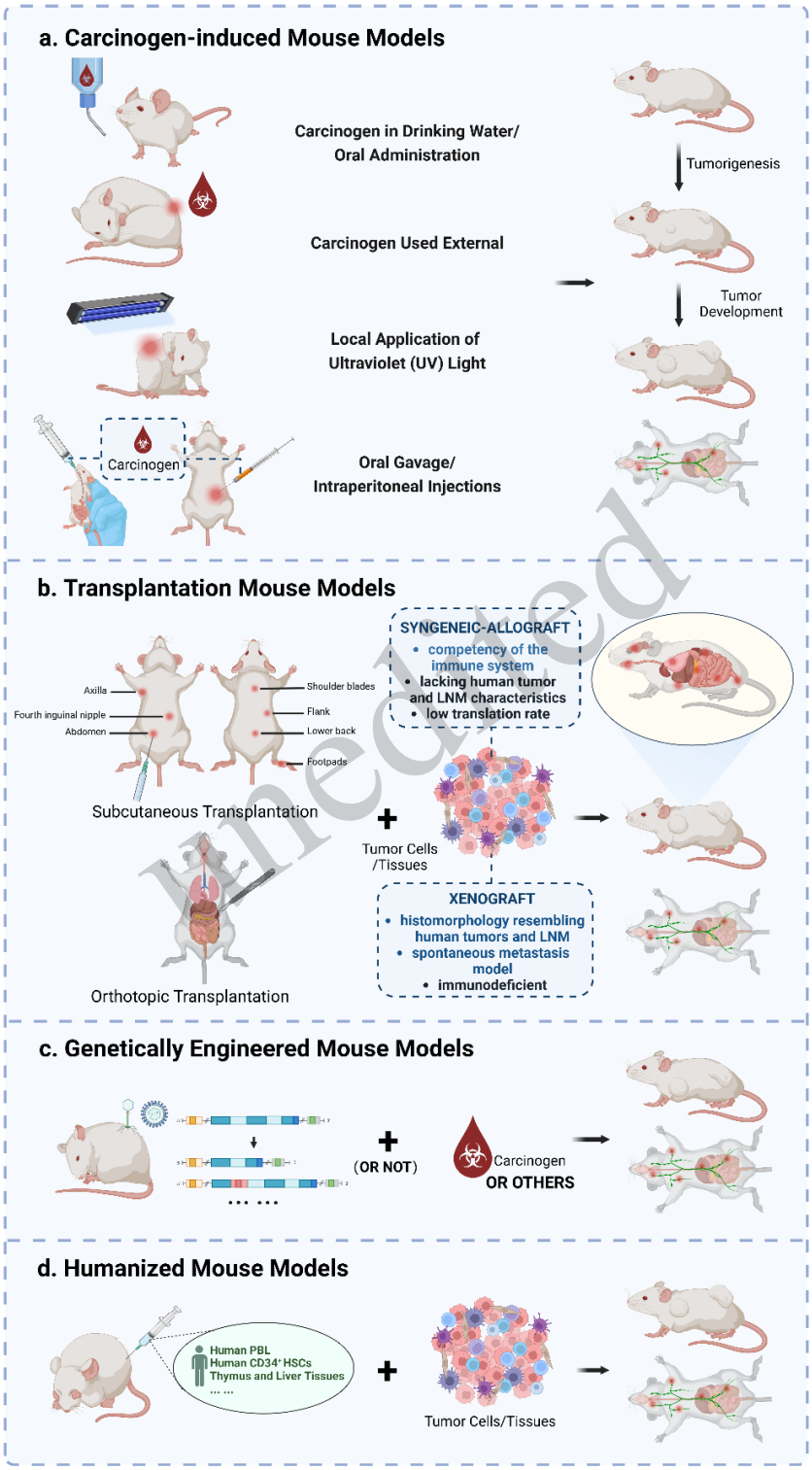

Fig. 2 Types of mouse models currently in use and their construction. Mouse models are still the main animal models used for studying lymph node metastasis. Mouse models are divided mainly into carcinogen-induced, transplantation, genetically engineered, and humanized mouse models. Created with BioRender.com.

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2.1 Carcinogen-induced tumors

Carcinogen-induced tumor models are commonly used in investigating tumor mechanisms and therapeutic strategies. Careful selection of appropriate carcinogens, treatments, and mouse strains enables the induction of tumors with pathogenic, genetic, histological, and immunohistological features akin to those found in human tumors (Tormo et al., 2006) (**Fig. 2a**). Notably, tumors induced in immunodeficient and immunologically competent mice exhibit variations in tumor growth, the TME, and other factors (Zitvogel et al., 2016). Appropriate model selection, aligned with the experimental objectives, is pivotal. 4NQO-induced tumors showed 93.9% similarity to smoking-associated HNSCC, whereas DMBA-induced tumors showed only 39.7% similarity, even though DMBA is a widely used tobacco carcinogen (Wang et al., 2019b). Also, although the treatments all induce tumor formation, different carcinogens induce different mutations. Ras oncogenes, a mutational target of carcinogens such as DMBA, are potent tumor driver genes, but they are not the dominant mutated genes in human HNSCC (Hoadley et al., 2018). 4NQO-induced tumors are not mutation-driven in genes such as HRas proto-oncogenes, Kirsten rats sarcoma viral oncogene homolog (KRAS), nor neuroblastoma ras oncogene (Nras) genes, and the driving process may be related to a more relevant and specific signaling pathway for human malignancies (Wang, et al., 2019b). Various models demonstrating lymph node metastatic ability have been successfully induced (**Table 1**).

Table 1 Carcinogen-induced tumors with LNM

Cancer	Mouse strain	Carcin-ogen	Method	Frequency or Time of Treatment	Incidence Rate of Cancer	LNM Related Information	Reference
OSCC	WT mice	4NQO	Drinking water	16 weeks	-	More and larger invasive tumors and more frequent LNM. The incidence rates of local LNM were 12.5% in the week 25–32 observation period and 100% in the week 33–40 observation period.	(Spenlé et al., 2020)
OSCC	BALB/c	4NQO	Drinking water	20 weeks	100%	Advanced OSCC developed during the week 33–40 observation period leads to mice displaying decreased food and water intake, causing weight loss, which could kill some mice and limits the use of this model for the study of LNM and evaluating therapeutic strategies of advanced stage.	(Li et al., 2013)
OSCC	p53 WT mice (K14 CreTg/+; p53wt/wt) (Cell line ROC1) and p53 R172H transgenic mouse (K14 CreTg/+; p53R172H/flox) (Cell line ROC3).	4NQO	Drinking water	8 weeks	-	ROC1 cells' Cervical LNM rate of orthotopic injection: 80% for 500,000, 40% for 100,000, 40% for 50,000, and 60% for 10,000. ROC3 cells: 60% for 500,000. These cell lines have genomic alterations similar to those found in tobacco-associated OSCC and present TP53 mutations consistent with early development of human oral cancer.	(Shi et al., 2022)
OSCC	C57Bl/6(Cell line 4MOSC2)	4NQO	-	16 weeks	-	Locoregional LNM was observed as early as 2 days postimplantation; a higher rate of LNM was observed 8	(Z. Wang et al., 2019)

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						days later. Established tumors exhibited a much higher density of lymphatic vessels staining LYVE-1 (+), aligning with the strong correlation between intratumoral lymphangiogenesis and metastasis in human HNSCC.	
ESCC	Ptpro− /− Wild-type (DBY747)	4NQO	Drinking water	16 weeks	-	More lymph node metastases (~20%) than wild-type (~10%).	(Dong et al., 2023)
HNSCC	B6;129S2-Trp53tm1Tyj p53+/- mice	DMBA	Oral administration	2 weekly	100%	LNM occurs 3 weeks after the onset of the primary tumor.	(Ku et al., 2007)
Colon Cancer	Tp53ΔIEC	AOM	i.p.	6 weekly	-	LNM in ~20 to 30% mice. Mutations of p53, the most frequently inactivated tumor suppressor gene in HNSCC.	(Neufert et al., 2021; Sarah Schwitalla et al., 2013)
Colon Cancer	Trp53ΔIECAktE17K	AOM	i.p.	6 weekly	-	More frequent LNM (80%) than Trp53ΔIEC mice. Gene expression profiles resemble human CMS4 subtype profiles.	(Varga et al., 2020)
Colon Cancer	Sprague-Dawley rats	MNU	intrarectal injections	15 weekly	100%	The first time that LNM from carcinogen-induced colonic cancer has been consistently and predictably documented in this outbred animal strain.	(Weese et al., 1983)
Melanoma	CDK4-mutant mice	DMBA, TPA	External used	Once (DMBA), twice a week for 5 weeks (TPA)	-	Distinct LNM and histology that are similar to human tumors.	(Tormo et al., 2006)
msSCC	FVB/N mice and Lgr5CREER/KrasLSL-G12D/Trp53fl/fl/Rosa-YFP mice	DMBA, TPA	External used (after shaving)	Single dose (DMBA),16 weekly (TPA)	-	22% LNM (6 of 27).	(Nassar et al., 2015)
Breast cancer	Sprague–Daley rats	MNU	i.p.	Single dose	-	Hormone-dependent and histopathologic features similar to human breast cancer tumors, presence of local LNM.	(Thordarson et al., 2001)

OSCC, oral squamous cell carcinoma; ESCC, esophageal squamous cell carcinoma; HNSCC, Squamous cell carcinoma of the head and neck; msSCC, mouse skin squamous cell carcinoma; 4NQO, 4-nitroquinoline 1-oxide; DMBA, 7,12-dimethylbenz[a]anthracene; MNU, N-methyl-N-nitrosourea; i.p., Intraperitoneal injection

For instance, in Ptpro-/- mice, invasive esophageal cancer was induced using 4NQO-treated drinking water (Dong, et al., 2023), while oral squamous carcinoma was induced in WT mice, BALB/c mice, p53 WT mice and p53 R172H transgenic mice and C57Bl/6 mice using the same method (Li et al., 2013; Wang et al., 2019a; Spenle et al., 2021; Shi et al., 2022). Head and neck squamous carcinoma was induced in B6;129S2-Trp53^{tm1Tyj} mice through oral treatment with 7,12-dimethylbenz[a]anthracene (DMBA) (Ku et al., 2007). Colon tumor models were obtained by intraperitoneal injection of Tp53$^{\Delta IEC}$ and Trp53$^{\Delta IEC}$Akt^{E17K} mice using Azoxymethane (AOM) (Schwitalla et al., 2013; Varga et al., 2020) and intrarectal injection of Sprague-Dawley rats using N-methyl-N-nitrosourea (MNU) (Weese et al., 1983).

As exemplified by these models, specific carcinogen-induced models exhibit similarities to human cancers in histomorphology, genetic features, etc. (Schwitalla, et al., 2013). Unlike transplantation models, these models

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demonstrate genetic instability, inducing a higher degree of tumor heterogeneity (Zitvogel, et al., 2016), thereby creating a physiologically more "realistic" TME (Sharma et al., 2023). These models can fully simulate the entire tumor growth and spread process. They are well-suited for drug experiments on LNM, exploring carcinogenicity prophylaxis, and studying the molecular mechanisms involved in various cancers. However, this instability can also lead to uncertainties in tumor characteristics such as epitaxy and latency (Sharma, et al., 2023), and the genetic heterogeneity still falls short of human tumors. Overall, carcinogen-induced tumor models have emerged as a promising category of preclinical models for studying LNM.

2.2 Transplantation tumors

Transplantation models can be categorized into syngeneic-allograft and xenograft mouse models based on the graft and donor relationship. Syngeneic models involve tumor cells or tissue allografts derived from inbred mice (**Fig. 2b**). Owing to the competency of the immune system, syngeneic models play a crucial role in investigating the interaction of the immune system, including the TME, with tumors. Nevertheless, due to the non-human origin of the transplants, tumors obtained from syngeneic models differ from those in humans. They often lack human tumor characteristics, making it challenging to generalize the heterogeneity of human tumors. Consequently, experimental results obtained from these models have a low translation rate. The emphasis here is on xenograft models.

In 1969, Rygaard and Poulsen first showcased the viability of xenograft models by subcutaneously implanting colon cancer tissue from patients into nude mice. They successfully obtained adenocarcinomas resembling those found in the donor patient (Rygaard and Poulsen, 1969). Within the transplantation model, human tumor cell lines or patient samples, presented as cell suspensions or tumor fragments, are transplanted *in situ* or subcutaneously into immunodeficient mice (e.g., nude mice or non-obese diabetic severe combined immunodeficiency mice (NOD-SCID mice)) to induce tumors like those seen in head and neck, breast, colon, and prostate cancers. The transplantation model addresses the shortcomings of carcinogen-induced models, overcoming issues like low tumor aggressiveness, prolonged tumor latency, and limited tumor specificity. With their simplicity of operation and histomorphology resembling that of human patients, they have found extensive application in lymphatic system-related studies. Notably, the spontaneous metastasis model is frequently used in LNM investigations.

Rashidi et al. (2000) developed an orthotopic transplantation model in nude mice to mimic LNM patterns observed in human colon cancer. Specifically, tumor cells in positive lymph nodes draining the liver originated from liver metastases of colon tumors. Kong et al. analyzed patient samples, proposing a hypothesis subsequently validated using the popliteal LNM model. This study confirmed the inhibition of LNM by circNFIB1 through the miR-486-5p/PIK3R1/VEGF-C axis in pancreatic cancer (Kong et al., 2020). Additionally, the fatty acid receptor CD36 amplification, highly correlated with poor prognosis in various human tumors, was investigated using a mouse-based *in situ* model of human oral squamous cell carcinoma. This study demonstrated that metastasis-initiating cells, in close association with dietary lipids, promote the process of tumor invasion, including LNM (Pascual et al., 2017). These studies fully demonstrate the utility and importance of transplantation models in mechanism research, drug discovery and preclinical trials.

Nonetheless, transplantation models have drawbacks, primarily from ethnic differences resulting from the evolutionary gap between mice and humans. These models are exclusively applicable to human tumor cell lines and can adapt to the *in vivo* environment of mice. They do not permit a comprehensive representation of early tumorigenesis and growth. Tumors formed in xenograft models comprise a mosaic of human tumor cells and mouse stromal cells. The species difference somewhat alters or prevents the interaction between the two (Cooper et al., 2003; De Wever and Mareel, 2003; Schmidt-Hansen et al., 2004). Additionally, differences in the immune systems of the experimental animal and humans contribute to this non-negligible effect in any experiments involving humans (Zitvogel, et al., 2016). To prevent rejection, xenograft models are established mainly in immunodeficient mice. This practice may overlook the antitumor or pro-tumor activity of the adaptive immune system (Kersten et al., 2016), thereby impacting the realism of the LNM process in the model.

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Moreover, the model's efficacy testing results often differ substantially from those observed in clinical trials (Gopinathan et al., 2015). To authentically replicate the lymph node metastatic characteristics of human tumors in transplantation models, it is imperative to meticulously select grafts and hosts, consider regional heterogeneity in the sample, and establish standardized engraftment volumes for tumor cells or tissues. Additionally, incorporating adjuvant substances requires standardization (Fu et al., 1991; Fu et al., 1992). Despite these challenges, the transplantation model remains highly favored in preclinical studies due to its economic efficiency and ease of operation.

2.2.1 Cell Line-Derived Xenograft (CDX) Models vs Patient-Derived Xenograft (PDX) Models

Based on the origins of tumor cells, transplantation models are divided into CDX built from *in vitro* tumor cell lines and PDX built from patient-derived tumors obtained from surgery or biopsy of human patients.

With the advantages of lower cost, broader tumor sources, and more accessible construction, there have been many CDX-based LNM research studies. For LNM research, the most outstanding feature of the CDX model is that it can be used to select appropriate cell lines according to the research needs, such as highly invasive lymph node cell lines. However, with a large number of inputs into preclinical experiments, its limitations have been increasingly recognized, including alterations in the original tumor characteristics of cell lines cultured *in vitro* for an extended period, the lack of phenotypic and genetic heterogeneity of human tumors, and the inability to simulate well the TME of human patients. The aggressiveness of CDX in brain tumors has been shown to be dissimilar to that of tumors of clinical patients in preclinical studies (Wang et al., 2009). Although many CDX-based tumor therapies are effective in preclinical studies, most have failed to make it through phase 3 clinical trials.

Since tumors in PDX models originate from patients, they offer a more accurate reflection of tumor heterogeneity, leading to clinical drug results that align more consistently with patient outcomes. Even after successive passages in mice, PDX tumors retain many characteristics of their human donors, encompassing histopathology, clinical tissue markers, chromosomal aberrations, gene expression profiles, aggressiveness, and various other traits (Kersten, et al., 2016). PDX models, particularly patient-derived orthotopic xenograft (PDOX) models, exhibit natural biological behaviors akin to human tumors, including high metastasis rates (Fu, et al., 1992). For instance, in pancreatic cancer, a PDOX model constructed by orthotopically implanting patient samples into nude mice not only maintains the histological integrity of tumors but also faithfully reproduces characteristics similar to those observed in human cancers. Notably, the expression of TAG-72 is well-maintained in metastatic foci, and carcinoembryonic antigen (CEA) shows similar expression, making it an excellent tool for related studies that preserve the natural structure and original antigenic phenotype of human tumors (Fu, et al., 1992).

Beyond directly transplanting patient-derived tumors into immunodeficient mice after treatment, alternative approaches offer intriguing avenues for further investigation. Lin et al., for instance, explored the construction of multiple transplantable tumor cell lines with distinct characteristics, such as clonally related metastatic potential and growth rate, by obtaining multiple biopsies from different foci of the same patient's primary tumor. They then compared the differences in microRNA expression among these cell lines (Lin et al., 2014). This comparative analysis holds promise for extension to the study of LNM.

While the PDX model has more advantages than the CDX model, substantial differences between mouse and human immune systems stemming from evolutionary distance and distinct lymphatic system structures and immune responses in humans result in a notable gap between the PDX model and actual human tumors. Data indicate that bone and brain metastases are more prevalent in human breast cancer, while lymph node and lung metastases predominate in PDXs (Murayama and Gotoh, 2019). Additionally, PDX has operational limitations, including high cost, technical difficulty, and a relatively low modeling success rate. In establishing PDX based on immunodeficient mice like NOG, Post-Transplant Lymphoproliferative Disorder (PTLD) poses a serious obstacle due to the absence of a T-cell immune response in immunodeficient mice. Detection measures, such as checking for lymphocyte marker adulteration, are necessary to exclude lymphocytic tumor modeling during the

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process (Bondarenko et al., 2015). Encouragingly, researchers have found that implanting tumors in nude mice before using the next generation of tumors for transplantation significantly reduces the incidence of PTLD (Choi et al., 2016), with similar effects observed with rituximab (Corso et al., 2018).

Despite its imperfections, the PDX model remains a crucial tool for current therapeutic evaluation and determining personalized medical regimens based on animal experiments. As early as 1992, Fu et al. proposed the construction of public databases from the data of established PDX models, including details of patients' tumors and drug response profiles (Fu, et al., 1992). As a relatively "real" model currently available, PDX will continue to play a significant role in lymphatic system diseases, especially those represented by lymph node metastases.

2.2.2 Orthotopic Transplantation vs. Subcutaneous Transplantation

As the most commonly used model, subcutaneous tumor transplantation models are extensively used in tumor-related preclinical studies, offering advantages such as operational simplicity, reproducibility, and easy tumor monitoring (Sano and Myers, 2009). Typical subcutaneous transplantation sites include the abdomen, inguinal region, and footpads. However, subcutaneous tumors poorly mimic the malignant characteristics of cancers due to their tendency to envelop and because of differences in subcutaneous tissue structure in mice compared to humans (Kubota, 1994). In a study by Sharkey and Fogh, the incidence of metastasis was only 1.3% in subcutaneously transplanted models out of 1,045 nude mice across 11 different tumor lineages (Sharkey and Fogh, 1979). Moreover, subcutaneous transplantation mice models for lung, gastric, and other tumors differ significantly from human patients in characteristics such as chemotherapeutic drug sensitivity, leading to challenges in anti-tumor drug development (Kubota, 1994; Suggitt and Bibby, 2005).

In contrast, orthotopic models can more closely mimic human cancers. Orthotopic transplantation involves delivering cancer cells or tissue to the site of tumor origin through surgery or injection to restore the tumor's original environment. Researchers have established whole tissue orthotopic transplantation models for various cancers, including head and neck, lung, gastric, liver, colon, pancreatic, breast, prostate, and bladder cancers. Orthotopic models share a similar microenvironment with the human body (Killion et al., 1998), exhibiting a higher tumorigenicity rate, a shorter latency period, resistance to envelope formation, and the ability to simulate the entire metastatic process (Kubota, 1994), including local vascular and lymphatic invasion, blood vessel and lymphatic flow, extravasation into relevant metastatic organs, and growth seeding metastasis at the metastatic site. They demonstrate a high degree of metastatic ability (Kerbel, 2003), and the metastatic behavior of transplanted tumors closely resembles that of tumors in clinical patients (Furukawa et al., 1993), offering a better simulation of advanced localized or metastatic disease as well as therapy response compared to subcutaneous models (Sano and Myers, 2009).

The main limitation of orthotopic models is the technical difficulty of model establishment. The morbidity or mortality rate of animals in the process of model establishment is high. Moreover, some deep tumors, such as cervical cancer and ovarian cancer, are challenging to access orthotopically, increasing the difficulty of constructing orthotopic models. Second, tumors inside the body are difficult to monitor continuously (Sano and Myers, 2009; Spenle, et al., 2021). Techniques such as small-animal magnetic resonance imaging, positron emission tomography, reporter genes with specific fluorescent properties, the luciferase gene, and other techniques have greatly aided monitoring of *in situ* tumor models (Sano and Myers, 2009).

2.3 Genetically Engineered Mouse Models (GEMMs)

Cancer development is associated with dysregulation of the relationship between proto-oncogenes and oncogenes. As mice have a genome similar to that of humans and which is less complicated to edit (Chinwalla et al., 2002), they are a prime target for gene editing (**Fig. 2c**). Presently, GEMMs are used in investigating LNM in lung, breast, colon, pancreatic, and prostate cancers, among others (Frese and Tuveson, 2007), recognizing various modeling genes, including KRAS mutations in pancreatic cancer (Almoguera et al., 1988), cyclin-dependent kinase inhibitor 2A (CDKN2A) inactivation, cellular tumor antigen 53 (TP53) mutation

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(Hruban et al., 2001), and driver genes B-type Raf kinase (Braf), NRAS, phosphatase and tensin homolog (Pten), and premelanosome protein (Pmel) in melanoma (Pérez-Guijarro et al., 2017). Additionally, human epidermal growth factor receptor-2 (Her2), polyoma middle T antigen (PyMT), wingless and integration site growth factor 1 (Wnt1), KRAS in breast cancer (Schlange et al., 2007; Anastas and Moon, 2013), and KrasG12D, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), human papillomavirus oncogenes E6 and E7 in head and neck cancer have been identified (Tasoulas et al., 2023). GEMMs have addressed the deficiency in tumor characterization resulting from immune deficiency in xenograft models and the lack of patient population heterogeneity in CDX models (Heyer et al., 2010), more realistically simulating the tumor microenvironment in the human body.

Integrating GEMM-based metastasis analysis data with human cancer molecular data across species plays a crucial role in understanding metastatic progression mechanisms and identifying therapeutic targets. For instance, Aytes et al. (2018) discovered that in NPK mice (Nkx3.1CreERT2; Ptenflox/flox; KrasLSL-G12D/+), unsupervised principal component analysis revealed highly similar expression profiles of post-metastatic primary tumor cells, all significantly differing from the expression profiles of hepatic, lung, and lymph node metastases. Dankort et al. (2009) proposed that the expression of BRafV600E, combined with Pten tumor suppressor gene silencing, can generate primary cutaneous melanoma characterized by 100% epitope, displaying high aggressiveness and metastasis to draining lymph nodes.

In conventional GEMMs, the genome is modified *in vivo* in experimental animals (or tissue-specifically in transgenic models driven by tissue-specific promoters). This leads to the simultaneous development of cancer associated with the altered genes at multiple sites in the animal. Therefore, compared to carcinogen-induced or spontaneous models, GEMMs exhibit a diversity of translational events and a lower burden of passenger mutations. However, the immune system may be overwhelmed (Ciampricotti et al., 2012), limiting their ability to model sporadic tumors effectively.

Ongoing research is focused on developing GEMMs that overcome these deficiencies. The earliest attempt was to generate a mouse colorectal cancer model by CreloxP-mediated inactivation of APC somatic cells, in which colorectal tumors have a short latency and closely resemble adenomas in patients with familial adenomatous polyposis coli (Shibata et al., 1997). KPC mice (LSL-KrasG12D; LSL-Trp53R172H; Pdx1-cre), one of the successful models of spontaneous tumors, exhibit genetic features, histological features, and features of advanced disease based on spontaneous metastatic tumors similar to those of human patients, which include hemorrhagic ascites as well as metastases to the lungs, liver, peritoneum, and lymph nodes (Hingorani et al., 2005). In the case of pancreatic cancer research, chemoprevention, early intervention, later intervention, and factors that influence response to therapy can be used throughout the study (Gopinathan, et al., 2015). Additionally, the rapid advancement of non-germline genetically engineered mouse models (nGEMMs), encompassing chimeric models, mouse in mouse models, classic xenograft models, and human in mouse models, has freed GEMMs from the constraints of excessive genetic alterations in traditional GEMMs (Heyer, et al., 2010).

Currently, the main limitations of GEMMs in lymphatic disease research are the high costs associated with their construction and validation and the necessity to develop models based on known cancer-related genes. Nonetheless, leveraging the swift progress in genetic engineering, the next generation of GEMMs for lymphatic system-related diseases holds the potential for further discoveries.

2.4 Humanized mouse models

Humanized models, based on severely immunodeficient mice, facilitate the implantation and functioning of the human immune system, significantly enhancing the exploration of the human immune system and its diseases. Constructing humanized mice involves transplanting human hematopoietic cells into immunodeficient mice, ideally, those in which the endogenous hematopoietic and immune systems have been spatially and functionally completely replaced by human grafts (**Fig. 2d**). This necessitates specific immunodeficiency in the recipient strain, providing the human graft with the appropriate space and environment for growth and ensuring

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harmonization with the recipient to achieve symbiosis (Theocharides et al., 2016).

The most prevalent humanized model systems include the Hu-PBL-SCID model (SCID mice injected intravenously or intraperitoneally with mature peripheral blood mononuclear cells and two types of hematopoietic stem cells), the Hu-SRC-SCID model (injection of human SCID regenerating cells or granulocyte colony-stimulating factor-mobilized peripheral blood cells), and BLT (bone marrow, liver, thymus) models (Eswaraka and Giddabasappa, 2017).

Despite generating a partial human immune response, these humanized mouse models still fail to replicate the tumor response observed in humans. Limitations of humanized models include restricted immune cell development, the presence of only MHC molecules (no HLA molecules), limited adaptive immune responses, and poor lymph node development (Cao et al., 1995; Chuprin et al., 2023). The development of lymph node progenitors is compromised due to the interleukin-2 (IL-2) receptor γ-chain locus in immunodeficient mice, hypoplasia of lymph nodes and lymphoid tissues, and poor lymph node architecture (Shultz et al., 2007; Shultz et al., 2012; Rongvaux et al., 2013a; Bryce et al., 2016): Peripheral lymph nodes other than mesenteric lymph nodes are small and structurally underdeveloped (Vuyyuru et al., 2011). In the Hu-PBL-SCID and Hu-SRC-SCID models, human T cells can express only murine H-2d Ags, preventing the recognition of Ag in an HLA-restricted manner. Consequently, these models fail to generate a human cytotoxic T lymphocyte (CTL) response and exhibit limited peripheral lymph node development. Although the BLT model shows improved peripheral lymph node development, the lymph nodes still lack generative centers, exhibit no Ig class switching, and have poorly developed lymphoid tissue structures in the liver, spleen, and thymus (Shultz, et al., 2007; Shultz, et al., 2012; Lang et al., 2013; Rongvaux et al., 2013b; Bryce, et al., 2016; Eswaraka and Giddabasappa, 2017). These deficiencies significantly restrict the application of humanized models in studying LNM and the related lymphatic system.

Researchers have proposed solutions to these challenges. Takahashi et al. (2018) introduced a NOG-pRORγt-γc model that exhibited significantly enhanced lymph node development. This model uses the endogenous promoter of RORγt, facilitating the expression of the γc gene across various lymphoid tissue inducers, resulting in an improved lymph node architecture. Other models, such as humanized Balb/c Rag2-/IL2rg-/-SirpaNOD TSLP (BRGST) mice, have demonstrated enhancements in lymph node and thymus structures, leading to improved adaptive immunity (Li et al., 2018). Additionally, administering human IL-7 to immunodeficient mice during lymph node development in neonatal individuals, along with supplying human lymphotoxin-β receptor at a crucial stage in lymph node maturation, has shown improvements (Chappaz and Finke, 2010) (Rennert et al., 1998). Furthermore, the NSG-HLA-A2/HHD model addresses the limitations observed in the Hu-PBL-SCID and Hu-SRC-SCID models, which struggle to generate a CTL response (Shultz, et al., 2012). To summarize, enhancing the human immune cell profile, increasing the proportion of human immune responses, and addressing developmental challenges in immune organs, particularly lymph nodes, constitute the central challenges in advancing humanized models.

Note that while lymphocyte translocation to HIV-infected regions (Lang, et al., 2013), sites of inflammation in arthritis (Murooka et al., 2012), and mucosal tissues (Sun et al., 2007; Denton et al., 2012) have been observed in these models, further investigation is needed to determine if their migration within lymphoid tissues and systemic transport align with human patterns.

A comparative analysis of the four models is shown in **Table 2**.

Table 2 Strategies to mouse models for LNM

Category	Description	Merits	Obstacles	Reference
Carcino-gen-induced Models	Induction of tumors with pathogenesis, genetic features, histo-logical features, and immunohistological features similar to those of human tumors	Similarity to hu-man cancers in terms of histo-morphology, ge-netic features, etc; A higher degree of genetic instability	Although immunodeficient mice can establish LNM models faster, immunodeficiency tends to inter-fere with studies of the immune response to tumors; the same carcinogen induction process in immunodeficient mice tends to	(S. Schwitalla et al., 2013; Sharma et al., 2023; Tormo et al., 2006; Zitvogel et al., 2016)

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		and tumor heterogeneity; Physiologically more "realistic" TME; Suitability for studying LNM, exploring carcinogenicity prophylaxis, as well as studying the molecular mechanisms involved in various cancers	result in faster tumor establishment and growth compared to immunocompetent mice. Uncertainties in tumor characteristics; Disparities in genetic heterogeneity from human tumors	
Transplantation Models	Transplantation of human tumor cell lines or human patient samples in the form of cell suspensions or tumor fragments in situ or subcutaneously into immunodeficient mice (nude mice, NOD SCID mice, etc.)	High tumor aggressiveness; Short tumor latency; High tumor specificity; Wide application in lymphatic system-related studies due to simple operation and histomorphology similar to that of human patients	Limited appliance to human tumor cell lines that can adapt to the in vivo environment of mice; Incapacity to allow a complete representation of early tumorigenesis and growth; Evolutionary distance between mice and humans; To prevent rejection, xenograft models are primarily established in immunodeficient mice while neglecting the antitumor or pro-tumor activity of the adaptive immune system resulting from immunodeficiency; Differences in efficacy testing; Non-standardized graft and host selection	(Kersten et al., 2017; Murayama & Gotoh, 2019; Overgaard et al., 2018; Zitvogel et al., 2016)
GEMMs	Transgenic expression of oncogenes, recombinant inactivation of tumor suppressor genes, and alteration of other immune-related genes in experimental animals	Excellent representation of altered tumor properties and genetic characterization; Realistic reproduction of patient population heterogeneity, natural tumor processes; High lymphatic invasive potential for metastasis	High costs for construction and validation; Reliance based on established cancer-associated genes; Failure to stimulate solitary tumor resulted from systematic cell lineage involvement Gene editing at key loci may lead to the immune system being overwhelmed.	(Aytes et al., 2018; Ciampricotti et al., 2012; Dankort et al., 2009; J. Heyer et al., 2010; Zitvogel et al., 2016)
Humanized Models	Transplantation of human hematopoietic cells into immunodeficient mice	Partial spatial and functional replacement of the endogenous hematopoietic and immune systems of mice with human-derived grafts	The model is based on immunodeficiency; however, immunodeficiency affects the immune system's response to tumors and influences the structure and function of lymph nodes, which limits its use in LNM research; Restriction of immune cell development; Lack of HLA molecule; Limited adaptive immune response; Impaired lymph nodes primordia; Poor development of lymph nodes and lymphoid tissue; Poor lymph node structure	(Bryce et al., 2016; Chuprin et al., 2023; Theocharides et al., 2016; Vuyyuru et al., 2011; Zitvogel et al., 2016)

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3 Other animal models

The hamster model, akin to the mouse model but with the advantages of small size, ease of operation, and lower cost, features the distinctive cheek pouch. Numerous tumor models in hamsters exhibit similarities to humans. Syrian golden hamsters with induced tongue cancer, for instance, display histological features akin to human tongue cancer. The process of LNM in establishing metastatic foci closely mirrors human tumor development. Ohtake et al. (1993) screened DMBA-induced squamous cell carcinoma of the tongue *in vivo*, transplanted positive lymph nodes from donor hamsters into the cheek pouch of recipient hamsters after treatment, and succeeded in constructing a reproducible and transgenerational O-l N tumor model with a LNM rate of 100%. The metastatic process was very similar to that of human tumors, which overcame the limitations of the well-differentiated, less invasive DMBA-induced model. This provides modeling ideas for a more "realistic" LNM in a hamster model. Despite its many applications, the hamster model has limitations. Muhanna et al. (2015) found that hamsters with multiple tumors in the buccal pouch experienced a high survival burden.

Rabbit models have been extensively used in lymph node-related studies due to the unique presence of buccal (facial), submental, preauricular, retroauricular, pretracheal, paratracheal, posttracheal, intercostal, and gastric cardiac lymph nodes, which are absent from mice and rats (Oshiro, 2014), as well as lymphatic drainage routes closely resembling those in humans (Hayakawa et al., 1986). The commonly used VX2 rabbit model maintains immune competence and its advantages include ease of tumor maintenance and metastatic patterns akin to human tumors (Coman et al., 1949). For instance, Wulong et al. (2008) established the RSCC-1 cell line and a corresponding rabbit model resembling human tongue cancer in metastatic ability and lymph node behavior. A metastatic breast cancer model injected into the rabbit mammary gland exhibited a 59% metastatic rate and behavior similar to that of human tumors (Junping et al., 2012). Despite its versatility, the rabbit model has limitations, including differences in pathophysiology between the VX2 cell line and human tumors and challenges related to the active feeding and long digestion times of rabbits, which can interfere with imaging-related studies (Lee et al., 2012).

In lymph-related research studies, felines stand out due to their unique advantage over standard animal models. Unlike rodents and other mammals, where lymphatic drainage converges in a single large lymph node, cats closely resemble humans in having multiple lymph nodes in the neck and axilla (Califano, 1970). With a 90% genetic match between humans and cats (Pontius et al., 2007), essential tumor genes and molecules such as TP53, EGFR, PTHrP, and NFκB align between the two species (Supsavhad et al., 2016). In gastrointestinal tumors, breast cancer, and HNSCC, felines offer a valuable opportunity to explore similarities in both clinical presentations and biological behaviors, including metastatic rates, heterogeneity factors, tumor receptor positivity, and post-treatment spontaneous relapses (Weijer et al., 1972; Queiroga et al., 2011; Savan et al., 2022).

Canines have significant anatomical, physiological, and genetic similarities to humans, and serve as valuable models for defining the safety of novel oncology drugs in phase I clinical studies (Khanna and Hunter, 2005). Canines can spontaneously develop various cancers, with some tumors having the ability to metastasize to lymph nodes, bone, and lungs. The prognostic impact of LNM in mammary tumors in canines mirrors that of humans (Queiroga, et al., 2011), including characteristics of tumor infiltrating lymphocytes (TIL), the CD4+/CD8+ T-cell ratio, and the relationship between the number of Treg cells and poor prognosis (Estrela-Lima et al., 2010).

While being ideal models for various human diseases and invaluable for drug research, non-human primates face ethical and cost challenges, limiting their application. They are used mainly for preclinical drug experiments at present. Ostrow et al. have reported a case of papillomavirus isolation from metastatic lymph nodes in rhesus monkeys suffering from primary squamous carcinoma of the penis (Ostrow et al., 1990). Zhong et al. (2021) successfully constructed primary and metastatic tumors by CRISPR/Cas9 in adult crab-eating monkeys. The model had high efficiency of optimal mutation of Pten and p53, and the incidence rate of primary and extensive metastatic hepatocellular carcinomas in lymph nodes, lungs, and spleens was up to 87.5%.

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Similar techniques could enable the construction of other human models, providing a precious tool for tumor research (Zhong et al., 2021).

These animal models contribute significantly to understanding the pathogenesis, molecular pathways, and therapeutic modalities of LNM. Higher-level animal models are vital in translating mouse-based research into human clinical outcomes. When selecting an appropriate model, it is crucial to consider the advantages and disadvantages by comparing their characteristics with various human tumors and analyzing their indications (**Fig. 3**).

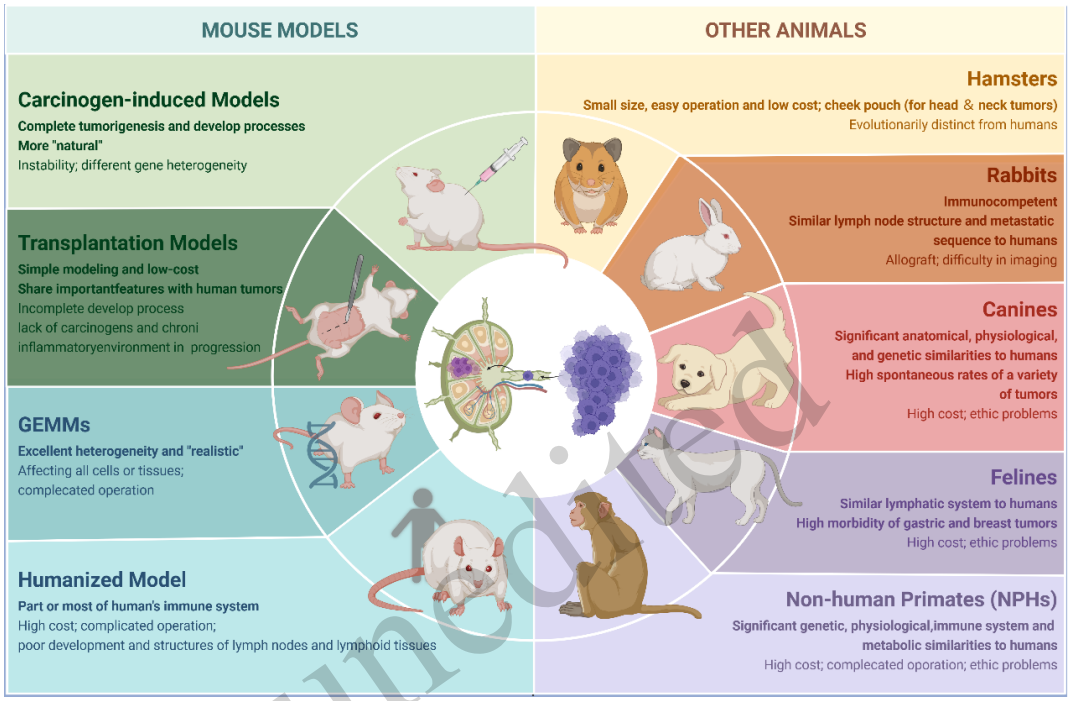

Fig. 3. A summary of the advantages and disadvantages of different models. It is crucial to compare the characteristics of mouse and other animal models with those of various human tumors and analyze their indications to consider their advantages and disadvantages. Created with BioRender.com.

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4 Preclinical trials

In human patients, the process of tumor LNM is intricate and continuous. Conducting studies on human-based LNM poses considerable challenges, due mainly to ethical constraints. Consequently, preclinical studies using animal models become a bridge from basic research to clinical translation. Nevertheless, the gap between preclinical models and humans cannot be ignored. Based on the massive gap between rodents and humans, many scholars have pointed out that more attention should be paid to the complexity of human diseases (Seok et al., 2013) rather than relying on mouse models to explore human diseases.

Most experimental animal species, including lower mammals, have significantly fewer lymphatic trunks and nodes than humans (Sovak, 1984). A substantial evolutionary gap exists between mice and humans, resulting in the absence of specific lymphoid centers, such as parotid, retropharyngeal, superficial cervical, ventral thoracic, dorsal thoracic, or superficial inguinal lymphocenters. Unlike humans, mice and rats lack various lymph nodes, including buccal, submental, preauricular, retroauricular, pretracheal, paratracheal, posttracheal, intercostal, and gastric cardiac lymph nodes. Furthermore, specific strains of mice exhibit deficiencies in certain lymph nodes, such as bronchial and gastric lymph nodes in DD-strain mice. The cranial ventral mediastinal lymph nodes of rats differ significantly from those of humans.

Additionally, in rats and mice it is difficult to establish a robust superficial and deep lymphatic drainage system in the upper branches, and the murine thoracic duct corresponds to only one of the human ductal subtypes, namely type VI (Oshiro, 2014). Moreover, the murine immune response differs from that of humans in several respects, including the balance of leukocyte subpopulations, defensins, Toll receptors, inducible NO synthase, the NK inhibitory receptor families Ly49 and KIR, FcR, Ig subpopulations, components of the B-cell (BLNK, Btk, 5) and T-cell (ZAP70 and cochaperone) signaling pathways, Thy-1, T-cells, cytokines and cytokine receptors, T-cells, and cytokines, and T-cells, and cytokine receptors, Th1/Th2 differentiation, co-stimulatory molecule expression, and function, Ag presentation function in endothelial cells, and chemokine and chemokine receptor expression (Mestas and Hughes, 2004). Rabbits, felines, and canines exhibit closer structural resemblances to humans than rats, yet notable disparities persist. These variations are likely to impact functions within the lymphatic system, particularly lymphatic drainage, thereby influencing the reliability of relevant preclinical experiments. Notably, the creation of transplantation and humanization models relies on immunodeficient mice. Even in immunologically robust mice, maintaining a specific pathogen-free (SPF) environment results in limited microbial exposure, and their growth conditions deviate significantly from conditions representative of human environmental exposures. This divergence further amplifies the contrast between their immune responses and those of humans (Tao and Reese, 2017). The immunological distinctions underscore the challenges that numerous animal-based preclinical trials face in translating successfully into clinically viable human treatments (**Fig. 4**). Consequently, preclinical experiments struggle to predict the efficacy and toxicity of oncology therapies accurately. The enactment of the FDA Modernization Act 2.0 in 2022 signifies the end of an era wherein experimental drugs had to undergo animal testing before clinical trials (Wadman, 2023).

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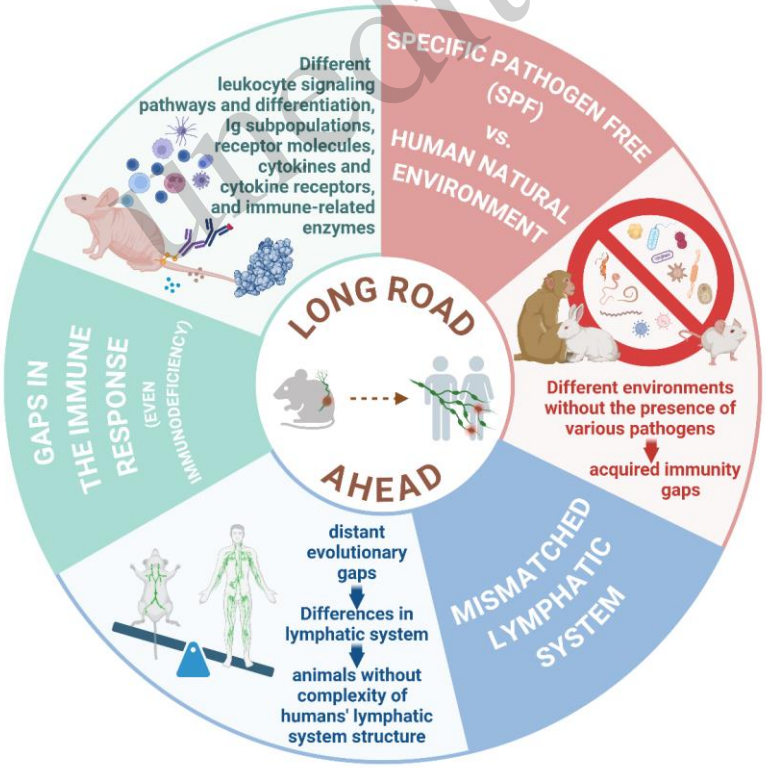

Fig. 4 The gap between mouse models and the human body. There is still a significant gap between mouse models and the natural environment of the human body, manifested in differences in the lymphatic system, immune response, and the impact of the animal feeding environment. These differences will be important factors affecting preclinical research. Created with BioRender.com.

5 Prospects

5.1 Effect of Age on Animal Models

In preclinical experiments, subjects often comprise healthy and youthful experimental animals. However, human patients undergo diverse environmental exposures, and non-genetic factors like age, body weight, nutritional status, health, and hygiene vary, leading to distinct metabolic and immune profiles. These variations consequently influence the outcomes of experiments and therapies associated with lymphatic system diseases. (**Fig. 5**). Bouchlaka et al. (2013) observed that immunotherapy-associated toxicity was minimal in mice under 6 months old but significantly increased in mice aged 9 months, indicating the need to establish preclinical models that can replicate human age groups when conducting studies on LNM and other lymphatic system-related diseases. Conversely, performing a side-by-side comparison of model responses at different ages offers novel insights into exploring the effects of age. Moreover, integrating the various influences mentioned above into the future development of more "realistic" modeling holds promise for improving the authenticity of preclinical models.

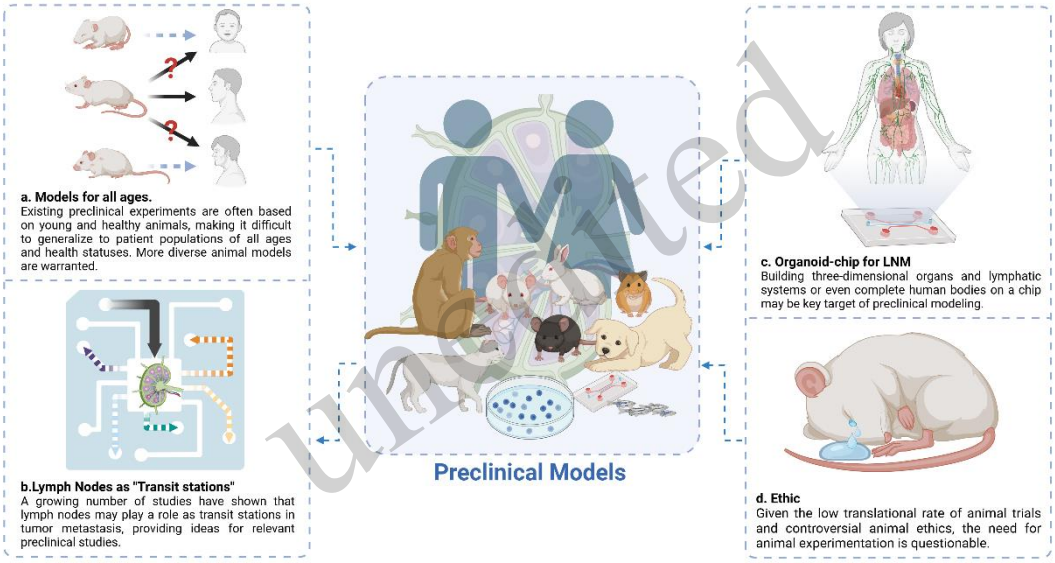

Fig. 5 Preclinical research still has a long road ahead. The differences between animal models and human tissues are still significant. Further exploration and development of suitable animal models are needed to assist in developing preclinical research. Created with BioRender.com.

5.2 Lymph Nodes as "Transit Stations"

LNM is not a single event of tumor dissemination but involves a complex regulatory mechanism of anti-tumor immunosuppression and dysfunction. Studies have shown that LNM can resist T cell-mediated cytotoxicity and induce the production of antigen-specific Tregs cells, thereby generating tumor-specific immune tolerance, which in turn promotes both the expansion of LN metastatic foci and distal colonization of the primary tumor (Baek, 2022; Reticker-Flynn et al., 2022; Ventre et al., 2022). This effect is manifested in the dysregulation of the body's resistance to tumors and impaired response to immunotherapy (Rahim et al., 2023). Therefore, any study of LNM must consider the profound impact of impaired immune function on the outcome, which requires that preclinical models mimic the disruption of systemic immunity by LNM to a greater extent. Comparative analysis of gene expression, biomolecular activity, histology, and immune response between "daughter cell lines" derived from *in vivo* selection of primary tumors and primary tumor cell lines could help to elucidate the mechanisms of LNM and identify potential therapeutic targets (**Fig. 5**).

5.3 Tertiary Lymphoid Structures

Tertiary lymphoid structures (TLSs) formed due to chronic inflammation, including tumors, consist of ectopic aggregates of immune cells gathered within or around the diseased area, similar in structure to secondary lymphoid organs. As with other levels of lymphoid organs, TLSs are regulated by the microenvironment (Jones et al., 2016; Kim et al., 2019). TLSs localized to tumors in certain human tumors are associated with better survival, and thus, research on the mechanism of formation and induction of TLSs has become a hot topic. Studies based on preclinical animal models have identified that the formation of TLSs involves the same signaling pathways as those involved in secondary lymphoid organ development by a variety of lymphotoxins. In addition, overexpression of chemokines important for recruiting immune cells is similarly associated with TLS formation in transgenic mouse models (Zhu et al., 2017). These studies provide a theoretical basis for artificial TLSs.

(Kobayashi and Watanabe, 2016) proposed that the use of a collagen sponge wrapped with slow-releasing Medgel beads loaded with lymphoid organogenesis-stimulating factors after implantation into preimmunized BALB/c mice successfully induced TLSs with native ectopic lymphoid tissue properties and their excision and reimplantation into immune-deficient mice increased in the production of antigen specific high-affinity antibody-forming cells. A macroporous poly lactic-co-glycolic acid matrix functionalized with granulocyte-macrophage colony-stimulating factor (Ali et al., 2009) and *in situ* crosslinkable hydrogels carrying chemokines and microparticle vaccines (Singh et al., 2011) exhibited similar tumor immune-enhancing effects.

Impediments to clinical application of artificial TLSs need to be addressed, including their biocompatibility, which mimics the dynamic processes of material exchange with cells and biomolecules *in vivo*. Advances in research on novel materials, large-scale production techniques, signaling pathways and cytokines associated with TLSs, and improved clinical translation of results from existing preclinical trials will provide therapeutic options for immunosuppressed tumor patients.

5.4 Closer to The Human Environment

The construction of preclinical models continually integrates new research findings to enhance model development, striving for more "realistic" simulations of the human environment. With advantages like low maintenance cost, high reproductive capacity, rapid modeling, and short experimental cycles, zebrafish are potent screening platforms for vasculature system-related drugs, including those affecting the lymphatic system (Okuda et al., 2016). Zaheer et al. used zebrafish patient-derived xenograft models, revealing 2.1 times more metastatic cells from lymph node-positive patients than from lymph node-negative patients, demonstrating the predictive potential of zebrafish models for LNM (Ali et al., 2022). However, the absence of lymph nodes and lymphatic valves in zebrafish limits their applicability in LNM studies (Hewson, 1769).

Cell lines are pivotal in current preclinical model construction, including GEMMs and humanized models. However, a comprehensive understanding of their origin and long-term *in vitro* passaging effects on survival mechanisms, metastatic capacity, and sensitivity to hormones or drugs is essential. Establishing open web platforms to collate information and encourage continuous discoveries about existing cell lines could enhance this understanding. In the prevailing SPF husbandry environment, neglecting environmental effects on the immune system of experimental animals hinders their mimicry of human environmental exposures. Combining specific genotypes with housing conditions may aid in constructing animal models that are more compatible with human phenotypes. For GEMMs, accelerating the two-way translation between preclinical model-based oncogene exploration and applying research results to constructing new GEMMs is imperative. With the rapid development of gene editing technology, personalized CRISPR-based editing holds promise for GEMMs that align more closely with human tumor specificity. Despite being less commonly used due to cost and ethical factors, non-human primates like rhesus monkeys and crab-eating monkeys could be promising candidates for LNM research owing to their physiological structures and immune systems being closer to those of humans than those of other animal models.

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5.5 Organoids and organoid-chips

Combining organoids and organoid-chip microenvironments that faithfully replicate tumor characteristics is emerging as an optimal alternative to *in vivo* models (Hofer and Lutolf, 2021), addressing the challenges posed by cancer heterogeneity in treatment (**Fig. 5**). Currently, organoid and organoid-chip preclinical models derived from head and neck tumors, colorectal cancer, breast cancer, and other tumors are readily accessible (Jeon et al., 2015; Al-Samadi et al., 2019; Zhu et al., 2023). Organoids are 3D multicellular tissue constructs originating from human pluripotent or adult stem cells. One of the significant features of organoids, compared to traditional cell culture and animal models, is that they allow preclinical simulation of the human body at the subtle *in vivo* environmental level (e.g., cytokines, extracellular matrix, etc.) (Kim et al., 2021), thus overcoming the heterogeneity between animal models and the human body. However, the results may also be unstable due to the heterogeneity of organoids derived from different patients.

Recent advances in organoid-on-a-chip technology have suggested various ways to address this issue using micro-engineered culture devices. Technologies such as artificial intelligence, multiple biosensors, multi-omics analysis, and real-time imaging monitoring can also be integrated into organoid chip systems, allowing for an integrated approach to improve real-time detection and analysis of biological signals (Wang et al., 2024). Combining technologies that detect cell migration, such as Transwell, with organoid-chips can help assess tumor metastasis. For example, Chen et al. (2024) developed a Transwell-integrated organoid-chip platform for accurate assessment of tumor metastasis, showing good potential for its use in LNM research. However, technical constraints, high cost, and limitations in chip raw materials persist as substantial obstacles to the advancement of this approach. Creating standardized assessment systems in specific disease states also remains an issue (Wang, et al., 2024). Enhancing the "realism" of organoid-chips to mirror the actual TME and human body structure closely is a crucial focus for further development.

5.6 Artificial intelligence in preclinical research

Unique algorithms, such as those from deep learning and artificial intelligence (AI) show excellent processing and mining capabilities for large datasets (Perez-Lopez et al., 2024). Based on this characteristic, AI is particularly suitable for assisting monitoring and identification in preclinical studies. The identification of LNM by AI has been extensively studied and is advancing from the preclinical to the clinical research stage (Elemento et al., 2021). Studies have shown that the assistance of AI can enhance the sensitivity of imaging and pathology in interpreting LNM and significantly reduce the risk of under-detection (Van Dooijeweert et al., 2024). In laboratory settings, it has shown sufficient accuracy to enhance the efficiency of preclinical studies substantially. Another area of interest for AI is the detection of specific key mutations directly from histopathology images, especially those that serve as biomarkers of response to targeted therapy (e.g., EGFR) (Bhinder et al., 2021). This would significantly reduce the cost and redundancy of preclinical studies. However, AI models are built on large datasets, so adequately capturing data from the entire population is critical to developing robust AI models. This creates the potential risk of significant data bias in its application (Bhinder, et al., 2021). Also, the regulation of AI is still unclear, and the ethical and legal issues arising from incorrect AI judgement deserve attention.

5.7 Ethics

With the increasing use of animal models in preclinical experiments, there is a growing discourse on the limitations and indispensability of animal studies (**Fig. 5**).

On the one hand, notwithstanding the rigorous ethical scrutiny and efforts to minimize animal suffering, most animal experiments inadvertently inflict disease and distress upon experimental animals, which should be avoided. According to the National Institutes of Health (NIH) Guidelines for Endpoints in Animal Study Proposals, animals are mandated to be euthanized upon presenting specific indicators. In tumor experiments, stringent criteria dictate that the tumor burden must not surpass 10%, and tumors must not exceed 20 mm in length, width, and height in mice and 40 mm in rats. Additional markers for endpoints include rapid or

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progressive weight loss, inability to eat and drink normally, and abnormal body temperature. An intriguing observation by Sano and Myers (2009) revealed that a diminished burden of primary tumors in mice, achieved through partial resection, could forestall death or extend the time to reach endpoints, thereby providing an extended window for metastasis to develop.

On the other hand, while animal models serve as a convenient means for humans to explore physiological responses and microenvironments in intact living organisms, the substantial physiological and immunological differences resulting from the evolutionary gap between animals and humans undermine the credibility of extrapolating behavior observed in animal models to humans. Animal models fall short of fully mimicking human clinical diseases. In 2023, the US Congress passed the FDA Modernization Act 2.0 (Wadman, 2023), signaling the cessation of animal testing, stating that animal testing is no longer imperative for preclinical testing when suitable alternatives are available. From this perspective, channeling more efforts into exploring "realistic" solutions such as organoid microarrays, computer modeling, and other emerging translational approaches may pave the way for LNM and related research.

6 Conclusions

Preclinical models, the bedrock of preclinical experiments, are pivotal in advancing basic human research and therapeutic development for lymphatic system diseases, particularly those involving LNM. These models contribute significantly to a profound understanding of tumors and form the cornerstone for translating fundamental theories into clinical advances (**Fig. 4**). Commonly used preclinical models for LNM encompass isolated models, represented by cell lines, and mouse-based *in vivo* models. Additionally, various animals, including rabbits, cats, dogs, and monkeys, offer platforms for LNM research that closely align with the human body's structure and physiological functions.

Despite the advantages of these animal-based preclinical models, the low translation rate from research results to clinical applications underscores their limitations. There is room for enhancing current animal models, emphasizing the pressing need of the medical community for more precise and accurate *in vitro* models, notably represented by organoid microarrays.

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Data availability statement

Not applicable.

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Author contributions

W-LY and L-ZZ established the review idea and the article structure. All authors reviewed published articles and collaborated with the first and revised draft. C-ZM, Z-NN, C-LM and XY, collected the references. W-GR realized the illustration. LB, CB and B-LL contributed to the general improvement of the writing and critical review of the figures. All authors have seen and approved the final version of the manuscript being submitted.

Compliance with ethics guidelines

All authors declare that they have no conflict of interest.

Not applicable.

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