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Single-cell omics in tumor lymph node metastasis: mechanisms and therapeutic implications

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Abstract

Lymph node metastasis (LNM) represents a distinctive stage in cancer progression, exerting a profound impact on patient prognosis. Accumulating studies have unveiled that tumor-draining lymph nodes (TDLNs) play a pivotal role in immunotherapy, functioning as an armory for anti-tumor immunity. Concurrently, LNM itself accelerates systemic metastatic progression, exacerbates systemic immunosuppression, and in turn fuels tumor progression. Recent advancements in single-cell sequencing technology have furnished insights into the mechanisms of LNM at single-cell resolution, unraveling the process of LNM in tumor cells and accompanying microenvironmental alterations from the perspectives of cellular heterogeneity, intercellular communication, and cellular evolutionary trajectories. Furthermore, single-cell sequencing studies have underscored the critical role of TDLNs in immunotherapy and their corresponding adaptive modifications. This article reviews the LNM microenvironment and immunosuppressive mechanisms revealed by single-cell sequencing technology, summarizes the novel targets for LNM and the current status of LNM-related immunotherapy research, and outlines the current challenges and future development directions from both technological and research content perspectives.

Keywords Single-cell sequencing, Lymph node metastasis, Tumor-draining lymph node, Immunotherapy, Microenvironment

Introduction

Lymph node metastasis (LNM) occupies a pivotal position in tumor progression and stands as a critical determinant of patient prognosis. Once LNM occurs, patient outcomes are significantly compromised. For oral cancer, the 5-year survival rate of patients is approximately 50%, but it drops to 30% when LNM is present [1–3]. Meanwhile, LNM induces immune tolerance in patients, impairs the efficacy of conventional immunotherapy, and may accelerate systemic metastatic progression [4, 5].

In current mechanistic studies of LNM, the "PUMP+" principle proposed by Bu et al. encompasses the entire process from pre-metastatic niche formation, lymph node (LN) colonization, to immune tolerance and distant metastasis [6], providing a fundamental framework and

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profound insights for subsequent LNM research. However, due to technical limitations and the difficulty in recapitulating the pre-metastatic microenvironment, the specific cell subsets involved and their underlying mechanisms remain elusive.

The rise of single-cell sequencing technology has offered a revolutionary tool to address this dilemma. By dissecting the expression profiles of individual cells, this technology enables the identification of heterogeneous cell subsets that are inaccessible via traditional methods. This technical breakthrough not only allows precise tracking of tumor cell migration trajectories but also facilitates the deciphering of dynamic crosstalk within the tumor-immune microenvironment [7, 8]. Compared with traditional bulk sequencing, single-cell technology exhibits multiple advantages in LNM research. Firstly, it can detect trace tumor cells, enabling accurate identification of occult lesions such as micrometastases. Secondly, single-cell T-cell receptor (TCR) sequencing can reveal the clonal expansion patterns and exhaustion trajectories of tumor-infiltrating T cells, providing direct evidence for the design of immunotherapeutic regimens. Finally, combined with pseudotime analysis and the construction of intercellular communication networks, it can dynamically simulate the molecular pathways of tumor cell migration from primary lesions to LNs. These breakthroughs are reshaping our understanding of LNM, transforming it from a static anatomical event to a dynamic process involving multi-dimensional interactions between tumor cells and microenvironmental cells via chemokines, adhesion molecules, and other mediators [9, 10].

In this review, we summarize the occurrence patterns of LNM, the single-cell-level microenvironments of primary tumors with LNM, tumor-draining lymph nodes (TDLNs), and metastatic lymph nodes (MLNs), immunosuppressive mechanisms and preclinical therapeutic targets, and the impact of immunotherapy on the LNM microenvironment at single-cell resolution. Finally, we highlight the current challenges and prospects of single-cell sequencing in LNM research, aiming to promote its translation from basic research to clinical applications in LNM management.

Overview of single-cell sequencing technology in LNM research

Traditional bulk analysis methods, while advancing biomedical research to some extent, average signals across large cell populations, masking intercellular heterogeneity. Single-cell sequencing technology overcomes this limitation by capturing genetic information at the single-cell level [11, 12], and has rapidly evolved into multi-omics (genomics, epigenomics, transcriptomics, proteomics, metabolomics), spatial transcriptomics, and CRISPR screening. Below, we summarize the advantages, disadvantages, and applications of major single-cell technologies in LNM research (Table 1).

Single-cell DNA sequencing (e.g., whole-genome, low-coverage whole-genome, whole-exome sequencing [23]) analyzes genomic variations (single-nucleotide variants, copy number variations) in individual tumor cells, facilitating insights into clonal evolution. Single-cell epigenomics sequencing (bisulfite-based methylation analysis, ATAC-seq, DNase-seq, ChIP-seq [24]) reveals epigenetic mechanisms (DNA methylation, chromatin accessibility, histone modifications) underlying cellular heterogeneity. Single-cell RNA sequencing, the most widely used technique, identifies differentially expressed genes and functional cell subsets associated with tumor metastasis and drug resistance [25, 26]; it is often combined with single-cell TCR/BCR sequencing to trace effector T/B cell origins and explore antigen receptor diversity [27]. Single-cell proteomics (mass spectrometry-based [28]) and metabolomics (LC-MS/GC-MS-based [29]) analyze protein expression/modifications and metabolite profiles, respectively, providing insights into cellular functions and metabolic reprogramming.

Notably, traditional single-cell technologies lose spatial information, which is addressed by spatial sequencing. Combined with single-cell annotation, it enables single-cell-resolution spatial mapping of tissues. Algorithms such as Cell2location [30], CMAP [31], and CARD [32] realize spatial deconvolution. Recent imaging-based spatial omics technologies (CosMx, MERSCOPE, Xenium [33, 34]) achieve nm-level resolution, albeit with lower throughput than sequencing-based methods. Given that cell-cell interactions in primary tumors and LNs synergistically drive LNM, we summarize pan-cancer spatial niche information based on spatial technologies (Table 2), which clarifies spatial crosstalk in the microenvironment and provides a solid theoretical basis for optimizing LNM therapy.

Single-cell deciphering of LNM mechanisms

LNM is a spatiotemporally coordinated process driven by tumor cell intrinsic properties and microenvironmental cues, which single-cell omic has uniquely unraveled at unprecedented resolution.

Overview and specialty of LNM

Cancer metastasis usually indicates a poorer prognosis, among which LNM is a unique metastatic pattern involving a series of complex processes—from the establishment of a pre-metastatic niche in the primary tumor to tumor colonization in LNs. Unlike distant metastasis, lymph node metastases exhibit high inter-tumor diversity and heterogeneity, a phenomenon observed in colorectal

Table 1 The advantages and disadvantages of single-cell sequencing technology and its application value in LNM research

Sequencing method	Advantages	Disadvantages	Applications in LNM research
Single-cell DNA sequencing	High-resolution genetic information: Allows for the detection of genetic mutations, copy number variations at a single-cell level. Identifying clonal evolution: Helps in tracing the clonal origin and evolution of cells.	Technical complexity: Requires sophisticated techniques for single-cell isolation and DNA amplification. High cost: The procedures involved, such as high-throughput sequencing and specialized reagents, make it an expensive approach.	Pinpointing driver mutations: Identifying specific genetic mutations associated with LNM [13]. Understanding metastatic clones: Determining the clonal relationship between primary tumor cells and metastatic cells in lymph nodes [14].
Single-cell epigenomics sequencing	Revealing regulatory mechanisms: Uncovers epigenetic modifications like DNA methylation, histone modifications at a single-cell level. Cell-specific epigenetic profiles: Allows for the identification of cell-specific epigenetic signatures related to LNM.	Limited throughput: Current techniques often have relatively low throughput, making it difficult to analyze a large number of single cells in a timely manner. Interpretation complexity: Epigenetic data is complex to interpret, as the relationship between epigenetic modifications and gene expression is not always straightforward.	Discovering epigenetic biomarkers: Identifying epigenetic biomarkers associated with LNM [15]. Identifying the regulatory mechanism of transcription factors: Identifying LNM-related cis-acting regulatory elements and key regulatory networks [16].
Single-cell RNA sequencing	Capturing transcriptional dynamics: Enables the identification of different cell subsets and their transcriptional states. Discovery of rare cell subsets: Finds rare cell subsets associated with the lesions.	RNA instability: RNA is relatively unstable, making sample collection and processing challenging. Data analysis challenges: The large amount of data generated requires sophisticated bioinformatics tools for analysis.	Defining cell-type-specific roles: Identifying the specific cell types that contribute to LNM [17]. Depicting dynamic changes in the microenvironment: Shows changes in the proportion and state of cells in primary tumors and lymph nodes before and after LNM [10].
Single-cell proteomics sequencing	Direct protein measurement: Provides direct information about the protein expression levels, post-translational modifications, and protein-protein interactions in individual cells. Functional insights: Helps in understanding the functional state of cells.	Technical limitations: Current proteomic techniques for single-cell analysis have limited sensitivity and throughput. Complex sample preparation: Protein extraction and purification from single cells are complex procedures, and the recovery rate may be low.	Identifying functional proteins: Pinpointing proteins that are directly involved in the process of LNM [18]. Understanding immune-tumor cell interactions: Analyzing the protein-level interactions in the context of LNM [19].
Single-cell metabolomics sequencing	Reflecting cellular metabolism: Measures the small-molecule metabolites in individual cells, providing insights into the metabolic status of cells. Cell-specific metabolism: Allows for the identification of cell-specific metabolic profiles related to LNM.	Analytical challenges: Metabolites are highly diverse in terms of chemical properties, making their separation and detection difficult. Sample complexity: The small amounts of metabolites in single cells and the presence of interfering substances in the cell matrix pose challenges for accurate analysis.	Metabolic target identification: Identifying metabolic pathways or metabolites that are essential for LNM. Identifying cellular metabolic subtypes: Identifying LNM-related cellular metabolic changes and metabolic subtypes [20].
Spatial omics sequencing	Spatial information preservation: Retains the spatial context of gene expression within tissue samples. Tumor microenvironment analysis: Allows for the study of the interactions between different cell types in their native spatial environment.	Technical complexity: The techniques for spatial omics sequencing are complex and require specialized equipment and protocols. Limited resolution: The current resolution may not be sufficient to precisely analyze the interactions at the single-cell level.	Identifying the distribution of cell subtypes: Identifying the distribution of cell subtypes associated with LNM [21]. Identifying metastasis-promoting microenvironments: Identifying the specific spatial regions related to LNM [22].

LNM Lymph node metastasis

cancer, lung cancer, and breast cancer [44, 45]. Numerous studies have found that cancer cells in distant metastases and regional lymph node metastases mostly do not share the same clones [46, 47], and distant metastases derived from cancer cells of the same origin may predict a worse prognosis. Furthermore, distant metastasis typically initiates with the formation of a pre-metastatic niche in distant organs, followed by the infiltration and colonization of circulating tumor cells [48]. However, LNs are not passive sites in the metastatic cascade but key hubs for inducing systemic immunosuppression; LNM induces tumor-specific immune tolerance in a conserved manner across multiple cancer types [49], thereby facilitating distant metastasis. A recent study similarly confirmed that, contrary to the previous notion that TDLNs are preconditioned by exogenous tumor signals to drive immunosuppression, LNs themselves possess intrinsic immune escape features independent of pre-stimulation, which

Table 2 Special niches associated with LNM found in single-cell omics studies

Location	Special niche components	Cancer type	Function	Significance in LNM	Ref
Primary tumor	PDGFRα + ITGA11 + CAFs, LECs	Bladder cancer	Interact through ITGA11-SELE axis; Secret CHI3L1 to activate MMP2	Lymphogenesis; ECM remodel	[17]
Primary tumor	CD36 + macrophages, CD8 + Tex, Tregs	Intrahepatic cholangiocarcinoma	Form a spatially adjacent immunosuppressive unit	Immunosuppression	[35]
Primary tumor	iCAFs, endothelial cells	Acral melanoma	Form a fibrovascular niche	Angiogenesis	[36]
Primary tumor	IGLL5 + B cells, HEVs	Bladder cancer	Disrupt the homeostasis of the tertiary lymphatic structure	Immunosuppression	[37]
Primary tumor	Treg, perivenous CCR7 + DC	Head and neck squamous cell carcinoma; non-small cell lung cancer; Colorectal cancer	Inhibit CD40 expression and weaken the activation capacity of DCs for T cells	Immunosuppression	[38]
Primary tumor	Treg, mregDC, lymphatic vessels	Colorectal cancer	Inhibit the transport of tumor antigens from mregDCs to TDLNs, suppress T cell activation, and create an immune tolerance microenvironment.	Immunosuppression	[39]
TDLN	CD25 + Tregs, CD8 + T cells	Melanoma; Lung adenocarcinoma; Colorectal adenocarcinoma; pancreatic ductal adenocarcinoma	CD25 + Tregs inhibit the differentiation of CD8 + T cells into cytotoxic phenotype by high expression of CD25 and competitive uptake of IL-2	Immunosuppression	[22]
TDLN	FRCs, PD-L1 + monocytes	Triple-negative breast cancer	High expression of PD-L1 and iNOS directly inhibits T cell proliferation and activation	Immunosuppression	[40]
MLN	C1QC + macrophages, CD8 + CXCL13 + Tex	Esophageal squamous cell carcinoma	Interact via the MIF-(CD74 + CD44) axis	Immunosuppression	[41]
MLN	Tregs, DCs, Tex	Head and neck squamous cell carcinoma	Block Tpex-Tex-int differentiation	Immunosuppression	[42]
MLN	PERK + macrophages, FAP + fibroblasts	Oral squamous cell carcinoma	Reject CD8 + T cells infiltration through ECM remodeling	Immunosuppression	[43]

CAFs Cancer-associated fibroblasts, *DCs* Dendritic cells, *ECM* Extracellular matrix, *FRCs* Fibroblastic reticular cells, *HEV* High endothelial venule, *LEC* Lymphatic endothelial cells, *LNM* Lymph node metastasis, *MLN* Metastatic lymph node, *TDLN* Tumor-draining lymph node, *Tregs* Regulatory T cells, *Tex* Exhausted T cells, *Tpex* Progenitor exhausted T cells, *Tex-int* Intermediate-exhausted T cells

play a critical role in LNM [50]. Meanwhile, since LNs are essential immune activation organs, LNM often impairs the responsiveness of intranodal T cells to immunotherapy [42]. Based on these characteristics, a deeper understanding of LNM-associated cancer cells and the involved metastasis-related microenvironment is imperative to propose more constructive strategies for LNM treatment in the future.

Clonal evolution and metastatic patterns of tumor cells

LNM originates from a subset of metastasis-initiating cells (MICs) within primary tumors, characterized by partial epithelial-mesenchymal transition (pEMT) and immune evasion potential. MICs are mainly located at the invasive front to prepare for lymphatic dissemination. In pancreatic ductal adenocarcinoma, intrahepatic cholangiocarcinoma and lung adenocarcinoma, MICs with high S100A2 expression display an invasive phenotype, activating epithelial-mesenchymal transition (EMT) signaling [51–53]. In head and neck squamous cell carcinoma, AXL/AURKB/STAT2-high MICs enrich EMT pathways and share transcriptomic similarity with MLN tumor cells [54], confirming their metastatic fate. Furthermore, MICs evade immune surveillance by reducing the expression of their own MHC-I molecules [55, 56]. Besides their own phenotypic alterations, active lymphangiogenesis within the primary tumor provides a pathway for MIC metastasis [57], while the formation of an immunosuppressive microenvironment within LNs clears obstacles [40, 58]. In summary, MIC metastasis is the result of the interaction and synergistic promotion of multiple mechanisms.

When tumor cells migrate to LNs, they often undergo mesenchymal-epithelial transition (MET) to colonize the LNs, resulting in reduced morphological plasticity, enhanced proliferative capacity, and participation in the reinforcement of the immunosuppressive microenvironment within the LNs [59, 60], to adapt to the environment within the LNs and form metastatic lesions. Cancer cells exhibit unique plasticity in their survival strategies during LN metastasis and colonization. For instance, in MLNs of melanoma, cancer cells adapt to the hypoxic microenvironment by upregulating FSP1 expression to resist ferroptosis [61]. Studies have identified two metastatic patterns of tumor cells in MLNs. Clonal selection: tumor cells in MLNs carry chromosomal copy number

variations distinct from those in the primary tumor, suggesting formation by seeding of genetically unique subclones from the primary tumor; Mass migration: tumor cells in MLNs share most chromosomal copy number variations with the primary tumor, with highly overlapping transcriptomes, indicating collective migration of primary tumor cells to the LNs [62]. Additionally, mitochondrial mutations can provide new markers for tracing metastatic clones [63]. A recent study based on single-cell exome sequencing technology revealed that the genetic heterogeneity of LNM is significantly higher than that of the primary tumor, carrying a large number of somatic mutations shared with the primary tumor along with a few unique mutations, supporting a polyclonal seeding model [14].

Metabolic reprogramming of mics

Notable metabolic reprogramming occurs in MICs during metastasis. Contrary to the glycolytic properties of traditional tumor cells, MICs exhibit significantly elevated oxidative phosphorylation activity [54, 64, 65]. Studies have found that MICs characterized by MYC + are enriched in mesenchymal development and stem cell-related pathways, relying on fatty acid oxidation for energy supply. They highly express fatty acid oxidation-related genes such as EPHX1, GAPDHS, and HSP90AA1, and their high expression of MITF drives metastasis by activating fatty acid oxidation pathways [36]. Notably, MYC can sustain malignant phenotypes of cancer cells such as proliferation and invasion by regulating pathways including glycolysis and glutamine metabolism, while inducing the expression of PD-L1 and CD47 to achieve immune evasion [66]. However, MICs with high MYC expression exhibit significantly elevated oxidative phosphorylation pathway activity [36, 64]. Previous studies have also reported that MYC may be involved in promoting intratumoral oxidative phosphorylation [67, 68], indicating that MYC may exert a unique mode of action in regulating cancer cell metastasis, which requires further in-depth research to confirm. Additionally, abnormal activation of nicotinic acid and nicotinamide metabolic pathways has been identified as a key metabolic alteration driving tumor cell metastasis. Research indicates that MICs in LNM-positive primary tumors highly express NNMT. As a "metabolic-epigenetic" regulatory hub in LNM of esophageal squamous cell carcinoma, NNMT inhibits Ecad expression by depleting S-adenosylmethionine, reducing H3K4me3 modification in the Ecad promoter region and m6A modification of mRNA, thereby promoting EMT and metastasis of MICs [69].

Microenvironmental alterations in LNM

Single-cell sequencing has uncovered dynamic remodeling of cellular components and interactions across primary tumors, TDLNs, and MLNs, which orchestrates metastatic progression.

Metastasis-priming niche

A series of adaptive changes occur within the primary tumor, forming a microenvironment conducive to LNM (Fig. 1). MICs within the tumor are distributed at the invasive front and harbor pEMT characteristics. Within the microenvironment, the numbers of helper T cells (Th) and regulatory T cells (Treg) are significantly increased, CD8 + T cells differentiate into an exhausted state, and the maturation of tertiary lymphoid structures (TLS) is impaired, collectively forming an overall immunosuppressive niche [70–72]. The dysregulated amino acid metabolic environment within the tumor further impairs the cytotoxic activity of effector T cells [73]. Moreover, a study combining single-cell transcriptomics and TCR sequencing in breast cancer found non-TDLN-derived expanded T cell clones in primary tumors. These cells exhibit high tissue retention capacity (CXCR6 + ITGAE+) and a unique co-inhibitory phenotype (CD39 + NKG2A+), with lower expression of IL7R and GZMK compared to shared clones present in both primary tumors and LNs [74].

During the initiation phase of LNM, alterations in intratumoral stromal components represent a crucial step. Multiple pro-metastatic stromal cell subsets exist in the primary tumor, among which CAFs are mainly involved in angiogenesis, stromal remodeling, and alterations in tumor cell phenotypes [75]—such as PDGFRα + ITGA11 + CAFs colocalized with lymphatic endothelial cells in bladder cancer [17], SOD3 + CAFs in lung adenocarcinoma [57], and inflammatory CAFs in acral melanoma [36], to name a few. Additionally, lymphatic endothelial cells participate in intratumoral lymphangiogenesis. Furthermore, myeloid-derived cell subsets also contribute to the formation of the metastatic phenotype of tumor cells, such as CCL2 + tumor-associated macrophages (TAMs) identified in pancreatic ductal adenocarcinoma [51].

Pre-metastatic niche formation

LNs serve as sites for lymphocyte retention and proliferation. In the presence of tumors, TDLNs undergo a series of adaptive changes (Fig. 2), including extensive lymphangiogenesis; dilation and dedifferentiation of high endothelial venules that impair lymphocyte recruitment; enhanced proliferative capacity of fibroblastic reticular cells (FRCs) mediating fibrotic remodeling of the conduit system, diminished cytokine secretion capacity that affects naive T cell survival, to name a few [76].

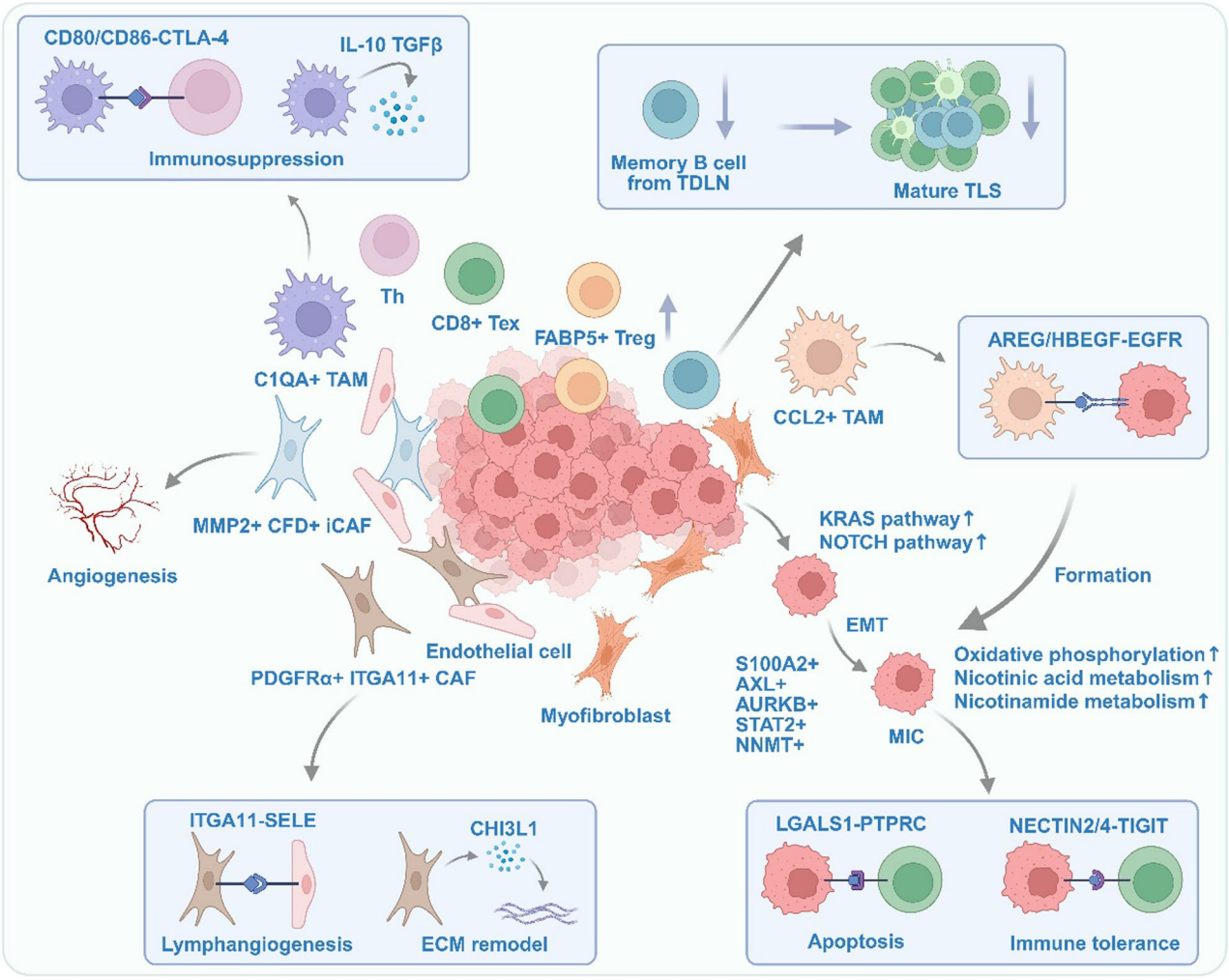

Fig. 1 The metastasis-promoting microenvironment of primary tumor with lymph node metastasis at single-cell resolution. CAF, cancer-associated fibroblast; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; iCAF, inflammatory cancer-associated fibroblast; MIC, metastasis initiating cell; TAM, tumor-associated macrophage; TDLN, tumor-draining lymph node; Tex, exhausted T cell; Th, helper T cell; TLS, tertiary lymphoid structure; Treg, regulatory T cell. Created with BioRender.com

Fibroblasts are key structural components of TDLNs. Among them, myofibroblasts constitute a small proportion, with the majority being FRCs. FRCs can be influenced by the primary tumor to recruit immunosuppressive cells and inhibit the function of intranodal T cells. Notably, compared with normal LNs, FRCs in TDLNs of breast cancer mice exhibit enhanced metabolic reprogramming, specifically characterized by activation of the oxidative phosphorylation pathway and enrichment of bile acid and fatty acid metabolic pathways [77]. However, the specific mechanism by which this change contributes to pre-metastatic niche formation remains unclear.

Immune cells are the predominant cell type in TDLNs, with a high proportion of naive T and B cells [78]. Single-cell sequencing studies have uncovered several unique immune cell subsets within TDLNs. TDLNs are the main reservoir for progenitor exhausted T cells (Tpex), which share clonal homology with terminally exhausted T cells (Tex-term) in tumors. This subset is primarily localized in T-cell zones, forming neighborhoods with DCs [42]. Meanwhile, a recent study identified a unique pre-exhausted CD4 + T cell subset in TDLNs of oral cancer mice, which participates in pre-metastatic niche establishment and mediates DC immune tolerance via the CTLA-4-CD86 axis [79]. In TDLNs of B16F10 melanoma mice, there is a subset of TIM-1 + B cells that highly express immune checkpoint molecules such as PD-1 and TIGIT, and limits antigen presentation and cellular activation by inhibiting type I interferon receptor signaling [80]. Additionally, TDLNs also harbor myeloid-derived suppressor cells with immunosuppressive properties [81, 82].

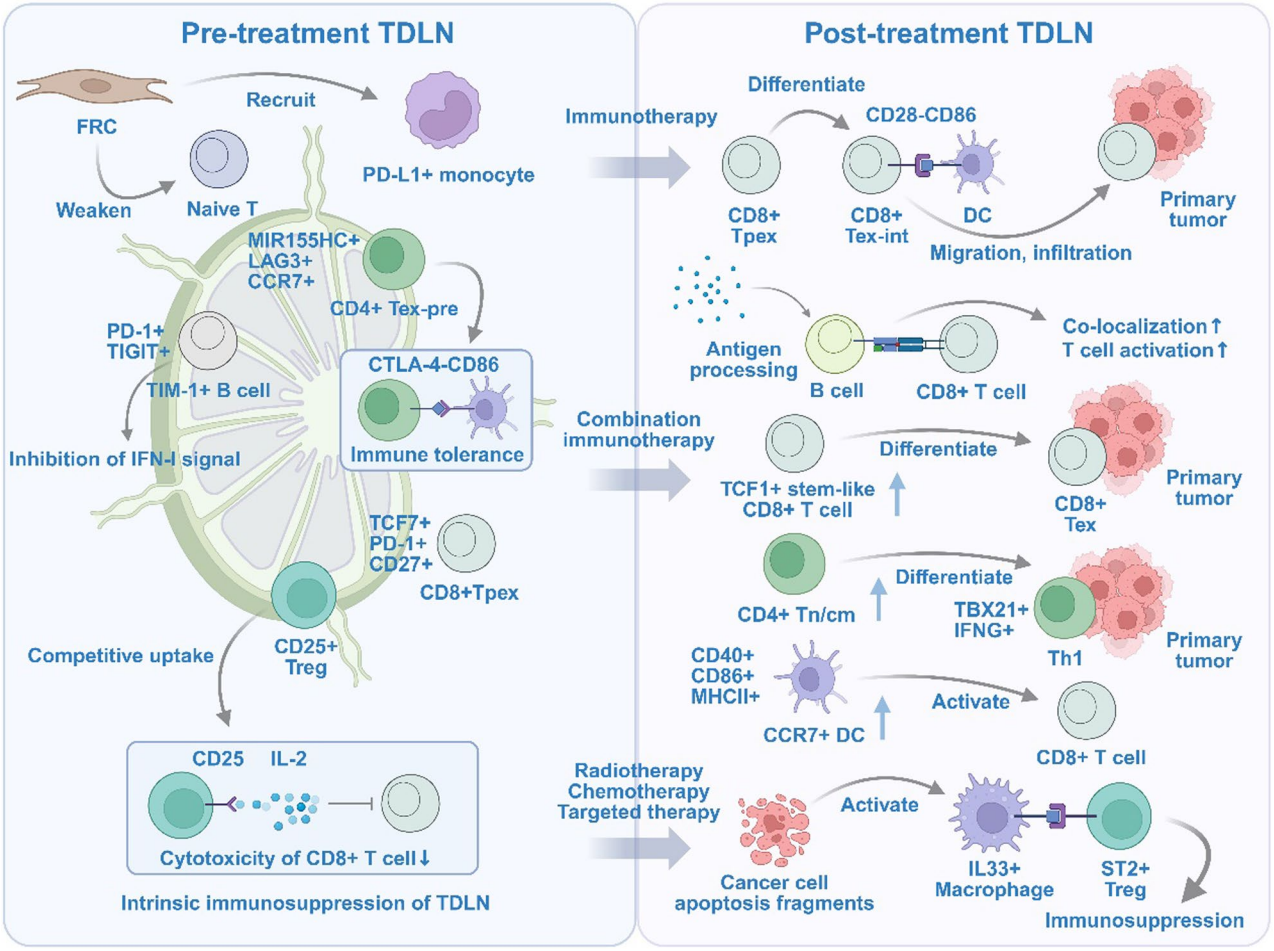

Fig. 2 The pro-metastatic microenvironment of tumor-draining lymph node and the adaptive changes after treatment at single-cell resolution. DC, dendritic cell; FRC, fibroblastic reticular cell; TDLN, tumor-draining lymph node; Tex, exhausted T cell; Tex-int, intermediate-exhausted T cell; Tex-pre, pre-exhausted T cell; Th, helper T cell; Tn/cm, naive/central memory T cell; Tpex, progenitor exhausted T cell; Treg, regulatory T cell. Created with BioRender.com

Metastasis-established niche

MLNs not only serve as a refuge for tumor cells but also possess the capacity to disseminate tumor cells themselves. Notably, compared with TDLNs, MLNs exhibit a more pronounced immunosuppressive state [6]. Meanwhile, significant metabolic reprogramming occurs within their microenvironment, leading to a substantial decline in adaptive immune regulation capacity, which further influences tumor development and outcomes (Fig. 3).

Single-cell sequencing analysis of mouse MLNs has shown that MICs colonizing LNs exhibit enhanced fatty acid oxidation activity to sustain energy supply, while promoting glutathione synthesis to markedly reduce lipid peroxidation levels and resist ferroptosis induced by oxidative stress [83]—representing an adaptive change of MICs to the LN microenvironment. Meanwhile, tumor cells colonized in LNs are also influenced by the MLN microenvironment, which reinforces their malignant phenotypes. Single-cell sequencing analysis of LNM in intrahepatic cholangiocarcinoma revealed that LN-resident tumor cells are regulated by lipid metabolism-related macrophages with high CD36 expression, which drive tumor cell invasion via the SAA1-CD36 axis [35]. Subcapsular sinus macrophages, localized in the subcapsular sinus region of MLNs, can secrete IL-1α by phagocytosing tumor cells, activating the IL1R1-STAT3 axis to directly promote tumor cell proliferation [84].

Furthermore, compared with TDLNs, MLNs are enriched in CAFs highly expressing FAP and POSTN, which are mainly involved in extracellular matrix remodeling. A subset of FRCs in MLNs is activated via the YAP/TAZ pathway to differentiate into myofibroblasts; during this process, they lose immune recruitment capacity (downregulation of CCL19) and acquire a contractile phenotype (upregulation of ACTA2). They may also degrade collagen fibers to form TACS-3 structures—fiber bundles perpendicular to the tumor boundary—inducing extracellular matrix remodeling and promoting extranodal extension (ENE) [85]. Additionally, myofibroblasts

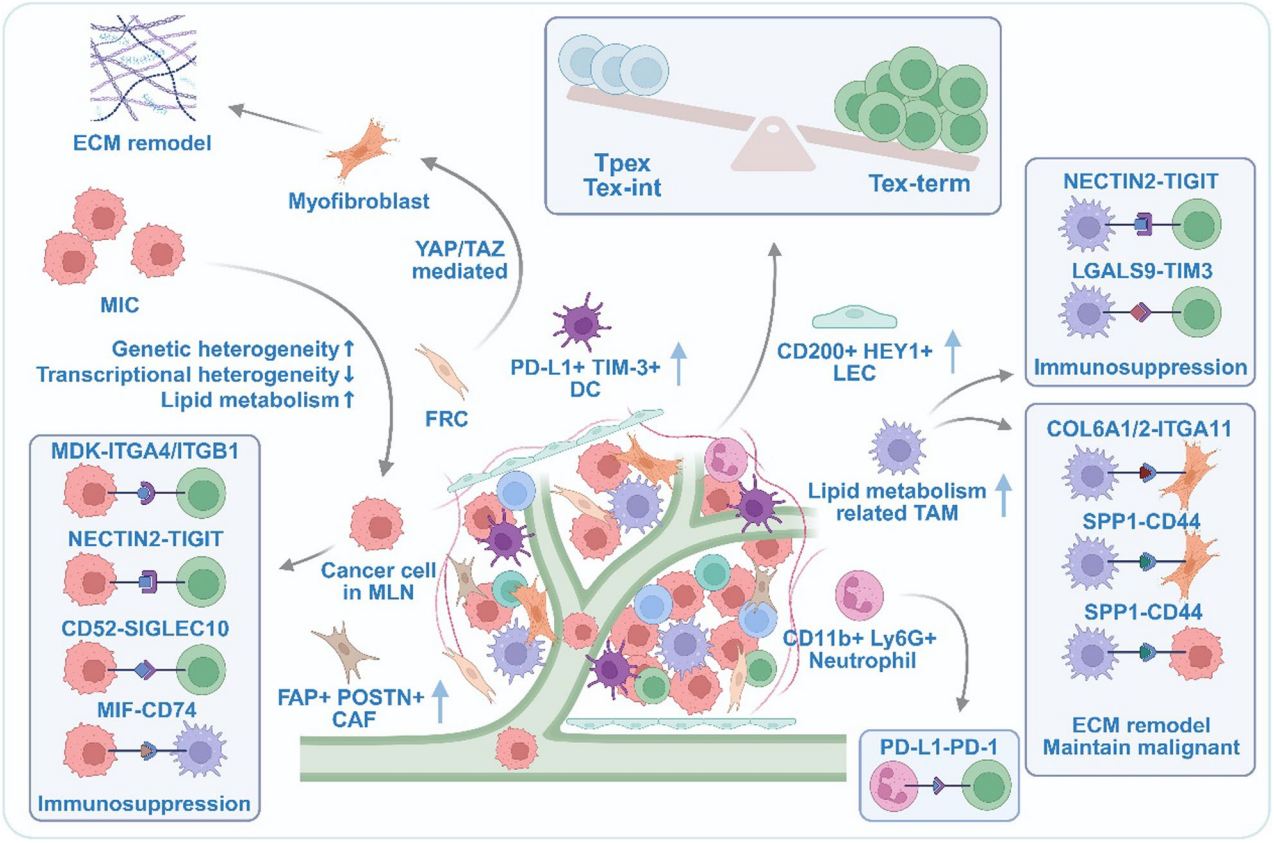

Fig. 3 The microenvironment of metastatic lymph nodes at single-cell resolution. CAF, cancer-associated fibroblast; DC, dendritic cell; ECM, extracellular matrix; FRC, fibroblastic reticular cell; MIC, metastasis initiating cell; MLN, metastatic lymph node; TAM, tumor-associated macrophage; Tex-int, intermediate-exhausted T cell; Tex-term, terminally exhausted T cell; Tpex, progenitor exhausted T cell; Treg, regulatory T cell. Created with BioRender.com

surround the tumor invasive front and colocalize with SPP1 + TAMs, potentially contributing to the formation of an immunosuppressive microenvironment in MLNs [86].

Most immune cells in MLNs exhibit an immunosuppressive phenotype. Compared with TDLNs, MLNs contain more myeloid-derived suppressor cells, though this may vary across cancer types. For example, Fusobacterium nucleatum in gastric cancer induces the specific recruitment of CD11b + Ly6G + neutrophils in MLNs, facilitating immune evasion [87]. Notably, MLNs are highly enriched in lipid metabolism-related TAMs, which are deeply involved in shaping the immunosuppressive microenvironment in MLNs and conserved across MLNs of lung cancer, breast cancer, osteosarcoma, and esophageal squamous cell carcinoma [81, 88–90]. Furthermore, MLNs show an increased proportion of CD8 + Tex, predominantly composed of highly exhausted, low-proliferative terminally exhausted T cells (Tex-term), with a lack of Tpex and intermediate exhausted T cells (Tex-int)—indicating further enhancement of immunosuppression in MLNs [42].

Immunosuppressive mechanisms in LNM

In 2013, Chen et al. proposed the concept of the Cancer-Immunity Cycle (CIC) [91], which was further refined in 2023 [92]. The CIC delineates a coordinated cascade encompassing antigen release, processing and presentation within the primary tumor, T cell activation and clonal expansion in LNs, effector T cell trafficking and intratumoral infiltration, and subsequent tumor cell recognition and elimination. More recently, the concept of an intratumoral cancer immunity sub-cycle has been incorporated into this framework, highlighting the critical role of TLS in the CIC [93, 94]. Current immunotherapies, including immune checkpoint inhibitors and CAR-T cell therapy, are developed and optimized within the CIC paradigm, underscoring its fundamental importance in cancer immunology research. Notably, the LN serves as a central hub for CIC execution. Recent single-cell sequencing studies have progressively elucidated the association between LNM and CIC dysfunction, advancing our understanding of this intricate biological process to the single-cell resolution (Fig. 4).

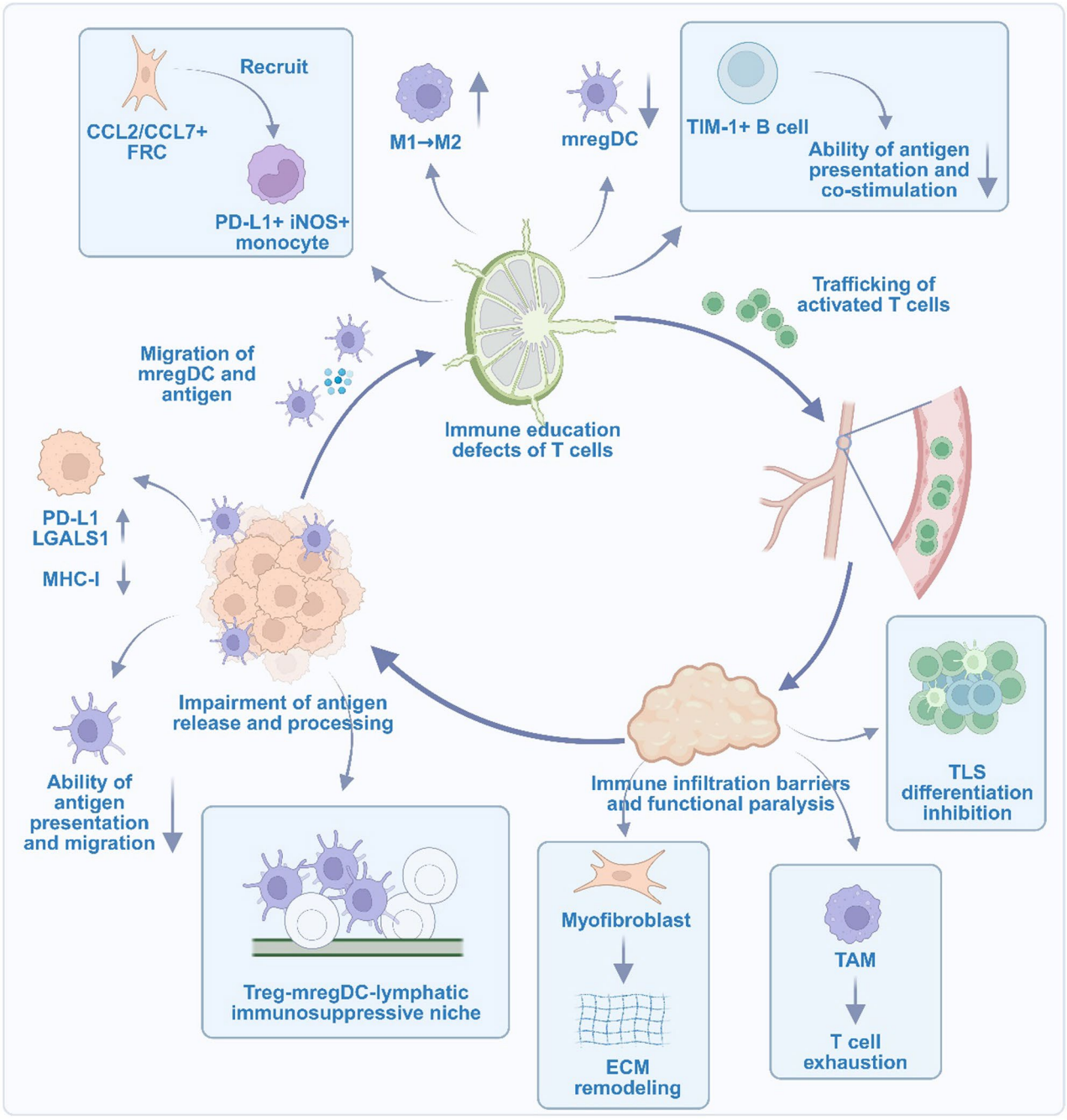

Fig. 4 Disruption of the cancer immunity cycle in LNM. DC, dendritic cell; ECM, extracellular matrix; FRC, fibroblastic reticular cell; TAM, tumor-associated macrophage; TLS, tertiary lymphoid structure; Treg, regulatory T cell. Created with BioRender.com

Antigen release and processing: tumor cell immune editing and APC capture failure

In normal conditions, MHC-I molecules participate in immune surveillance, assisting T cells in recognizing and eliminating abnormal cells; simultaneously, their high expression inhibits NK cell function. In LNM cell lines from melanoma mice, MHC-I expression is upregulated—a feature conserved in pancreatic cancer and head and neck squamous cell carcinoma, with cross-species LN-specificity [49]—and promotes LNM by inducing NK cell dysfunction. Meanwhile, tumor cells can also inhibit effector T cell activity by expressing immune checkpoint genes such as PD-L1 and LGALS1 [95], reinforcing pre-metastatic immune evasion.

Within the CIC, DCs play a pivotal role in antigen presentation, guiding and activating T cells in LNs. Recent single-cell sequencing studies have identified a subset of DCs characterized by maturation, activation, and

migratory properties, termed mregDCs or migDCs [96, 97], which are key subsets mediating antigen presentation and anti-tumor immune activation in the CIC. A single-cell combined with spatial sequencing analysis of colorectal cancer revealed that CCR4-mediated Tregs migrate toward mregDCs and form a Treg-mregDC-lymphatic vessel immunosuppressive niche in tumor stromal regions [39]. Specific depletion of Tregs or blockade of Treg-mregDC interactions in mice enhanced the transport of tumor antigens to TDLNs. Notably, not all mregDCs are capable of migrating to TDLNs. Single-cell sequencing analysis of mouse subcutaneous tumor models showed that tumors harbor a subset of CCR7-expressing resident DCs; compared with migratory DCs, this subset exhibits markedly reduced antigen presentation function [98]. They have also been found predominantly distributed in perivascular regions of veins in head and neck squamous cell carcinoma, endometrial cancer, and non-small cell lung cancer [38], potentially involved in mediating local immune activation. Impaired DC migration profoundly impairs anti-tumor responses in TDLNs. Encouragingly, recent studies have identified the therapeutic potential of PDE5 inhibitors in restoring the migratory capacity of murine and human DCs [99], providing a novel direction for immunotherapies targeting mregDCs.

Lymphocyte activation and clonal expansion: T cell functional exhaustion and suppression

Impaired immune education in TDLN

Immune education refers to a critical process in TDLNs, where antigen-presenting cells (APCs) present tumor antigens to naive T cells and provide co-stimulatory signals to induce T cell activation and functional differentiation, which directly dictates the magnitude of anti-tumor immune responses. Traditional studies have been confined to the binary classification of LNs as "metastatic/non-metastatic", lacking systematic insights into their functional heterogeneity and the mechanisms underlying impaired immune education. However, technological breakthroughs such as single-cell sequencing and spatial transcriptomics have enabled precise dissection of these complex processes.

The gradual molecular subtype transition of TDLNs represents the core pathological basis for impaired immune education. A large-sample multi-omics study on colorectal cancer first established a TDLN molecular subtype system, identifying 4 non-metastatic subtypes and 2 metastatic subtypes that exhibit a progressive shift from immune-active to immune-dysfunctional states [100]. The core mechanism involves the downregulation of FDCs and transcription factors SPIB/RELB, which disrupts the FDC-B cell interaction network; notably, the dysfunctional subtypes are significantly correlated with poor tumor prognosis. Future validation of the pan-cancer conservation of this classification system will provide novel insights for personalized immunotherapy.

Aberrant signals derived from primary tumors further exacerbate impaired immune education in TDLNs by affecting APCs. In triple-negative breast cancer, single-cell RNA sequencing (scRNA-seq) combined with spatial transcriptomics has uncovered a TLR4-dependent FRC-monocyte axis in TDLNs [40]. Tumors secrete SAA1 and S100A9 to activate FRCs in TDLNs, which then secrete CCL2/CCL7 to recruit monocytes; these recruited monocytes suppress T cells via PD-L1 and iNOS, a mechanism validated in human samples. A CODEX imaging and scRNA-seq study on head and neck squamous cell carcinoma revealed that tumors activate nociceptive neurons through the ATF4-SLIT2 axis, which release calcitonin gene-related peptide (CGRP) to remodel TDLNs into an immunosuppressive state, characterized by reduced CCL5 secretion and enhanced activation of M2-like immunosuppressive TAMs [58]. Additionally, DC migration defects represent a conserved impairment mechanism across cancer types. Integrating in vivo CRISPR screening, scRNA-seq, and analyses of patient samples from pancreatic cancer, breast cancer, and colon cancer, a study identified the sGC-PDE5-cGMP axis as a regulator of mregDC motility [99]. Tumors suppress the NOS2-NO-sGC axis to reduce intracellular cGMP levels in DCs, leading to impaired migration to TDLNs and decreased antigen presentation efficiency.

Dysregulated T/B cell signaling further aggravates these impairments. In mouse model studies, 3D immunofluorescence combined with single-cell TCR analysis demonstrated that PD-1 fine-tunes TCR signaling to maintain the self-renewal of high-affinity stem-like CD8 + T cells in TDLNs [101]; complete blockade may lead to the loss of this subset, challenging conventional wisdom. Regarding B cells, single-cell RNA/BCR sequencing revealed that the TIM-1 + B cell subset in TDLNs highly expresses co-inhibitory molecules and impairs antigen presentation by suppressing type I interferon responses [80]; the synergistic effect of targeting this subset with PD-1 inhibitors has been verified in mouse models. Currently, most studies on B cells in TDLNs only describe their proportions and basic subset characteristics, warranting further in-depth investigations in future research.

Furthermore, the local intrinsic immunosuppressive microenvironment of TDLNs and therapy-induced tolerance exacerbate immune education impairment. Single-cell spatial analysis showed that Tregs and CD8 + exhausted T cells are closely adjacent in TDLNs; Tregs competitively sequester IL-2 via high CD25 expression, thereby inhibiting T cell differentiation [22]. After chemotherapy or targeted therapy, medullary sinus macrophages in TDLNs activate ST2 + Tregs through the

TLR9-IL-33 axis; these activated Tregs migrate to the primary tumor and suppress CD8 + T cell function, ultimately leading to therapy resistance [82].

Immunosuppression in MLN

As a central hub for tumor lymphatic metastasis, the malignant remodeling of the MLN immune microenvironment represents a key step in tumor immune evasion. This process is primarily mediated through three interconnected mechanisms: dysfunction of APCs, cell-intrinsic immunosuppression by tumor cells, and microenvironmental regulation by stromal cells, which collectively disrupt the normal operation of the cancer-immunity cycle.

As core mediators of immune education, APCs in MLNs are specifically reprogrammed into an immunosuppressive phenotype, directly blocking T cell activation and clonal expansion. Pan-cancer scRNA-seq has revealed that MLNs are selectively enriched in a Galectin-9 + IL-10 + macrophage subset. This subset activates the BTK/STAT3 signaling axis via Galectin-9 to sustain IL-10 secretion, directly inhibiting the proliferation and cytotoxicity of CD8 + T cells, and thus serves as a conserved, cross-cancer APC subset driving MLN immune tolerance [102]. Additionally, the presence of mregDCs in MLNs suggests that these nodes may retain residual immune education potential. However, these mregDCs co-express immunosuppressive molecules (e.g., PD-L1, CD39) and co-stimulatory molecules (e.g., CD40, CD86), inducing T cell tolerance while mediating antigen presentation [103]. Furthermore, tolerogenic DCs (highly expressing CD39, IDO, PD-L1, and TIM-3) are enriched in the vicinity of Tex-term, consolidating T cell exhaustion by forming localized immunosuppressive niches [42]. These findings indicate that APC subsets in MLNs are not merely functionally deficient, but are precisely reprogrammed into an "immune-regulatory" phenotype that disrupts immune education through multiple signaling axes.

Tumor cells within MLNs actively establish an immunosuppressive microenvironment via cell-intrinsic expression of immune checkpoint molecules, secretion of effector molecules, and direct cell-cell interactions. Single-cell spatial transcriptomics and flow cytometry analyses have shown that MLN-resident tumor cells highly express MHC-II molecules and immune checkpoint molecules such as CD276 and LGALS9, directly suppressing cytotoxic T cell activity [52]. Concurrently, these tumor cells secrete midkine (MDK), which binds to ITGA4/ITGB1 on T cells to activate the NF-κB pathway, thereby upregulating PD-1 and CD39 expression and enhancing interactions with Tregs, exacerbating T cell exhaustion [54]. Moreover, tumor cells mediate direct immunosuppression through surface molecule interactions: they engage with CD8 + effector T cells via the NECTIN2-TIGIT and CD52-SIGLEC10 pathways to block T cell activation [95]; they also bind to macrophages via MIF-CD74, inhibiting macrophage M1 polarization and promoting Treg recruitment, thus forming a "tumor cell-immune cell" inhibitory loop. These cell-intrinsic regulatory mechanisms enable tumor cells to acquire immune privilege in MLNs, laying the foundation for their colonization and subsequent metastasis.

Stromal cells in MLNs provide physical and molecular support for immunosuppression through structural remodeling, signal modulation, and ECM reconstruction. Lymphatic endothelial cells (LECs) are key regulators in this process. In breast cancer MLNs, combined TGF-β and VEGF signaling drives LEC subset remodeling: PD-L1$^+$ subcapsular sinus LECs are reduced, while immunosuppressive CD200 + HEY1 + capillary-like LECs are enriched. The latter directly suppress T cell activation (via downregulation of CD69/CD25) through CD200 signaling; additionally, LECs highly express matrix Gla protein (MGP), which enhances tumor cell adhesion to endothelium and promotes lymphatic metastasis [104]. Single-cell technologies have for the first time revealed that this LEC remodeling pattern is not simply a result of abnormal lymphangiogenesis, but a subset-specific functional reprogramming event. Fibroblasts and associated stromal networks are also critical: in osteosarcoma MLNs, osteoblast-like CAFs form physical barriers that separate malignant cells from T cells, blocking immune recognition [89]. Lipid metabolism-related macrophages enhance the ECM remodeling capacity of myofibroblasts via the COL6A1/2-ITGA11 signaling pathway, impeding effector T cell infiltration; they also form an immunosuppressive network through NECTIN2-TIGIT and LGALS9-TIM3 interactions [35, 78]. Furthermore, due to differences in microenvironmental characteristics, MLNs harbor cancer type-specific immunosuppressive cell subsets—for example, *Fusobacterium nucleatum*-induced recruitment of CD11b + Ly6G + neutrophils in gastric cancer MLNs [87].

Following tumor metastasis, the cancer-immunity cycle function of MLNs is severely impaired. The synergistic action of these three immunosuppressive mechanisms transforms MLNs from "immune activation stations" into "immune tolerance niches", where effector T cells are predominantly in a state of terminal exhaustion that is difficult to reverse with conventional immunotherapy due to epigenetic barriers [105]. Although studies have suggested that IL-10-mediated metabolic reprogramming can restore the activity of Tex-term [106], further evidence is required to support its clinical translation, which may broaden the horizons for MLN-targeted therapeutic strategies.

Effector cell trafficking and tumor cell killing: infiltration barriers and functional paralysis

Stromal cells and their mediated microenvironmental remodeling represent the core drivers of physical and signaling barriers to T cell infiltration, primarily through aberrant ECM deposition and dysregulated chemokine/adhesion signaling. As key stromal components, CAFs exhibit subtype-specific barrier functions across different cancer types. In non-small cell lung cancer, MYH11 + αSMA + CAFs form a monolayer envelope surrounding tumor nests and secrete type IV collagen to construct a basement membrane-like structure, whereas FAP + αSMA + CAFs highly express fibrillar collagens such as COL11A1 and COL12A1, forming a dense ECM network that directly impedes T cell migration [107]. In pancreatic cancer, the desmoplastic stroma driven by FAP + CAFs further restricts perivascular T cell infiltration and extravasation, a barrier that can only be specifically disrupted by FAP-targeted CAR-T cells [108]. In oral squamous cell carcinoma, myofibroblasts are enriched at the tumor invasive front; they abnormally recruit immune cells via the CXCL12-CXCR4 axis and dysregulate chemokine gradients, indirectly hindering the directional infiltration of T cells [85]. Furthermore, in breast cancer, TAMs synergize with CAFs to strengthen this barrier, participating in collagen synthesis under the drive of stiff ECM and TGF-β, thereby collectively shaping the immune infiltration defect [109].

The primary tumor is enriched with multiple cancer-specific immunosuppressive cell subsets, among which TAMs have been precisely identified and extensively investigated using single-cell omics technologies. C1QA + TAMs and CCL2 + TAMs block T cell activation and recruit Tregs via the CD80/CD86-CTLA4 axis and CXCL16-CXCR6 axis, respectively, to reinforce the immunosuppressive microenvironment [110, 111]. In non-small cell lung cancer, SPP1 + TAMs colocalize with COL11A1 + CAFs, reducing the secretion of T cell-attracting chemokines such as CCL5; meanwhile, the hypoxic microenvironment inhibits the activation of TLS, further weakening immune-recruiting signals [112]. In breast cancer, TAMs deplete arginine from the microenvironment and secrete ornithine, directly impairing the cytotoxic function of CD8 + T cells [109]. In addition to TAMs, Treg subsets also play a critical immunosuppressive role. In esophageal squamous cell carcinoma, FABP5 + Tregs enhance their immunosuppressive activity through lipid metabolic reprogramming and are specifically enriched in tumors with positive LNM [69]. These findings delineate the subtype specificity and diverse immunosuppressive mechanisms of these cell populations, with single-cell technologies facilitating the clarification of the transcriptional signatures and functional specialization of each subset.

Impaired cancer-immunity cycle in TLS

TLSs are ectopic lymphoid tissues formed under pathological conditions such as chronic inflammation. Composed of a fibroblastic reticular network as a scaffold for lymphocyte aggregation, TLSs serve as core functional units of the intratumoral cancer-immunity cycle. They can form germinal centers containing follicular helper T cells and follicular DCs to mediate antigen presentation and T/B cell activation, directly regulating the intensity of anti-tumor immune responses [94, 113]. TLSs exhibit distinct maturation heterogeneity: single-cell sequencing has confirmed their core components include B cells, T cells, and high endothelial venules, with enrichment of clonal T cells of non-TDLN origin (e.g., CCL4 + CD8 + T cells), suggesting TLSs may act as initiation sites for unique T cell clones within the primary tumor [74]. In bladder cancer, single-cell combined with spatial transcriptomics revealed that the IGLL5 + B cell subset can target high endothelial venules via the CCL19-CCRL2 axis, leading to its dysfunction and disrupted TLS homeostasis, which ultimately reduces CD8 + T cell activation and mediates immune checkpoint blockade resistance—this mechanism is conserved across pan-cancers such as kidney cancer and lung cancer [37].

Pan-cancer and cancer-type-specific studies have further delineated the complex associations between TLS maturation, the cancer-immunity cycle, and LNM. As a critical supportive component for TLS germinal center formation, TDLNs serve as key sources of memory B cells, yet memory B cell differentiation is impaired during LNM. In a non-small cell lung cancer mouse model, inhibition of IFN-γ signaling blocks the B cell IFNGR1-STAT1 pathway, impairing memory B cell differentiation and ultimately hindering TLS maturation [72]. Immature TLSs weaken immune checkpoint blockade therapeutic responses and fail to initiate an effective intratumoral cancer-immunity cycle [114, 115]. In lung adenocarcinoma, patients with TLS + tumors exhibit significantly better disease-free survival and overall survival than those with TLS- tumors; a dysregulated B cell ratio between the primary tumor and TDLNs directly affects TLS formation efficiency, while the TIM-1 + B cell subset—an immunosuppressive population in TDLNs—specifically impedes TLS maturation [116]. Meanwhile, mature TLSs are independently associated with a low LNM rate and favorable prognosis: they can directly inhibit tumor lymphatic metastasis by increasing cytotoxic lymphocyte infiltration in TDLNs, demonstrating a "protective role" against LNM [117]. These studies suggest a potential bidirectional regulatory relationship between TLS maturation and LNM, but relevant mechanistic research remains scarce, and no unified conclusion has been reached. Additionally, immature TLSs are often accompanied by enrichment of an immunosuppressive

microenvironment and cannot effectively initiate the cancer-immunity cycle, further highlighting the decisive role of TLS maturation status in immune function [114]. Collectively, these findings indicate that impaired TLS-mediated cancer-immunity cycle is a key link in tumor immune evasion, and the unclear complex associations between TLSs and LNM as well as their formation mechanisms represent critical directions for future research.

Novel therapeutic targets and pre-clinical research for LNM

Single-cell omics technologies have revolutionized LNM therapeutic target discovery by precisely identifying functionally distinct cell subsets, uncovering intercellular interaction networks, and localizing regulatory nodes—enabling targeting of previously unrecognizable LNM-related mechanisms. These single-cell-derived targets (detailed in Table 3) have demonstrated promising preclinical efficacy. Notably, all targets in Table 3 are validated in animal models, with interventions such as immune checkpoint inhibitors, pathway antagonists, and cell-specific depletors consistently reducing LNM incidence, suppressing metastatic burden in TDLNs or MLNs, and restoring antitumor immunity.

Spatial omics has gradually emerged as a powerful tool for deciphering the spatial architecture of LNM-related niches, which is critical for clarifying spatial-dependent regulatory mechanisms. However, spatial omics studies specifically focusing on LNM remain scarce; future in-depth exploration of TDLN/MLN spatial landscapes will facilitate more precise subtype-specific targeting.

In terms of delivery strategies, intranodal injection has become a promising direction for LNM therapy. By directly delivering agents to TDLNs/MLNs, it achieves precise targeting, enhances local drug concentration, and minimizes systemic toxicity. Currently, this method has been applied to natural DCs therapy in patients with stage IIIB/C melanoma (NCT02993315), significantly improving the patients' adaptive immune response, although no benefit was observed in the recurrence-free survival rate [119]. Further preclinical studies are needed to optimize the drug delivery strategy.

Despite encouraging preclinical results, clinical translation of single-cell-derived targets still faces challenges, including tumor-type-specific target heterogeneity, LN microenvironmental drug penetration barriers, and the lack of reliable LNM-specific biomarkers for patient stratification. Future efforts integrating single-cell and spatial omics to decode the "cellular-spatial" regulatory network of LNM, combined with the development of intranodal precision delivery systems, will accelerate the translation of these targets from preclinical studies to clinical applications.

Research on LNM and immunotherapy at single-cell resolution

As a key immune organ, LNs significantly impact patient survival and treatment efficacy. Currently, numerous therapeutic approaches aim to restore LN immune function and inhibit metastasis to improve patient survival rates. Immunotherapy, a treatment modality that harnesses the immune system to combat diseases, activates immune effector cells by blocking immune checkpoints, among other mechanisms. However, the issue of low response rates remains unresolved. Single-cell high-throughput sequencing technology has accelerated research into LNM-related immunotherapy and brought new insights and hope.

Current status of immunotherapy research related to LNM

Immunotherapeutic sensitivity varies substantially across different cancer types and individuals, which is closely associated with the heterogeneity of the TME [120–123]. However, whether this heterogeneity is linked to the functional status of TDLNs—a pivotal organ for immune priming and anti-tumor immunity—remains poorly elucidated. Currently, most predictive biomarkers for immunotherapy response are derived from the primary tumor [124], while LN-specific molecular indicators are largely lacking. Nevertheless, LNs exhibit distinct pathological responses to immunotherapy compared with primary tumors. In studies of breast cancer metastases, PD-L1—a major biomarker for immunotherapy—shows significantly differential expression in MLNs versus primary lesions [125]. Notably, a recent large-sample LN sequencing study on colorectal cancer has uncovered the heterogeneity of TDLNs among different patients, highlighting the potential of LN molecular signatures in clinical diagnosis and therapeutic efficacy prediction [100]. Given the critical role of TDLNs in mediating immunotherapeutic efficacy and the uniqueness of their microenvironment, preoperative sentinel lymph node biopsy (SLNB) and postoperative molecular profiling of resected LNs hold great promise for predicting patient responses to immunotherapy.

Notably, accumulating clinical and preclinical evidence indicates that LNM is a key factor associated with reduced immunotherapeutic sensitivity [42, 126]. The prevailing view attributes this to the poor responsiveness of MLNs to immunotherapy, which is manifested at the single-cell level by the accumulation of Tex-term with stable epigenetic features and a reduction in circulating Tex-int [42]. However, it remains unclear whether APC-mediated immune education dysfunction in MLNs is reversible. Meanwhile, a clinical trial (NCT03247712) demonstrated that neoadjuvant stereotactic body radiation therapy (SBRT) combined with nivolumab achieved pathological lymph node downstaging in approximately

Table 3 Summary of novel therapeutic targets for LNM identified by single-cell omics

Therape-utic target/*p*-athway	Cancer type	Core mechanism	Omics meth-ods	Therap-eutic agents	Validation model	Ref
IL1α-STAT3 axis (SSM)	Melanoma	SSM secretes IL1α, which activates STAT3 in tumor cells and promotes LNM	scRNA-seq	IL1α neutralizing antibody; STAT3 inhibitor (static)	In vivo model (local injection, mouse model)	[84]
CCR7 + OX40 + DCs; OX40L signaling	Glioma	Induces Treg amplification, inhibits CD8 + T cell cytotoxicity, promotes immune tolerance and LNM	scRNA-seq	OX40 agonist (OX-86)	In vivo model (local injection, mouse model)	[118]
PDGFRα + ITGA11 + CAFs (ITGA11-SELE axis; CHI3L1-MMP2 axis)	Bladder cancer	ITGA11 binds to SELE on the LEC surface, promoting lymphangiogenesis; secretion of CHI3L1 activates MMP2, remodeling the ECM arrangement	scRNA-seq; ST-seq	ITGA11 neutral-izing antibody; CHI3L1 neutral-izing antibody	In vivo models (tail vein injection, mouse model)	[17]
Fusobacterium nucleatum-IL17/ NF-κB/RelB-TAN axis	Gastric cancer	Activation of the IL17/NF-κB/RelB sig-naling axis recruits TAN, promoting LNM and immune escape	scRNA-seq	Anti-IL17A anti-body; anti-Ly6G antibody; PD-L1 inhibitor	In vivo models (Subcutaneous injection/intraperi-toneal injection, mouse model)	[87]
Oleic acid (OA)-PPARγ-FABP4 loop	Cholangiocarcinoma	OA induces PPARγ nuclear transloca-tion, promotes FABP4 expression, enhances FAO and induces CD8 + T cell exhaustion, to promote LNM	scRNA-seq	PPARγ inhibi-tors (T0070907); FABP4 inhibitors (BMS309403); PD-1/PD-L1 inhibitors	In vivo models (in-tranodal injection, mouse model), organoids	[65]
FAO-fueled OXPHOS; NRF2-SLC7A11 axis (MICs)	Esophageal squa-mous cell carcinoma	MICs rely on FAO-driven OXPHOS for energy, enhancing stress resistance and stem cell-like properties, driving LNM and chemotherapy resistance	scRNA-seq	FAO inhibitor (etomoxir); NRF2 inhibitor (ML385); SLC7A11 inhibitor (IKE); cisplatin	In vivo model (local injection, mouse model)	[83]
MITF-FAO axis (MYC + melanoma cells)	Acral melanoma	In MYC + cancer cells, MITF induces metabolic reprogramming to shift towards FAO, driving LNM	scRNA-seq; ST-seq	FAO inhibitor (Etomoxir)	In vivo models (footpad injection, mouse model)	[36]
IGLL5-LTβR axis (IGLL5 + B cells)	Bladder cancer	IGLL5 + B cells target HEVs via CCL19-CCRL2 chemotaxis, inhibit non-canonical NF-κB signaling, disrupt HEV function and TLS formation, and promote LNM	scRNA-seq; ST-seq	IGLL5 neutralizing antibody (αIGLL5); PD-1 inhibitor (ICB); CD40L (non-classical NF-κB agonist)	In vivo models (intraperitoneal injection/intratu-moral injection, mouse model)	[37]
CCR7-CCL19 axis; Treg-CCL22-CCR7 + DC axis	Head and neck squa-mous cell carcinoma; non-small cell lung cancer; Colorectal cancer	Tregs interact with CCR7 + DCs via CCL22, inhibiting CD40 expression and T cell activation, thus promoting im-munosuppression and LNM	scRNA-seq; ST (MERFISH)	Anti-PD-1; Treg depletion agent	In vivo models (intraperitoneal injection, mouse model)	[38]
CCL22/17-CCR4 axis; Treg-mregDC crosstalk	Colorectal cancer	mregDCs secrete CCL22/17, which recruits and activates Tregs via CCR4. Tregs inhibit the migration of mregDCs to TDLNs and antigen transport, weakening anti-tumor immunity and promoting LNM	scRNA-seq; ST-seq	Anti-CTLA-4 anti-body; anti-MHC-II antibody; Treg depletion agent	In vivo models (intraperitoneal injection/intratu-moral injection, mouse model)	[39]
Galectin-9-BTK-STAT3-IL-10 axis (Galectin-9high macrophages)	Pan-cancer, 11 types	Macrophages activate the BTK/STAT3 pathway to promote IL-10 secretion, inhibits CD8 + T cell cytotoxicity, pro-motes LNM	scRNA-seq	STAT3 inhibitor; PD-1 inhibitor (combination therapy)	In vivo models (intraperitoneal injection, mouse model)	[102]

Table 3 (continued)

Therape-utic target/*p*-athway	Cancer type	Core mechanism	Omics meth-ods	Therap-eutic agents	Validation model	Ref
TLR4-CCL2/CCL7-CCR2 axis (FRC-monocyte)	Triple-negative breast cancer	Tumor-derived TLR4 ligands induce FRC to secrete CCL2/CCL7, recruiting monocytes, and suppress T cells through PD-L1/iNOS, promoting immune escape and LNM	scRNA-seq; ST-seq	TLR4 inhibitor (TAK-242); MyD88 inhibitor (TJ-M2010); PD-1 inhibitor (combination therapy)	In vivo models (local subcutaneous injection/intraperitoneal injection, mouse model)	[40]
PDE5-cGMP-RhoA-ROCK-myosin-II axis (mregDC)	Melanoma; Colorectal cancer; Pancreatic cancer; Breast cancer	Tumor progression inhibits the NOS2-NO-sGC axis, reduces cGMP synthesis, decreases the interstitial motility of mregDCs, leads to insufficient homing to TDLNs, impairs T cell activation, and promotes LNM	scRNA-seq	PDE5 inhibitors (sildenafil); PD-1 inhibitors (combination therapy)	In vivo models (intraperitoneal injection/subcutaneous injection/gavage, mouse model)	[99]

CAF Cancer-associated fibroblast, *DC* Dendritic cells, *FAO* Fatty acid oxidation, *FRC* Fibroblastic reticular cell, *HEV* High endothelial venule, *MICs* Metastasis-initiating cells, *LEC* Lymphatic endothelial cells, *LN* Lymph node, *LNM* Lymph node metastasis, *MLN* Metastatic lymph node, *scRNA-seq* single-cell RNA sequencing, *SSM* Subcapsular sinus macrophages *ST* Spatial transcriptomics, *TAN* Tumor-associated neutrophils, *TDLN* Tumor draining lymph node, *TLS* Tertiary lymphoid structure, *Treg* Regulatory T cells

80% of head and neck squamous cell carcinoma patients [127]. Additionally, over 70% of non-small cell lung cancer patients experienced lymph node downstaging after receiving immunotherapy combined with chemotherapy [128]. These findings suggest that although MLN responsiveness to immunotherapy may be attenuated, the immunosuppressive state is potentially reversible, indicating that targeted interventions may restore anti-tumor immunity in these nodes.

Clinically, surgical resection remains the mainstay for LNM-positive patients, with lymph node dissection (LND) strategies primarily guided by clinician experience, clinical N staging, and pathological findings. However, this approach poses unresolved controversies: Is prophylactic neck dissection necessary for early-stage patients without clinical evidence of LNM? Should selective or radical LND be preferred for LNM-positive patients? In numerous preclinical models, LND has been shown to impair local and systemic immunity in mice, thereby attenuating the efficacy of immunotherapy [129–131]. A multicenter retrospective analysis of non-small cell lung cancer patients revealed that excessive LND leads to diminished immunotherapeutic efficacy and poorer prognosis in recurrent non-small cell lung cancer [132]. In recent years, the concept of LN preservation has gained increasing attention [5, 133, 134]. Meanwhile, SLNB has been confirmed to achieve non-inferiority in overall survival compared with LND in multiple clinical trials, including those for melanoma (NCT00297895), breast cancer (NCT00072293), and oral squamous cell carcinoma (UMIN000006510) [135–137].

Single-cell sequencing technology has provided novel insights for personalized LND decision-making. For instance, an integrated pan-cancer single-cell sequencing study identified 177 core metastatic genes that can effectively predict metastasis risk in early-stage lesions [138]. Another single-cell sequencing study of primary tumors and MLNs across 11 cancer types identified Galectin-9 as a key mediator of MLN immunosuppression, providing a novel targeted strategy and holding promise for guiding future MLN downstaging therapies [102]. In summary, in the era of immunotherapy, simple radical LND is no longer sufficient to meet al.l clinical needs; particularly, treatment strategies for early-stage LNM require comprehensive evaluation. The clinical application of single-cell technology has gradually demonstrated its advantages [139]. In the future, evaluating metastasis-related molecular/spatial features of primary tumors and immune niches in LNs based on single-cell technology will be more conducive to immunotherapy sensitization, LN preservation, and prognosis improvement.

TDLN adaptive remodeling induced by immunotherapy

Immunotherapy (both monotherapy and combination therapy) reshapes the immune microenvironment of TDLNs to regulate anti-tumor efficacy, with adaptive changes centered on immune cell function, clonal dynamics, and intercellular crosstalk.

For monotherapy, PD-1/PD-L1 blockade drives tumor-specific Tex—a core anti-tumor subset—from TDLNs to primary tumors; TDLNs serve as the primary "armory" due to the higher proportion and phenotypic consistency of Tex clonotypes compared to peripheral blood [140]. Additionally, anti-PD-L1 therapy induces the differentiation of Tpex into Tex-int in TDLNs, which interact with DCs via the CD86-CD28 costimulatory pathway and infiltrate tumors to amplify immune responses [42]. TCR clonal analysis also reveals enhanced bidirectional migration and colocalization of T/B cell clones between tumors and TDLNs, with matched V gene usage in B cell IGHV and T cell TRAV/TRBV families suggesting antigen-specific interactions that promote T cell proliferation and tumor infiltration [141].

Although immunotherapy has shown promising efficacy, the low response rate of monotherapy remains a critical challenge, and combined immunotherapy has gradually demonstrated its superiority in both preclinical studies and clinical trials. The application of single-cell sequencing technology provides a clearer and more comprehensive perspective on changes in the LN microenvironment after combined immunotherapy. In a murine model of colon cancer, adenoviral vaccine plus anti-PD-1 significantly enriches Tcf1 + stem-like CD8 + T cells in TDLNs—this self-renewing subset acts as a functional reserve, with distinct differentiation states (stem-like in TDLNs vs. exhausted in tumors) highlighting TDLNs as key sites for maintaining T cell stemness [142]. In a prospective clinical trial of immunotherapy for head and neck squamous cell carcinoma (NCT03784066), single-cell combined with spatial omics revealed that anti-PD-L1 plus CTLA-4 combination therapy is characterized by increased clonal diversity of naive/central memory CD4 + T cells in TDLNs; these cells migrate to tumors, differentiate into TBX21+/IFNG + Th1 cells, and colocalize with CD8 + T cells, forming a synergistic immune network with migratory DCs and plasma cells [19]. Moreover, in a murine model of oral cancer, radiotherapy combined with immunotherapy converts TDLNs into an "immunomigratome". Tumor-directed radiotherapy activates DCs and promotes CXCR3 + CD8 + T cell infiltration, while subsequent PD-1 inhibition drives MMP9-dependent migration of CCR7 + DCs to TDLNs, enhancing antigen cross-presentation via high CD40/CD86/MHCII expression and inducing durable tumor responses [143].

Perioperative therapy decision-making related to LNM

Neoadjuvant therapy can often eliminate micrometastases, reduce primary tumor volume, and improve the rate of complete tumor resection [144]. For neoadjuvant regimens incorporating immunotherapy, the primary tumor—serving as a large antigen pool—activates systemic and durable anti-tumor immunity, generating long-term memory effects [145, 146]. Notably, LNM is frequently associated with shorter recurrence time and poorer prognosis following neoadjuvant therapy [126, 147, 148], indicating that LNM is a key factor contributing to neoadjuvant therapy resistance. Single-cell level studies have facilitated a deeper characterization of LN microenvironmental features linked to neoadjuvant therapy resistance. Zhang et al. performed single-cell sequencing on breast cancer and MLNs refractory to neoadjuvant chemotherapy, identifying a subset of mast cells with low BTG2 expression enriched in MLNs; these cells induce therapy resistance by promoting Treg generation and intranodal collagen fiber formation [149]. Furthermore, single-cell technology provides guidance for optimizing neoadjuvant sequential therapy. In a murine model of oral cancer, single-cell sequencing revealed the migratory activation of CCR7 + DCs along the tumor-TDLN axis following neoadjuvant immunotherapy combined with radiotherapy, explaining the molecular advantages of administering immunotherapy after tumor-directed radiotherapy at single-cell resolution [143].

Clinically, immunotherapy is primarily applied as preoperative neoadjuvant therapy or postoperative adjuvant immunotherapy, with substantial differences between the two strategies. Neoadjuvant immunotherapy, administered preoperatively, reduces tumor burden, achieves clinical downstaging, and improves surgical resectability; simultaneously, it converts the in-situ tumor into an autologous vaccine to activate the patient's intrinsic anti-tumor immune response. In contrast, adjuvant immunotherapy is administered postoperatively, targeting residual micrometastases and reducing recurrence risk by modulating the postoperative immunosuppressive state [145]. Clinical evidence confirms the superiority of neoadjuvant immunotherapy in improving prognosis: In the OpACIN and OpACIN-neo trials, neoadjuvant immune checkpoint blockade increased the 5-year overall survival rate of patients with stage III melanoma to 90%, compared with 70% in the adjuvant therapy group [150]. Similarly, in the SWOG S1801 trial, patients with resectable stage IIIB-IV melanoma who received neoadjuvant pembrolizumab achieved a 2-year event-free survival rate of 72%, versus 49% in the adjuvant-only group [151].

Preoperative neoadjuvant immunotherapy should be regarded as a strategy to preserve TDLN function and enhance systemic anti-tumor immunity. By retaining the immune priming hub function of TDLNs, it induces a more extensive, effective, and durable immune response against primary tumors and micrometastases, thereby achieving LNM downstaging and improving long-term prognosis. In contrast, postoperative adjuvant immunotherapy is limited by TDLN loss and insufficient antigen exposure, resulting in relatively limited efficacy in controlling residual micrometastases; it may be more suitable for patients without LNM or with completely resected LNM. Recently, the CheckMate 77T clinical trial demonstrated that non-small cell lung cancer patients receiving neoadjuvant chemoimmunotherapy combined with perioperative adjuvant immunotherapy achieved significantly prolonged EFS [152]. In the KEYNOTE-689 clinical trial, neoadjuvant and adjuvant pembrolizumab treatment improved EFS in patients with locally advanced head and neck squamous cell carcinoma [153]. However, immune-related adverse events still occurred in 10% of patients. In summary, recent clinical study results highlight the effectiveness of neoadjuvant combined with adjuvant immunotherapy, which may effectively improve survival

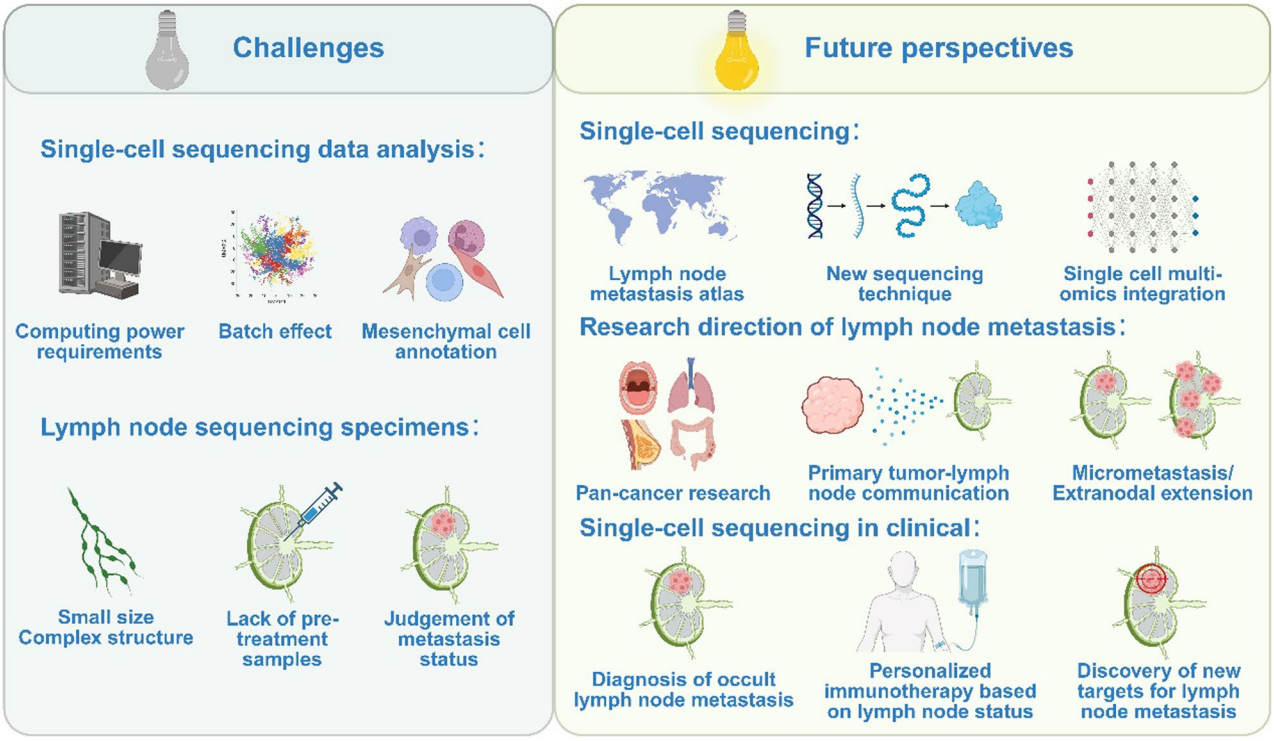

Fig. 5 Challenges and future perspectives of the application of single-cell sequencing technology in lymph node metastasis research. Created with BioRender.com

in advanced cancer patients with metastases. Single-cell omics technologies will further refine patient selection for perioperative immunotherapy—for example, identifying patients most likely to benefit from neoadjuvant therapy through specific single-cell biomarkers (e.g., SPP1 + macrophages, CCL3 + neutrophils, CD103 + CD8 + T cells) [154–156].

Research on immunotherapy resistance in MLN

LNM is closely associated with reduced patient survival rates. Recent studies have shown significant differences in regression characteristics between LNs and primary tumors after immunotherapy, with metastases exhibiting significantly lower regression than primary tumors [43, 128, 157, 158]. Against this background, a single-cell sequencing study identified that the macrophage-fibroblast axis in MLNs constructs a T cell-excluding microenvironment, which is the core mechanism of immune resistance in LNM of oral squamous cell carcinoma. Specifically, after anti-PD-1 therapy, macrophages in MLNs highly expressing SPP1, CD68, and APOE significantly activate the unfolded protein response and PERK/ATF4 signaling pathway. This subset binds to ITGAV/ITGB1 in fibroblasts via SPP1, promoting ECM remodeling. Fibroblasts highly express FAP, COL1A1, and POSTN, with significantly activated pathways for ECM organization and collagen fiber formation. They are spatially adjacent to PERK + macrophages, collectively forming a dense fibrous microenvironment that excludes CD8 + T cell infiltration [43]. This highlights the potential of a future "PERK inhibitor + anti-PD-1" combination strategy in specifically reversing immune resistance in LNM, providing new targets for clinical treatment.

Challenges and perspectives

Challenges

Current research on LNM at single-cell resolution is confronted with multiple challenges, where technical constraints and the intrinsic characteristics of LN specimens collectively constitute major obstacles (Fig. 5).

Technically, the massive volumes of data generated by large-sample single-cell sequencing impose exorbitant demands on computational capacity, and complex data analysis and processing necessitate more efficient algorithmic support. Integration of multi-platform data is plagued by significant batch effects; technical biases in data arising from different sequencing technologies or experimental batches may obscure genuine biological variations, undermining the reliability of results [159]. Additionally, there is a lack of unified and universally applicable standards for mesenchymal cell annotation. Divergent annotation strategies based on expression markers, developmental states, or functional traits often lead to ambiguities in cell subpopulation delineation, making it difficult to achieve consensus across studies.

In terms of LN sequencing, the small volume and intricate anatomical architecture of LNs hinder the procurement of samples from distinct anatomical compartments for single-cell analysis, precluding a comprehensive representation of the heterogeneous intranodal microenvironment. Given that anti-tumor therapies alter LN status and repeated sampling is highly invasive to patients, longitudinal comparison of single-cell-level changes in the same LN before and after treatment remains challenging, impeding in-depth investigation of treatment response mechanisms. Furthermore, determining the metastatic status of cancer cells in TDLNs is problematic; the cryptic presence of minimal residual tumor cells or variations in metastatic potential render it difficult to precisely distinguish true metastatic events solely via single-cell sequencing, compromising accurate interpretation of the metastatic process. These challenges restrict the in-depth application of single-cell technologies in exploring LNM mechanisms, necessitating technological innovations and methodological optimizations for breakthroughs.

Perspectives

Based on the existing research and technology, we put forward the future prospects of LNM research (Fig. 5). With the growing accessibility of single-cell sequencing technologies, an increasing number of studies are focusing on constructing large-scale single-cell atlases, including cross-species organ atlases [160], large-cohort pathological atlases [161], and pan-cancer single-cell atlases [162]. In the future, the establishment of large-cohort single-cell atlases of LNM will facilitate the identification of cellular heterogeneity, the exploration of evolutionarily conserved regulatory factors in metastatic cells, and the elucidation of functional plasticity in immune cells.

Moreover, emerging single-cell sequencing technologies hold promise for advancing LNM research. Conventional single-cell sequencing captures only unimodal information, failing to effectively distinguish cell populations with similar functions but distinct molecular signatures. In contrast, single-cell multimodal profiling technologies such as STAMP [163] and UDA-seq [164] enable simultaneous detection of chromatin states, CRISPR perturbations, RNA, proteins, and spatial information, potentially offering multi-dimensional insights into LNM research. Integration of single-cell sequencing with three-dimensional structural analyses (e.g., scMicro-C) can provide novel 3D perspectives on gene regulation [165], serving as a powerful tool for investigating fine-scale chromatin spatial architecture. Additionally, live-cell sequencing technologies like Live-seq [166], unlike traditional single-cell sequencing, possess the key advantage of non-destructive cellular profiling, facilitating the analysis of cellular state trajectories and dynamics.

Recent advances in multi-omics integration methodologies have facilitated a deeper understanding of cellular biological behaviors. Machine learning algorithms enable the extraction of single-cell features across multiple layers, facilitating the establishment of LNM prognostic prediction models based on multimodal single-cell data [167]. Recently, researchers developed a unified benchmarking platform (SCMMIB), which comprehensively evaluated six core single-cell multimodal integration tasks and identified optimal methodologies for each, propelling the in-depth advancement of single-cell omics research [168].

Significant exploration potential remains in single-cell sequencing-based LNM research. In cross-cancer conservation and evolutionary studies, there is a current lack of systematic comparisons of metastatic mechanisms across different cancer types. Integration of single-cell transcriptomic and epigenomic data from multiple cancers could help identify conserved cell communication pathways in LNM and cancer-specific driver gene mutation signatures, revealing the evolutionary principles underlying metastatic mechanisms.

In studies investigating crosstalk between primary tumors and TDLNs, existing findings are mostly limited to static descriptions. It is necessary to combine single-cell spatial transcriptomics and trajectory analysis to track spatiotemporal migration trajectories of tumor cells, immune cells, and mesenchymal cells between them, decipher mechanisms of pre-metastatic microenvironmental remodeling mediated by exosomes and cytokines, and delineate critical signal transduction nodes. The recent rise of single extracellular vesicle sequencing technology is also expected to provide subcellular-level insights into how the primary tumor shapes the pre-metastatic microenvironment in the TDLN [169].

For specific phenotypes of LNM (e.g., micrometastasis and extranodal extension), enhanced detection sensitivity is required. Utilizing single-cell full-length sequencing combined with proteomics, molecular switches governing the dormancy-activation of micrometastatic cells can be captured [170]. Simultaneously, deciphering stromal degradation pathways and immune evasion mechanisms by which tumor cells breach the lymph node capsule in ENE will provide a basis for identifying early diagnostic markers and targeted therapeutic targets, advancing clinical precision intervention.

Single-cell sequencing technology exhibits broad prospects in clinical applications for LNM. Among these, single-nucleus RNA sequencing (snRNA-seq) offers the advantage of profiling frozen specimens, providing a pivotal tool to overcome bottlenecks in clinical sample processing and promising to become the next frontier breakthrough [171]. In the diagnosis of occult LNM, traditional pathological detection often misses

micrometastases due to insufficient sensitivity. snRNA-seq can accurately identify molecular signatures of rare tumor cells by analyzing single-cell transcriptomic features of frozen LN specimens; combining this with spatial transcriptomics to localize their distribution can significantly improve early diagnostic rates, providing more precise guidance for clinical staging and surgical decision-making.

In the personalized formulation of immunotherapeutic regimens and efficacy evaluation, single-cell sequencing enables in-depth dissection of the heterogeneity and functional states of immune cell subsets within the LN microenvironment, predicting patient responses to immune checkpoint inhibitors and guiding individualized medication. Dynamic monitoring of transcriptomic changes in immune cells during treatment allows real-time efficacy assessment and prompt regimen adjustments, avoiding ineffective treatments.

Furthermore, this technology provides a powerful means to discover novel therapeutic targets for LNM, accelerating the translation of single-cell sequencing from basic research to routine clinical practice, and ultimately achieving precision diagnosis and treatment of LNM.

Conclusion

Single-cell sequencing technology has established itself as a transformative tool for deciphering the mechanisms of cancer LNM. At single-cell resolution, it has systematically revealed the molecular characteristics of tumor cell subsets with metastatic potential in primary tumors, the rules governing pre-metastatic remodeling of the immunosuppressive microenvironment in TDLNs, and the dynamic networks of tumor-immune cell interactions within MLNs. Meanwhile, it offers core therapeutic targets and mechanistic rationale for developing strategies targeting critical metastatic nodes, demonstrating substantial academic value and translational prospects in bridging basic research and clinical practice.

Abbreviations

APCs	Antigen-presenting cells
BCR	B cell receptor
CAFs	Cancer-associated fibroblasts
CIC	Cancer-immunity cycle
DCs	Dendritic cells
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ENE	Extranodal extension
FRCs	Fibroblastic reticular cells
LN	Lymph node
LND	Lymph node dissection
LNM	Lymph node metastasis
MDSCs	Myeloid-derived suppressor cells
MICs	Metastasis initiating cells
MLNs	Metastatic lymph nodes
scRNA-seq	Single-cell RNA sequencing
SLNB	Sentinel lymph node biopsy
TAMs	Tumor-associated macrophages
TCR	T cell receptor
TDLNs	Tumor-draining lymph nodes
Treg	Regulatory T cells
Tex	Exhausted T cells
Tpex	Progenitor exhausted T cells
Tex-int	Intermediate-exhausted T cells
Tex-term	Terminally exhausted T cells
TLSs	Tertiary lymphoid structures

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Authors' contributions

**XL: ** Conceptualization, Methodology, Formal Analysis, Validation, Investigation, Data Curation, Writing - Original Draft, Visualization. **XM: ** Validation, Formal Analysis, Data Curation, Writing - Original Draft, Visualization. **ZL: ** Investigation, Methodology, Validation, Formal Analysis, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. **LC: ** Validation, Formal Analysis, Data Curation, Writing - Original Draft, Visualization. **LG: ** Validation, Formal Analysis, Investigation, Writing - Original Draft, Visualization. **YH: ** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Funding Acquisition. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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